Variation of iron loading expression in C282Y homozygous haemochromatosis probands and sib pairs

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EDITOR—Hereditary haemochromatosis (HH), a common autosomal recessive disease of iron metabolism, is more prevalent among populations of northern Europe with an affected rate of 1 in 200 to 400 and a carrier frequency of around 1 in 10.[1 2] The disease is characterised by progressive iron overload, and the clinical onset usually appears after middle age. The phenotypic manifestations of HH are variable, and the severity of the disease is related to the iron loading; the most common symptoms of iron overload are fatigue, lethargy, arthropathy, and skin pigmentation, often along with more serious organ damage including cirrhosis, diabetes mellitus, myocardiopathy, and endocrine dysfunction. The assessment of iron loading is currently based on the levels of biochemical iron markers such as transferrin saturation percentage, serum ferritin, and serum iron concentrations. However, the diagnosis of haemochromatosis can now be confirmed using direct HFE mutation testing. The prognosis depends on early diagnosis and therapeutic venesections. Thus, population screening would allow early diagnosis during the asymptomatic phase and prophylactic treatment by repeated venesection to prevent the irreversible damage of iron overload,[3] but predictive diagnosis requires a well established phenotype-genotype correlation.

The identification of the haemochromatosis gene,[4] now referred to as *HFE*,[5] enables the performance of direct genetic testing for diagnosis. The role of the HFE protein in iron metabolism has not yet been clearly established, but it seems that the complex of HFE with β2-microglobulin interacts with the transferrin receptor (TfR) on the cell surface, which decreases the affinity of TfR for transferrin.[6–9] Some mutations characterised in the *HFE* gene and leading to a functional defect have been correlated with HH. Two missense mutations, 845G→A (C282Y) accounting for 80-90% of HH chromosomes,[4 10–13] and 187C→G, (H63D) representing 40-70% of non-C282Y HH chromosomes,[4 12–15] leading to the absence and decrease of HFE activity, respectively, have been described.[16 17] Another variant, 193A→T, leading to the missense substitution S65C, has been reported to be increased in HH chromosomes, accounting for 7.2% of HH chromosomes, neither 845A (C282Y) nor 187G (H63D).[18]

The considerable heterogeneity of iron loading observed in HH patients has been correlated with their genotype. Several studies have confirmed that HH patients homozygous for the C282Y mutation are associated with a more severe form of the disease than those carrying other genotypes (H63D/H63D, C282Y/H63D, C282Y/S65C).[12 13 19] Phenotype-genotype correlation studies have shown discrepancies. Some reports have mentioned patients diagnosed with haemochromatosis who did not carry known HFE mutations on both chromosomes, accounting for up to 21% of the HH population.[4 12 13] Thus, the aetiology of the iron loading in these patients remains unclear. Non-HFE related patient cases may have been included in these HH subjects because of misdiagnosis owing to secondary iron overload or atypical juvenile haemochromatosis linked to chromosome 1q[20–21]; this point still needs to be clarified. In addition, despite the prominent role of the C282Y mutation in HH, population screening indicates that 17.6% of homozygotes for C282Y were asymptomatic patients.[22] Thus, C282Y penetrance confronts one with a problem and requires more investigation.

In the present study, we assessed the biochemical expression of iron loading in *HFE* C282Y homozygotes. We thus examined the parameters indicative of iron loading in a series of probands homozygous for the C282Y mutation. Then we conducted a family case study of *HFE* identical sibs enrolled because one of them had HH. The whole study showed a variable biochemical expression of iron overload related to the patients' age and sex, which was not correlated in subjects with an identical inherited genotype at the *HFE* locus.

Patients and methods

SUBJECTS
A series of 545 unrelated probands, all homozygous for C282Y, showing various symptoms of clinical haemochromatosis and referred from clinicians to our blood centre for treatment by venesection, was included in this study. Before treatment the diagnosis was confirmed by their iron status markers, and all of them showed at least two of the following criteria: (1) transferrin saturation higher than 60% in males and 50% in females, (2) serum ferritin concentration exceeding 400 µg/l in males and 300 µg/l in females, and (3) serum iron above 20 µmol/l. These iron status markers were measured by standard techniques.

SIB PAIR STUDY
As part of genetic counselling a family study was conducted. Partners and sibs of C282Y HH probands were screened for *HFE* mutations and biochemical iron markers. The study

Table 1 Iron status in 545 probands homozygous for the C282Y mutation

Sex	Age	No	Transferrin saturation (%)	Serum ferritin (µg/l)	Serum iron (µmol/l)
F	<30	11	68 (11.8) [55–82]	262 (104) [108–398]	37 (5.6) [28–42]
M	<30	22	79.6 (16.7) [51–98] NS	598 (506) [83–2000] NS	38.4 (8.8) [21–49] NS
F	<40	30	79 (14.7) [57–99]	663 (944) [69–4000]	37 (11.3) [14–62]
M	<40	105	78.2 (15.5) [34–100] NS	1341 (1078) [240–4800] p=0.02	38.8 (7.8) [22–69] NS
F	<50	50	76.6 (14.6) [54–95]	588 (764) [36–3300]	33.2 (6.6) [22–53]
M	<50	117	82.5 (12.1) [44–100] p=0.05	1822 (1672) [95–8890] p=0.0003	38.4 (6.3) [22–51] p=0.055
F	<60	52	70 (16.9) [27–96]	847 (657) [77–2500]	31.8 (6.4) [22–48]
M	<60	71	81.8 (12) [47–97] p=0.001	2133 (1601) [450–5368] p=7.10⁻⁶	44.7 (25.2) [27–57] p=0.01
F	>60	54	75.8 (18.4) [37–98]	1649 (1525) [195–6680]	36.3 (8.4) [14–55]
M	>60	33	79.6 (18) [37–95] NS	2349 (2059) [215–8800] p=0.033	38.8 (6) [28–49] NS

Transferrin saturation, serum ferritin, and serum iron values are expressed as means (SD) and range (NS = not significant).

group consisted of 53 subjects from 24 unrelated HH families in whom at least one sib had exhibited symptoms and an increased total body iron loading, indicative of HH. This allowed the examination of 18 same sex sib pairs, homozygous for C282Y, and composed of 13 male pairs and five female pairs; eight others were opposite sex pairs. At the time of the biochemical diagnosis, all these sibs were 32 to 64 years old. The mean difference in age within a set of sibs was 5.6 years.

HFE MUTATION ANALYSIS
DNA was extracted from peripheral blood leucocytes. C282Y, H63Ds, and S65C substitutions were analysed as previously described.[18]

STATISTICAL ANALYSIS
Measurements of transferrin saturation, serum ferritin, and serum iron are expressed as means (SD) and range is indicated. Comparisons between groups of subjects were made with Student's *t* test and correlation between iron parameters was assessed. The relationship between the iron parameters and the age of patients was studied separately in males and females using linear regression analysis.

Results
A total of 545 unrelated subjects including 197 females and 348 males, all diagnosed with HH and homozygous for the C282Y mutation, were studied. Thus, the male to female sex ratio was 1.7:1 showing a reduced penetrance of the C282Y mutation in females compared to males. The difference in age at onset was recorded according to the sex of probands. The mean age was 44.1 (SD 10.9) and 49.8 (SD 12.5) in males and females, respectively. The difference in mean age at onset calculated between the males and the females using Student's *t* test was significant (t=4.57, p=3.3 × 10⁻⁶) showing that, at onset, females were significantly older than males. Approximately
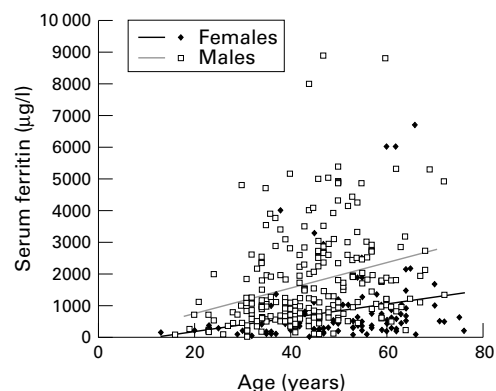

Figure 1 Distribution and correlation of serum ferritin concentration in 348 males (r=0.29) and 197 females (r=0.23) according to age. The equation of the fitted regression line was Y=39.9X-43.4 in males (light line) and Y=21.1X-217.2 in females (dark line).

70% of the males were diagnosed with HH before the age of 50, whereas only 48.6% of females were; 90% of the males and 76.5% of the females were diagnosed with HH before the age of 60 (table 1). These results show that the biochemical expression of haemochromatosis strongly depended on both sex and age in C282Y homozygotes. Iron marker values ranged between normal and significantly increased compared with control values and regardless of the proband's sex and age. However, transferrin saturation, serum ferritin, and serum iron values as a whole were significantly higher in males than in females (p=1.2 × 10⁻³, 6.7 × 10⁻⁸, 9 × 10⁻³, respectively).

According to the age range, above 30 years old serum ferritin and serum iron concentrations were significantly higher in males than in females; transferrin saturation was significantly increased with age only after 40 years in males compared with females (table 1). A correlation analysis showed that, whereas transferrin saturation and serum iron remained stable with age in both sexes, there was a progressive increase of serum ferritin concentration with age in males (r=0.29) and females (r=0.23) (fig 1); it increased from 262 to 1355 µg/l in females and from 598 to 2349 µg/l in males aged from 30 to over 60 years of age. The linear regression analysis indicated a significant mean annual progression of serum ferritin of 39.9 µg/l (p<10⁻³) in males and 21.1 µg/l (p=10⁻²) in females.

The biochemical data of families were reviewed following the discovery of one sib pair *HFE* identical by descent, in which one sib exhibited total body iron overload and was clinically diagnosed as HH. Sex matched sibs, homozygous for the C282Y mutation, were reviewed to determine the degree of iron loading in the other sib through transferrin saturation percentage and serum ferritin and serum iron concentrations. Opposite sex sibs were not compared because iron overload is known to be higher in male subjects compared to females. Therefore, 18 C282Y homozygous same sex sib pairs were examined; this showed that transferrin saturation ranged between 39 and 98%, serum ferritin between 159 and 4900 µg/l, and serum iron from 12.5 to 48 µmol/l.

Table 2 Comparison of 18 same sex sib pairs homozygous for the C282Y mutation

	Transferrin saturation (%)	Serum ferritin (μg/l)	Serum iron (μmol/l)
Correlation	r=0.47 p=0.07	r=0.10 NS	r=0.23 NS
Mean difference	14 [0–35] t=5.1 p=0.00017	1026 [90–4243] t=3.12 p=0.0065	7.5 [0–20] t=6.1 p=0.00002

Transferrin saturation, serum ferritin, and serum iron means in probands were 79.4% (SD 13), 1382 μg/l (SD 1348), 39.5 μmol/l (SD 6.4), respectively, and in sib cases 74.2% (SD 19), 1069 μg/l (SD 1166), and 38.1 μmol/l (SD 8.1), respectively; these results were not significantly different. The concordance of these parameters between sib pairs was also assessed. There was no correlation of serum ferritin or serum iron levels between the HH diagnosed sibs and other sibs, while transferrin saturation level tended to be correlated, but remained non-significant (p=0.07); the mean differences in transferrin saturation, serum ferritin, and serum iron values in sib pairs were all significant (table 2). In addition, no significant correlation was found between the oldest and the youngest sibs for the three iron markers when all same sex pairs were considered, indicating that, in this case, the lack of correlation was not related to the age of the subjects. This intrafamilial study showed a variable level of iron overload for subjects with *HFE* genotype identical by descent. In addition, among the 18 same sex sibs, two, six, and one sibs were in the normal range of values for transferrin saturation percentage (<43%), serum ferritin concentration (<300 μg/l), and serum iron concentration (<20 μmol/l), respectively.

Moreover, in a family of five *HFE* identical sibs homozygous for the C282Y mutation, ranging in age from 53 to 61 years old, the sibs displayed variable biochemical expression of iron loading (fig 2). One 60 year old female had no significant increase of any of the iron parameters. Her two sisters (mother of four and two children, respectively) and two brothers, who were C282Y homozygotes, were affected with HH and showed iron overload according to their iron status parameters.

Another case of discrepancy between genotype and phenotype was discovered in a female homozygous for the C282Y mutation; she had five children. Her husband was genotyped to evaluate the potential risk for the children of having HH. He was found to be homozygous for C282Y and his biochemical iron status at 61 years of age did not show any sign of iron loading (fig 3). Their son, 35 years old and C282Y homozygous, showed signs of iron overloading whereas of their four daughters, aged 30 to 40 years old, only the oldest showed raised transferrin saturation and serum iron concentration.

Discussion

The present study reports on the relationship between the biochemical expression of iron loading and the homozygous genotype for the C282Y mutation. Iron loading was first examined in a series of 545 probands homozygous for the C282Y mutation. The iron loading was significantly lower in females than in males whatever the parameter investigated; moreover the study confirmed the reduced penetrance of C282Y in females with a male to female sex ratio of 1.7:1 in probands with clinical HH. The biochemical expression of HH, lower in females than in males, indicated that some C282Y homozygous females may not develop signs of iron overload. In a family study, one case of a female identical by descent to four sibs homozygous for C282Y and diagnosed with HH did not reach the threshold values for iron

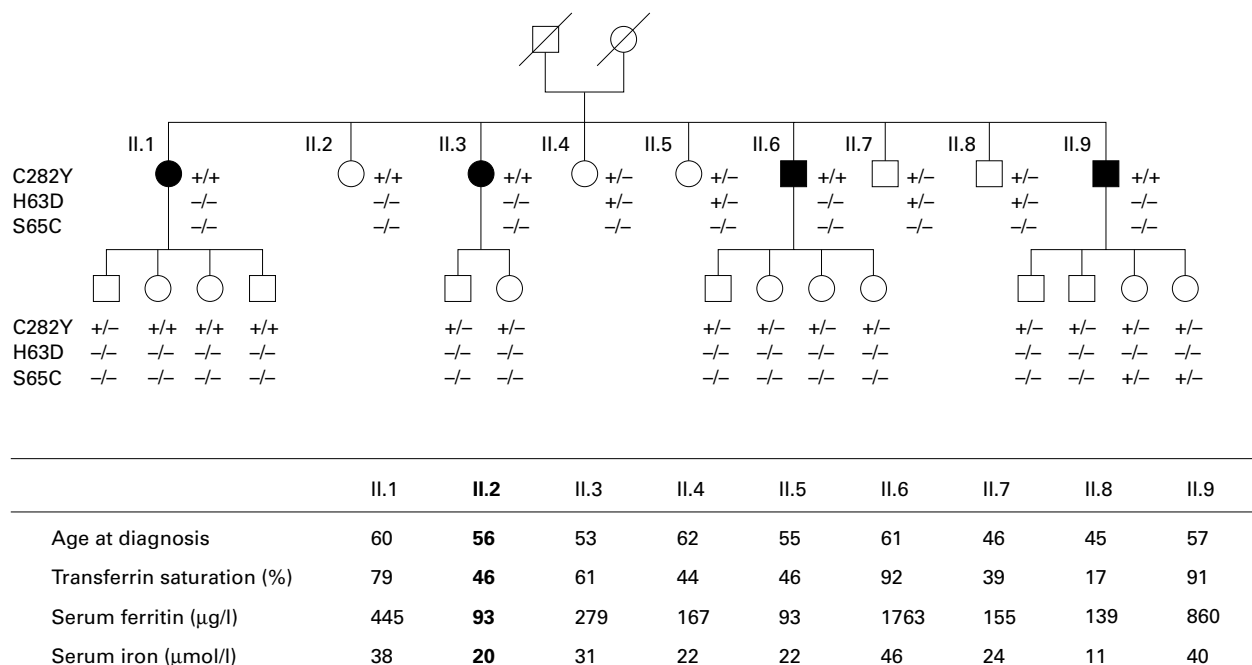|		II.1	II.2	II.3	II.4	II.5	II.6	II.7	II.8	II.9
Age at diagnosis		60	**56**	53	62	55	61	46	45	57
Transferrin saturation (%)		79	**46**	61	44	46	92	39	17	91
Serum ferritin (μg/l)		445	**93**	279	167	93	1763	155	139	860
Serum iron (μmol/l)		38	**20**	31	22	22	46	24	11	40

Figure 2 A familial case of a female (II.2) homozygous for the C282Y mutation without haemochromatosis. The genotypes are given in order C282Y, H63D, and S65C. + indicates the presence of the mutant allele and − the presence of the wild type allele.
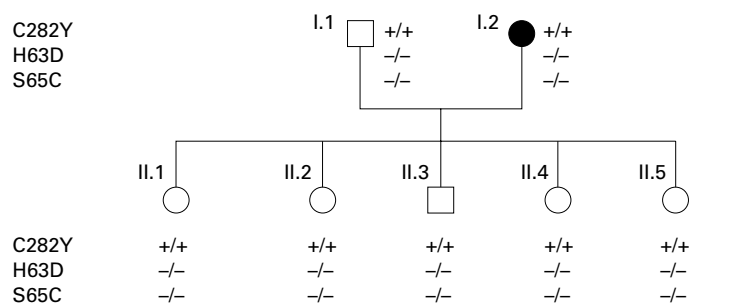
Figure 3   A male case (I.1) homozygous for the C282Y mutation without reaching iron status level for haemochromatosis. The genotypes are given in order C282Y, H63D, and S65C. +indicates the presence of the mutant allele and − the presence of the wild type allele.

overload expression defined for haemochromatosis, which indicated that the C282Y mutation did not show complete penetrance in females.

In this series, iron marker values ranged between normal and significantly increased; transferrin saturation percentage seemed to be the best parameter to predict haemochromatosis in young C282Y homozygous subjects whereas serum ferritin, the only value to increase progressively, was proved better to show overloading extent. This study thus confirmed that the extent of iron loading in haemochromatosis C282Y homozygotes is directly related to the age and sex of probands.[23–25] However, when considering age range, large variations in iron status values were observed in subjects homozygous for C282Y mutation; serum ferritin showed the largest variation. We thus checked for intrafamilial variation of iron markers in sib pairs homozygous for the C282Y mutation. The lack of correlation between sibs and the significant differences of the iron marker values between sib pairs clearly showed a variable biochemical expression of iron overload in sibs with genotype identical by descent at the *HFE* locus. Although the expression of the disease is strongly influenced by the C282Y mutation, an intrafamilial study of subjects also confirmed that an identical genotype for the *HFE* gene can show variable iron loading; thus, intrafamilial variations in iron loading do not only represent a variation in the genetic expression of the *HFE* gene. The cases of non-expressing C282Y homozygous males aged over 60 years old, and displaying iron parameters in the normal range, as reported here and by others,[26] showed that the biochemical expression of HH is under the influence of other factor(s). In the families studied, transferrin saturation, serum ferritin, and serum iron variations could result from environmental and non-genetic factors or other genetic factors than those examined. Recent population studies have shown that,

compared with non-carriers, any genotype group defined as carriers of a *HFE* mutation had higher mean serum iron concentration and mean transferrin saturation, whereas only C282Y homozygotes showed higher serum ferritin concentration[23]; they also highlighted that some C282Y heterozygotes could be diagnosed as HH.[22] Thus, despite a good correlation between HFE defined genotypes and phenotype in population studies, *HFE* genotyping does not show a clear level of iron loading in subjects and may have implications in genetic counselling.

- Hereditary haemochromatosis (HH) is a common autosomal recessive disease characterised by progressive iron overload. The identification of the *HFE* gene and mutations involved in haemochromatosis allows direct genetic testing for diagnosis. However, correlations between phenotype and HFE genotypes showed discrepancies and mutation penetrance raises questions.

- We examined the iron loading status in 545 probands and 18 same sex sibs, all homozygous for the C282Y mutation.

- Our data support transferrin saturation percentage and serum ferritin concentration as the best biochemical iron marker for HH phenotype in young subjects and extent of overload in these patients, respectively. The results also confirm a clear correlation of the iron loading level with age and sex of the patients. However, the lack of correlation of the iron marker status between pairs of sibs, homozygous for C282Y identical by descent, indicated a variable phenotypic expression of iron loading independent of HFE genotype.

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	I.1	I.2	I.3	I.4
Age at diagnosis	**67**	55	35	32
Transferrin saturation (%)	**25**	50	51	72
Serum ferritin (µg/l)	**41**	352	252	116
Serum iron (µmol/l)	**14.8**	22.2	29	33

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Haptoglobin genotype as a risk factor for postmenopausal osteoporosis

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EDITOR—Some epidemiological and experimental data have shown a correlation between iron metabolism and calcium, phosphate, and magnesium turnover.[1][2] In particular, previous reports have shown that iron availability can play a fundamental role in bone metabolism and that iron depletion can lead to bone demineralisation. For example, in patients who underwent gastrectomy[3–5] or in rats treated similarly,[6] osteoporosis was accompanied by laboratory and clinical signs of iron deficiency and was prevented by the administration of fructo-oligosaccharides, a substance that promotes iron absorption from the gut. In oophorectomised rats (a condition mimicking the oestrogen levels commonly found in the menopause), a wide range of cells, including osteoblasts, displayed a reduced number of transferrin receptors and hence a reduced iron uptake.[7] In humans, it has been assessed that out of 14 nutrients tested (including calcium), iron was the best positive predictor of BMD in the femoral neck,[8] and furthermore a negative correlation between ascorbic acid content of the diet and osteoporosis has been found[9][10]; it is notable that ascorbic acid in the diet affects iron absorption increasing it by a factor of 2-3. A severe nutritional iron deficiency anaemia provokes significant alterations in the metabolism of calcium, phosphorus, and magnesium in rats with a noticeable degree of bone demineralisation, even in the presence of normal serum levels of calcium, phosphorus, and magnesium.[1]

On the basis of the above evidence, we searched for a genetic marker of iron disposal (haptoglobin genotype) as a risk factor for postmenopausal osteoporosis.

Only about 5% of daily iron turnover comes from intestinal absorption, most of it coming from haemoglobin turnover, which requires three proteins, haemopexin, haptoglobin, and haem oxygenase. We focused our attention on haptoglobin since it is the only one with a well known polymorphism.

Haptoglobin (HP) is a serum α2 glycoprotein that exists as a tetramer, composed of two smaller identical alpha (α) and two larger identical beta (β) chains. At present, three main different genotypes of haptoglobin in normal adult plasma have been identified. Differences among the three haptoglobin genotypes are given by light alpha subunit structures: type 1.1, type 2.2, which has homozygous α1 (9 kDa) and α2 (18 kDa) subunits, and type 2.1, which has heterozygous α1 and α2 subunits, with a shared β subunit in all three genotypes (38 kDa). The β chain is a glycoprotein which does not exhibit polymorphism but only some rare variants.