Prevalence of C282Y and E168X HFE mutations in an Italian population of Northern European ancestry

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Background and Objectives. The prevalence of C282Y is lower in Italy than in Northern European countries. We hypothesized a higher prevalence of C282Y in Northern Italian populations than in Southern Italian ones. We previously identified a nonsense mutation (E168X) in hemochromatosis probands originating from a region in the north-west of Italy. We aimed to define the prevalence of C282Y and E168X in that region and the origin of the E168X mutation by haplotype analysis.

Design and Methods. Six hundred and six blood donors were investigated for C282Y, H63D, S65C and E168X mutations by polymerase chain reaction (PCR)-restriction assays. Three hundred were also tested for rare *HFE* and *TFR2* mutations by reverse-hybridization test strips. D6S265, D6S105 and D6S1281 microsatellites were analyzed to define E168X 6p-associated haplotypes.

Results. One C282Y homozygote, thirteen C282Y/H63D compound heterozygotes, four E168X heterozygotes and three E168X/H63D compound heterozygotes were found. The allele frequencies of C282Y, H63D, S65C, and E168X were 4.7%, 14.9%, 0.74% and 0.58%, respectively.

Interpretation and Conclusions. The prevalence of C282Y in the region investigated was much higher than that previously reported in Italy. This finding is probably due to the heavy Celtic component of this north-western population and suggests that in populations of Northern Italian descent screening studies for hemochromatosis could be cost-effective. The prevalence of E168X in this region, although low, suggests that the mutation probably originated here many years ago and its frequency increased as a result of a local founder effect. Given its severity, we suggest that the E168X mutation should be searched for in all hemochromatosis patients of Northern ancestry with an incomplete *HFE* genotype.

Key words: hemochromatosis, *HFE,* C282Y, E168X, 6p haplotype, blood donors.

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Hereditary hemochromatosis (HH) is a common hereditary disorder in populations of European descent.[1,2] Two common mutations in the *HFE* gene account for the majority of the patients with HH. Most of patients are homozygous for the C282Y mutation, 5-7% are compound heterozygotes for the C282Y allele and a H63D mutation and less than 2% are H63D homozygotes.[3-7] Another *HFE* mutation, S65C, was implicated in the development of a mild form of HH, its allelic frequency being estimated at about 1% in the general population.[8,9] Other very rare or private *HFE* mutations have also been described in affected probands.[10-13] Two nonsense *HFE* mutations (E168X and W169X) were found in the compound heterozygous state with C282Y in 5 unrelated Italian patients with HH.[14] The E168X mutation is the result of a G to T transition creating a stop codon at nucleotide 502 of the open reading frame (GAG → TAG, Glu168Stop), leading to a protein that lacks the α 3 domain, transmembrane domain and cytoplasmic tail. Since all the probands carrying E168X originated from the same region (*Ossola*) in the north-west of Italy and since the mutation was associated with a single chromosome 6p haplotype we hypothesized that E168X originated in this region many years ago and that its frequency increased as a result of a local founder effect.[14]

HH is a genetic disorder that fulfills the WHO criteria for large-scale population screening.[16] One of the main issues is to know, in any given population, the frequency of the mutations potentially able to cause the disease.[17,18] Unlike the situtation in Northern European countries, in which the prevalence of HH is rather homogeneous, in Italy the frequency of HH seems to decrease from north to south.[7] The same study provided evidence that the prevalence of C282Y was much lower in HH patients from Central and Southern Italy, than in those originating from Northern Italy.[7] Two more recent studies performed in populations from Central Italy or of mixed (north and south) origin confirmed the relatively low prevalence of the C282Y allele, ranging from 1.6% to 2.1%,[19,20] but also the existence of differences in allele frequency in samples from Northern (2.7%) and from Central-Southern Italy (1.7%).[19] Since these differences likely reflect regional differences in the frequency of this allele, we aimed to test the hypothesis of whether the frequency of C282Y is higher in a restricted region in Northern Italy. This information is relevant to know whether screening programs for HH in Italy should be designed on a regional-based cri-

terion, limiting large-scale screening studies to specific (Celtic-related) areas of Northern Italy.

The aim of the study was: a) to define the prevalence of C282Y, H63D and S65C mutations in an Italian population of Northern European descent; b) to establish the frequency of E168X in the region in which it was originally identified and to extend the analysis of 6p haplotype associated with E168X to the new cases identified; c) to evaluate the effect of *HFE* genotype on ferritin levels and on the number of donations.

Design and Methods

Subjects. From March to September 2000, 606 unrelated blood donors (401 men and 205 women) from the Blood Bank of Domodossola were enrolled in the study. Twenty were first-time donors and the others had an average (± SD) history of donation of 134 (±91) months, range 6-432 months. Men had given a mean of 22.3 (±14.4) units of whole blood and women 12 (±11).

Molecular study. A blood sample was obtained from each donor for *HFE* analysis. All of them had at least one grandparent originating from the geographical region of interest (Ossola Valley). A family study was performed in the donors who were found to carry the E168X mutation in order to define the chromosome 6p haplotype associated with the mutation. All blood donors gave their written informed consent to the study. Eight drops of peripheral whole blood from each blood donor were collected on a Guthrie's card and dried. Genomic DNA was extracted using GenomicPrep Blood DNA Isolation kit (Amersham Pharmacia Biotech Inc., Piscataway NJ, USA). C282Y, H63D, S65C and E168X mutations were detected in all samples using standard polymerase chain reaction (PCR) and restriction enzyme digestion as previously described.[3,8,14] In addition, 300 samples were analyzed for a total of 12 known *HFE* and transferrin receptor 2 (TFR2) mutations (*HFE*: V53M, V59M, H63D, H63H, S65C, Q127H, E168Q, E168X, W169X, C282Y, Q283P; *TFR2*: Y250X) using reverse-hybridization test strips (Haemochromatosis StripAssay, ViennaLab, Austria)[21] and an automated strip processor (profiBlot IIT TECAN AG, Hombrechtikon, Switzerland). Analysis of microsatellites centromeric (D6S265, D6S306, D6S105 and D6S1558, in the order) and telomeric (D6S1281) to *HFE* was performed in donors carring the E168X mutation and in their relatives as previously reported.[14,15] Haplotypes were constructed manually on the basis of intrafamilial segregation of the marker alleles.

Iron study. Baseline ferritin (before first donation) was available for 516 donors because 90 individuals had given their first donation before 1982 when serum ferritin was first introduced as a routine test for donors at the Domodossola blood Bank. From that year a specific protocol was defined to control the development of iron deficiency anemia in the donors, based on annual measurement of serum ferritin and proportional and individualized modification of the frequency of donations.[22] For 586 donors serum ferritin values were available during the course of blood donations and the serum ferritin value at the last donation was taken as an index of the individual iron balance between iron losses and iron absorption at that moment. Serum ferritin was measured by standard methods. Serum iron and trasferrin saturation data were not available. The number and the frequency of donations, expressed as a donation/month ratio, was calculated for each donor who had made more than one donation.

Statistical analysis. As serum ferritin concentrations were not normally distributed, differences between groups were evaluated using non-parametric tests. Frequencies of *HFE* genotypes and alleles were compared with Fisher's exact test or the χ^2 test with Yates' correction. Computations were performed using the statistical package In Stat 2.01 (GraphPad Software, San Diego, CA, USA, 1993).

Table 1. Frequency of HFE genotypes and alleles* in 606 screened blood donors.

Genotypes	*Genotype frequency*	*95% confidence interval (%)*
C282Y $^{+}/^{+}$	1 (0.16%)	0-0.49
C282Y $^{+}/^{-}$	42 (6.93%)	4.91-8.95
C282Y $^{+}$/- H63D+/$^{-}$	13 (2.14%)	0.99-3.30
H63D $^{+}/^{-}$	145 (23.93%)	20.53-27.32
H63D $^{+}/^{+}$	8 (1.33%)	0.41-2.23
S65C $^{+}/^{-}$	5 (0.82%)	0.11-1.55
S65C $^{+}/^{-}$ H63D$^{+}/^{-}$	4(0.66%)	0.63-0.69
E168X $^{+}/^{-}$	4 (0.66%)	0.63-0.69
E168X $^{+}/^{-}$ H63D $^{+}/^{-}$	3 (0.49%)	0.47-0.52
Wild Type	381 (62.88%)	59.02-66.72
Allele	*Allele frequency*	*95% confidence interval (%)*
C282Y	57 (4.71%)	3.52-5.87
H63D	181 (14.93%)	12.92-16.93
S65C	9 (0.74%)	0.25-1.22
E168X	7 (0.58%)	0.15-1.00
Wild Type	958 (79.04%)	74.24-83.84

**allele frequency was calculated on number of chromosomes.*

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Table 2. Comparison of HFE genotype and allele frequencies in three Italian screening studies.

Genotypes	*Present study (n=606)*	*Barosi et al.[24] (n=1050)*	*Cassanelli et al.[19] (n=2100)*
C282Y $^{+/+}$	1 (0.2%)	0	0
C282Y $^{+/-}$	42 (6.9%)*	39 (3.7%)	66 (3.1%)
C282Y $^{+/-}$ H63D $^{+/-}$	13 (2.1%)°	7 (0.7%)	2 (0.1%)
H63D $^{+/-}$	152 (25.1%)	264 (25.1%)	452 (21.5%)
H63D $^{+/+}$	8 (1.3%)	22 (2.1%)	52 (2.5%)
Wild Type	390 (64.4%)	718 (68.4%)	1528 (72.8%)

**p=0.0044 and p<0.0001, °p=0.01 and p<0.0001 vs. Barosi et al. and Cassanelli et al., respectively. H63D $^{+/-}$ includes also compound heterozygotes with S65C (n=4) or E168X (n=3); Wild Type also includes heterozygotes for S65C (n=5) or E168X (n=4) (see Table 1 for more details).*

Alleles	*Present study*	*Barosi et al.[24]*	*Cassanelli et al.[19]*
C282Y	57 (4.7%)*	46 (2.2%)	68 (1.6%)
H63D	181 (14.9%)	315 (15%)	558 (13.3%)
Wild Type	974 (80.4%)	1739 (82.8%)	3574 (85.1%)

**p<0.0001 and p<0.0001 vs. Barosi et al. and Cassanelli et al. respectively. Wild Type also includes alleles positive for S65C (n=9) and E168X (n=7) mutations (Table 1).*

Figure 1. Chromosome 6p haplotypes associated with the E168X mutation.

Haplotype	*D6S265*	*D6S306*	*D6S105*	*D6S1558*	*E168X*	*D6S1281*	*No. of haplotypes*
1	3	3	5	3	2	6	5
2	5	3	5	3	2	6	2
3	5	5	5	3	2	6	1
4	6	5	1	3	2	3	1
5	6	3	1	4	2	5	1

Results

Molecular study. Table 1 shows the frequency of *HFE* genotypes and alleles of the population studied. One C282Y homozygous woman and 13 C282Y/H63D compound heterozygotes (7 men and 6 women) were identified. Four individuals (one man and three women) were heterozygotes for E168X and 3 (one man and two women) were E168X/H63D compound heterozygotes. Overall, in the studied population, the frequency of C282Y allele was 4.7%, that of H63D was 14.9%, that of S65C was 0.74% and that of E168X was 0.58%. Among the selected 300 samples tested for 12 different *HFE* and *TFR2* mutations, results for C282Y, H63D, S65C and E168X confirmed previous typings by restriction enzyme digestion and no other mutation was identified. In Table 2 the frequencies of C282Y and H63D genotypes and alleles in our series are compared with those of already published screening studies in Italy. A statistically significant difference was observed for C282Y-containing genotypes and the C282Y allele. In the seven E168X carriers, family studies allowed definition of the chromosome 6p haplotypes associated with the mutation. The haplotypes associated with E168X in the seven donors and in the three previously described patients with HH carrying this mutation are shown in Figure 1.[14] A common haplotype (D6S265-3, D6S306-3, D6S105-5, D6S1558-3, D6S1281-6) accounted for 50% of the chromosomes carrying the mutation. Two chromosomes (20%) changed at D6S265 locus, one (10%) at D6S306 locus and one (10%) retained only the D6S1558-3 allele. The fifth haplotype differed at each locus but a slippage mispairing cannot be excluded at the D6S1558 and D6S1281 loci.

Iron study. At baseline, 15 subjects (14 men and 1 woman) out of 516 individuals (2.91%) had serum ferritin above the cut-off value (> 300 mg/L in men and > 200 mg/L women). Table 3 shows serum ferritin levels and number of subjects with elevated serum ferritin according to sex and *HFE* genotypes. Excluding the C282Y homozygote who maintained an increased serum ferritin level even after 20 years of donation, the serum ferritin during of the other subjects normalized during the course of the blood donations.

In men, baseline serum ferritin was higher in C282Y/H63D compound heterozygotes, H63D homozygotes and C282Y heterozygous donors than in donors with the other *HFE* genotypes, but the differences were not statistically significant. A similar trend was observed in women. In men, serum ferritin at last donation was slighty higher in the C282Y/H63D compound heterozygotes (median 54 mg/L, range 26-136 mg/L), C282Y heterozygotes (median 53 μg/L, range 20-285 μg/L) and H63D homozygotes (median 64,5 μg/L, range 20-111 μg/L) than in donors carrying the wild type genotype (median 46 μg/L, range 7-168 μg/L), but again the differences did not achieve statistical significance. The number of individuals carrying the E168X and S65C mutations either alone or in combination with H63D was too small to perform valuable statistical analysis. There was no significant difference in the frequency of blood donations according to *HFE* genotype.

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Table 3. Serum ferritin level and frequency of increased serum ferritin according to HFE genotypes and sex.

Genotype	*N. of subjects*	*Median (mg/L)*	*Serum ferritin Elevated (Men: >300 mg/L Women: >200 mg/L)*
Men			
C282Y^{+}/$^{+}$	0	0	0 (0%)
C282Y^{+}/$^{-}$	25	102	2 (8%)
C282Y^{+}/- H63D $^{+}$/$^{-}$	4	171.5	1 (25%)
H63D^{+}/$^{-}$	71	62.5	2 (2.8%)
H63D^{+}/$^{+}$	5	140	0 (0%)
S65C^{+}/$^{-}$	4	62	0 (0%)
S65C^{+}/$^{-}$ H63D $^{+}$/$^{-}$	2	NC	1 (50%)
E168X^{+}/$^{-}$	1	NC	0 (0%)
E168X^{+}/$^{-}$ H63D^{+}/$^{-}$	1	NC	0 (0%)
Wild Type	201	86.5	8 (4%)
Total	314		14 (4.5%)
Women			
C282Y^{+}/$^{+}$	1	NC	1 (100%)
C282Y^{+}/-	11	13	0 (0%)
C282Y^{+}/- H63D^{+}/$^{-}$	6	34.5	0 (0%)
H63D^{+}/$^{-}$	45	14	0 (0%)
H63D^{+}/$^{+}$	3	34.5	0 (0%)
S65C^{+}/$^{-}$	1	NC	0 (0%)
S65C^{+}/- H63D^{+}/$^{-}$	1	NC	0 (0%)
E168X $^{+}$/$^{-}$	3	18	0 (0%)
E168X^{+}/$^{-}$ H63D^{+}/$^{-}$	2	NC	0 (0%)
Wild Type	129	13.5	0 (0%)
Total	202		1 (0.5%)

NC: not calculated because of the small number of subjects.

Discussion

The first finding of the present study is that the prevalence of C282Y mutation in an Italian population from the north-east of the country (*Ossola* region) is much higher than that previously reported in Italy and similar to that in Northern European populations.[1,7] The expected frequency of C282Y homozygotes and heterozygotes is 1:454 and 1:10.6, respectively. A second finding is that E168X, a nonsense mutation previously described in three unrelated HH probands originating from the *Ossola* region,[14] was also present in the healthy population from the same geographical area. It is possible that the prevalence of C282Y and E168X mutations might even be underestimated in this population. In fact, the Blood Bank of Domodossola has been involved in early screening of hemochromatosis since 1985 and at least two donors (one C282Y homozygote and one C282Y/E168X compound heterozygote), who had been diagnosed as affected by HH and excluded from the donors' list according to the Italian recommendations on the use of blood for donation, were also excluded from the present study. The *Ossola* region is a subalpine geographical area situated on the route that connects Switzerland to the *Padana* plain through Alpine passes. In ancient times this route was one of the main passages for trading between Celtic populations and ancient Italian inhabitants and also for military invasions. There is historical and archeological evidence of important Celtic settlements along and around this route.[23] These observations suggest that the high prevalence of the C282Y mutation that we observed in the population originating from this area is related to the Celtic component being more substantial in this population than in populations from Central and Southern Italy. A similar high prevalence of the C282Y mutation was reported in a small cohort of subjects originating from another subalpine region in the north-east of Italy.[24] The *Ossola* region, as all the subalpine valleys, was characterized by very low migratory fluxes for a long time until the second half of the last century, favoring the expansion of the C282Y mutation in this relatively isolated geographical area. The relative isolation of this population might also explain the spread of the E168X mutation in the region. The presence of a prevalent haplotype (D6S265-3, D6S306-3, D6S105-5, D6S1558-3, D6S1281-6) associated with the E168X mutation suggests a local founder effect. The other haplotypes probably originated from recombination events centromeric or telomeric to *HFE*, although it cannot be excluded that some modifications could have originated simply by slippage mispairing at single loci and not by recombination (Figure 1). Overall, these findings suggest that E168X originated in the area several centuries ago. Thus, we cannot exclude that this mutation might have spread into the neighboring districts, or even far from the Ossola region due to more recent migrations. However, no E168X mutation was found in 300 DNA samples, analyzed by reverse hybridization test strips (*see Methods*), belonging to individuals originating from another region in the North, around the city of Monza (*data not shown*). In the population studied the allele frequency of E168X was 0.57 % and the carrier frequency was estimated to be 1:86, indicating that E168X is a rare mutation, but also that its prevalence is not negligible in this region. The E168X mutation was slightly less frequent than the S65C one but, unlike S65C, which is a very mild mutation,[8] E168X leads to complete disruption of *HFE* function and to a severe HH phenotype when associated in the compound heterozygous state with C282Y.[14] As far as we know, the E168X mutation is the only inactivating *HFE* mutation, besides C282Y, that is present in a measurable frequency in a healthy population. Overall, these findings allow the following conclusions to be drawn: first, in selected populations of Northern Italian descent large screening studies for HH could be cost-effective, and second, the E168X mutation should be searched for in all Italian HH patients of Northern ancestry with an incomplete *HFE* genotype.

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A third finding of the study is that 2.91 % of donors (4.5 % of men and 0.5 % of women) had an increased level of serum ferritin at their first donation and that only two (one C282Y/H63D and one H63D/S65C compound heterozygote) of the 14 men with an increased level of serum ferritin had a known *HFE* genotype at risk for hemochromatosis. This figure is similar to that reported by Cassanelli *et al.*[20] in a recent screening study in Central Italy in which they also found a high prevalence of increased transferrin saturation in the *HFE* wild type individuals. They suggested the existence of genetic determinants other than *HFE* to explain their results, a hypothesis also supported by Barosi *et al.*[25] who recently found a highly prevalent *HFE*-unrelated, mild idiopathic iron overload in a population of 1,050 potential Italian blood donors. In our series we could not measure transferrin saturation since this is not part of the routine tests at the Blood Bank of Domodossola; we cannot, therefore, compare our data with those of Barosi *et al.*[25] All the male donors with high serum ferritin at baseline developed normal values during the course of the donations indicating that their body iron overload was mild and that possibly other factors, such as metabolic alterations or increased alcohol intake, were involved. Although the number of individuals carrying the E168X mutation is small, there is no evidence that E168X heterozygotes had an increased risk of accumulating iron, confirming our previous findings.[14]

In our small series we cannot demonstrate statistically significant differences in serum ferritin values, either at baseline or at last donation, or in the frequency of blood donations between genotype groups. However, serum ferritin values, both at baseline and at last donation, were higher in subjects with the following *HFE* genotypes: C282Y/H63D, H63D/H63D and C282Y/wild type, in accordance with results from previous studies.[20,25,26] This suggests that iron losses, as a result of donations, are more easily compensated in male individuals carrying these genotypes and, as recently demonstrated, even more so in C282Y homozygotes.[26] The hypothesis that an *individualized* phlebotomy regimen can be applied according to serum ferritin levels in blood donors, particularly in men, was proposed before the discovery of *HFE* [27-29] and could now be addressed in *ad-hoc* studies.

In conclusion, the high prevalence of *HFE* mutations in regions of North Italy, if confirmed, should prompt the National and Local Health Services in Italy to promote the development of screening guidelines for iron overload and hemochromatosis and to increase the understanding of these conditions in these areas. Furthermore, a revision of the restrictions endorsed by the National Italian committee for blood donation on the use of blood from otherwise healthy hemochromatosis patients is needed, considering the recent demonstration that this population does not represent a greater risk to blood safety than other donors.[30] These policies may help foster further changes that will promote more blood donations.

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Contributions

AS: conception and design, analysis and interpretation of data, drafting the article; RM: conception and design, analysis and interpretation of data, drafting the article; CO: conception and design, analysis and interpretation of data and final approval of the version to be published; AM: conception and design, analysis and interpretation of data, drafting the article; VM: conception and design, analysis and interpretation of data, drafting the article; CA: drafting the article; PC: conception and design, analysis and interpretation of data, drafting the article and final approval of the version to be published; AP: conception and design, analysis and interpretation of data, drafting the article, final approval of the version to be published. Primary responsibility for the paper: AP; primary responsibility for Table 1: AS, SP; Table 2: RM, AR; Table 3: AS, RM; primary responsibility for Figure 1: SP, AR. The authors thank all the blood donors of the Blood Bank of Domodossola for their co-operation.

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Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received August 14, 2002; accepted February 6, 2003.

In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

In Southern Europe HFE-related genetic hemochromatosis accounts for only about two thirds of all cases of genetic iron overload syndromes.

What this study adds

This study confirms that ethnic components are relevant to the prevalence of the C282Y HFE mutation, and shows that the E168X HFE mutation should be investigated in hemochromatosis patients of Northern ancestry with an incomplete HFE genotype.

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