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HFE mutations, iron deficiency and overload in 10 500 blood donors

H. A. Jackson,[1] K. Carter,[2] C. Darke,[2] M. G. Guttridge,[2] D. Ravine,[3] R. D. Hutton,[2] J. A. Napier[2] and M. Worwood[1] [1]*Department of Haematology, University of Wales College of Medicine and University Hospital of Wales, Cardiff,* [2]*Welsh Blood Service, Pontyclun, and* [3]*Institute of Medical Genetics, University Hospital of Wales, Cardiff, UK*

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Summary. People with genetic haemochromatosis (GH) accumulate iron from excessive dietary absorption. In populations of northern European origin, over 90% of patients are homozygous for the C282Y mutation of the *HFE* gene. While about 1 in 200 people in the general population have this genotype the proportion who develop clinical haemochromatosis is not known. The influence of *HFE* genotype on iron status was investigated in 10 556 blood donors. The allele frequencies of the C282Y and H63D mutations were 8·23% and 15·3% respectively. Heterozygosity for C282Y occurred in 1 in 7·9 donors, for H63D in 1 in 4·2 donors, and 1 in 42 were compound heterozygotes. Homozygosity for H63D occurred in 1 in 42 donors and 1 in 147 (72) were homozygous for C282Y. Mean values increased for transferrin saturation (TS) and serum ferritin (sFn), and decreased for unsaturated iron binding capacity (UIBC) in the order: donors lacking the mutations, H63D heterozygotes, C282Y heterozygotes, H63D homozygotes, compound heterozygotes and C282Y homozygotes, but serum ferritin (sFn) concentrations were no higher in H63D heterozygotes and C282Y heterozygous women than in donors lacking mutations. The percentage of donors failing the screening test for anaemia or of those with sFn < 15 μg/l did not differ among the genotype groups. C282Y and H63D heterozygotes and donors homozygous for H63D were at no greater risk of iron accumulation than donors lacking mutations, of whom 1 in 1200 had both a raised TS and sFn. The risk was higher for compound heterozygotes (1 in 80, $P = 0{\cdot}003$) and for C282Y homozygotes (1 in 5, $P < 0{\cdot}0001$). There was no correlation between sFn and either age or donation frequency in C282Y homozygotes. None of the 63 C282Y homozygous donors interviewed showed physical signs of overload or were aware of relatives with haemochromatosis. The Welsh Blood Service collects blood from about 140 000 people each year including an estimated 950 who are homozygous for *HFE* C282Y. They are probably healthy and unaware of any family history of iron overload.

Keywords: blood donation, *HFE* mutations, iron deficiency, iron overload, population screening.

Genetic haemochromatosis (GH) is an autosomal recessive disorder in which those affected accumulate iron as a result of increased absorption from food (Bothwell & MacPhail, 1998). GH, defined in terms of iron accumulation, is common in countries in which the population is largely of northern European origin (Worwood, 1994a). If patients are diagnosed in the precirrhotic stage and treated by phlebotomy to remove the excess iron, life expectancy is normal (Niederau *et al*, 1996). However, once cirrhosis has developed patients have a shortened life expectancy and a high risk of liver cancers, even when iron depletion has been achieved.

Correspondence: Professor Mark Worwood, Department of Haematology, University of Wales College of Medicine, Cardiff CF14 4XN, UK. E-mail: worwood@cardiff.ac.uk

In 1996, the *HFE* gene was located on the short arm of chromosome 6 about 5 Mb telomeric to the human leucocyte antigen (HLA) class I region (Feder *et al*, 1996). It codes for a HLA class I-like protein and over 90% of chromosomes from patients with GH carry a single mutation, C282Y, on this gene (Feder *et al*, 1996). There is convincing functional evidence for the involvement of the *HFE* protein in iron transport across cell membranes, as the *HFE* protein forms a complex with the transferrin receptor and decreases the affinity of the receptor for transferrin (Feder *et al*, 1998; Lebron *et al*, 1998). *HFE* knockout mice develop a similar form of iron overload to humans (Zhou *et al*, 1998). The C282Y mutation abolishes a disulphide bridge, which prevents binding to β_2 microglobulin and cell-surface expression (Feder *et al*, 1997). A second mutation, H63D, is common in the general population, but may cause

iron accumulation if present with the C282Y mutation (Feder *et al*, 1996). In Europe, Australia and the USA, from 60% to 100% of patients with GH are homozygous for the C282Y mutation of the *HFE* gene (Worwood, 1999). In the general population, the C282Y mutation frequency varies from 0·5% in Italy to over 9% in Ireland and parts of Brittany. The mutation is not found in non-European populations. The H63D mutation is more widespread and in Europe the frequency is 12–15% (Merryweather-Clarke *et al*, 2000). In the UK, over 90% of patients with GH are homozygous for the C282Y mutation of the *HFE* gene and another 4% are compound heterozygotes (The UK Haemochromatosis Consortium & Worwood, 1997). Other forms of non-*HFE*-related haemochromatosis include those patients with clinical haemochromatosis who lack detectable mutations in the *HFE* gene and any HLA haplotype association (Pietrangelo *et al*, 1999; Roetto *et al*, 2001).

On first investigation, most homozygous relatives of a haemochromatosis patient show evidence of iron accumulation (a raised transferrin saturation; TS) and about 50% of men and a lower proportion of women display at least one of the clinical manifestations of haemochromatosis: hepatomegaly, abdominal pain, skin pigmentation, weight loss, fatigue, arthropathy, hypogonadism, impotence, liver disease and cirrhosis (Bradley *et al*, 1996).

There are now several reports about clinical penetrance in subjects homozygous for C282Y detected by genetic testing. Olynyk *et al* (1999) found similar figures for penetrance in a population-based study of 3011 unrelated, white adults in Busselton, Australia, where homozygotes for haemochromatosis were detected by genetic testing. However, other population-based studies of both blood donors (Adams *et al*, 2000) and non-blood donors (Beutler *et al*, 2000) have revealed a lower clinical penetrance for homozygosity for C282Y.

Early population studies suggested an incidence of 1 in 5000 to 1 in 10 000 for clinical haemochromatosis (Sheldon, 1935; Finch & Finch, 1955). In the island of Jersey, where the C282Y allele frequency is 8·4% (Merryweather-Clarke *et al*, 1998), about 1 in 140 people would be homozygous for C282Y yet the population prevalence for clinical haemochromatosis was 1 in 4700. Haemochromatosis or iron overload was mentioned in about 1 in 6000 death certificates in the USA from 1979 to 1992 (Yang *et al*, 1998). The frequency of heterozygosity and homozygosity for C282Y was not significantly increased in patients with type II diabetes (Braun *et al*, 1998; Frayling *et al*, 1998; Florkowski *et al*, 1999) and liver disease (Bhavnani *et al*, 2000; Willis *et al*, 2000). It is therefore already apparent that the majority of C282Y homozygotes are not being detected clinically.

Motulsky (1979) suggested that the haemochromatosis gene may have spread because of a selective advantage – protection of heterozygotes against iron deficiency. About 25% of heterozygotes, identified by HLA typing of family members of haemochromatosis patients, have either a raised TS or a raised serum ferritin (sFn) (Powell *et al*, 1990; Adams, 1994; Bulaj *et al*, 1996). Slightly but significantly higher values for serum iron (sFe) and TS have been found in heterozygotes for either C282Y (Burt *et al*, 1998; Datz *et al*, 1998; Beutler *et al*, 2000) or H63D (Burt *et al*, 1998; Beutler *et al*, 2000) compared with subjects lacking these mutations. The differences in ferritin levels were smaller and not significant. In compound heterozygotes, there are greater differences (Beutler *et al*, 2000; Rossi *et al*, 2000). In heterozygotes for C282Y (Datz *et al*, 1998; Beutler *et al*, 2000) and H63D (Beutler *et al*, 2000), Hb levels were slightly higher than in subjects lacking mutations. Beutler *et al* (2000) have noted a lower prevalence of anaemia among women carrying either mutation, but the differences are small and only significant if all subjects carrying mutations were compared with those lacking mutations. Such changes may indicate an increase in iron absorption that might permit C282Y heterozygotes to donate blood more frequently than donors without the mutation.

We have studied the effect of *HFE* mutations on iron status by examining *HFE* genotypes and measures of iron status in blood donors. Blood donors are relatively young and fit and this largely removes the influence of disease on measures of iron status. By studying large numbers, the dietary influences on genotype are equalized. Blood donors also have greater iron losses as a result of donation of blood. However, this additional iron loss can be quantified along with the number of times that a donor has not been permitted to give blood because of possible anaemia.

SUBJECTS AND METHODS

Subjects. Blood samples were collected during a period of 7 months beginning in July 1998. People attending Welsh Blood Service sessions in South Wales at which more than 100 donations were expected were invited to take part in the study. A donor assistant offered an information sheet to those attending and invited them to sign the consent form. The assistant was available to answer questions about the project but had no knowledge of the medical or donation histories of the potential donors. Samples were available from all potential donors who signed the consent form, including those who failed the 'copper sulphate' screening test for anaemia and were therefore deferred as blood donors. After the session, samples corresponding to the completed consent forms were identified and both blood and serum were stored in microtitre plates (1 ml well volume) at −20°C. The protocol was approved by the Bro Taf Research Ethics Committee. Donors were able to withdraw their consent for testing at any time. Only donors homozygous for the *HFE* C282Y mutation (or who had both abnormal TS and sFn results) were informed of the genotype result. These donors were contacted and offered an interview with one of the authors in order to provide counselling, confirm the test results, provide treatment if necessary and advice on family testing. If results were not obtained from a blood sample, for any reason, this was explained to the donor in a letter and he or she was invited to attend a donor session to give another sample. Results were made available to any other donors requesting this information.

Genotyping. DNA was prepared using the Dynabead DNA Direct System 1 (Dynal, UK) and samples were genotyped

using the polymerase chain reaction–single-stranded polymorphism (PCR–SSP) method of Guttridge *et al* (1998). This single-step method does not use the reverse primer for C282Y recently implicated in possible mistyping (Jeffrey *et al*, 1999; Merryweather-Clarke *et al*, 1999) and also permits detection of 63D and 282Y in *cis*-linkage. 63H and 282Y did not occur on the same chromosome. For donors homozygous for *HFE* C282Y, results were confirmed on a second sample by heteroduplex analysis (Worwood *et al*, 1999). There were no discrepancies.

Iron and ferritin assays. Serum iron (sFe) concentrations and unsaturated iron binding capacity (UIBC) were determined by a microtitre plate version (Worwood, 2001) of the method of Persijn *et al* (1971). The total volume required was 160 μl. The total iron binding capacity (TIBC) was calculated as the sum of sFe and UIBC and the percentage TS as the sFe/TIBC × 100. Serum ferritin (sFn) concentrations were determined as described by Worwood (1995). Data from the microtitre plate reader were transferred to a PC for calculation of sFe and UIBC using Minitab (v 11).

Serum samples were added directly to microtitre plates from the storage trays using a multichannel pipette. Fifty-five assays for sFe, UIBC and sFn were carried out over a period of 3 months. Each batch of assays for sFe and UIBC consisted of a plate containing standards and control preparations in quadruplicate and three microtitre plates containing serum samples (assayed singly). For ferritin assays, the first plate included standards and two control sera (in duplicate) followed by up to three plates containing samples (assayed singly). Intrabatch variation was determined using a control serum from a patient with genetic haemochromatosis undergoing therapeutic phlebotomy. This provided an estimate of imprecision close to the diagnostic thresholds for iron overload. The sFe concentration was 27·1 ± 1·4 μmol/l (mean ± SD, $n = 96$), the UIBC 20·9 ± 1·0 μmol/l ($n = 96$), the TIBC was 48·0 ± 1·6 μmol/l and the TS 56·5 ± 1·9%. The coefficient of variation (CV) for sFe was 5·2%, for UIBC 4·8%, for TIBC 3·3% and for TS 3·4%. At a ferritin concentration of 130 μg/l, the intrabatch CV was 7·8% ($n = 96$). Interbatch variation was assessed using Biorad Lyphochek, assayed clinical chemistry control 1, for sFe, UIBC and TS. For 55 assays the mean ± SD for sFe was 36·5 ± 2·0 μmol/l, UIBC 10·8 ± 2·9 μmol/l and TS 77·3 ± 5·1%. The respective values for CV were 6·5%, 26·9% and 6·6%. For control 2, sFe was 12·1 ± 1·5 μmol/l, UIBC 21·6 ± 3·0 μmol/l and TS 36·0 ± 4·3%. The respective values for CV were 12·4%, 13·9% and 11·9%. A pooled, local control and Biorad Anaemia Control were assayed for sFn. Mean values were 176 ± 17·6 and 9·4 ± 1·7 μg/l (CV 10·0 and 18·1%).

Statistics. Statistical calculations including multiple, linear regression analysis were made using Microsoft Excel and Minitab v 11. sFe, UIBC, TIBC and TS were expressed as mean ± SD and differences between groups of subjects evaluated using the two-sample *t*-test. As serum ferritin concentrations were not normally distributed, results were expressed as median and 95% range, and differences between groups were evaluated using the Mann–Whitney test. Differences were regarded as significant if $P < 0·05$. For the genotype comparisons, differences were regarded as significant if $P < 0·01$ (using the Bonferroni correction for five comparisons). Differences in frequency of iron overload for the various genotypes were assessed using Fisher's exact test.

RESULTS

HFE genotypes

Almost all donors approached agreed to take part in the study. There were 10 556 samples available for genotyping – 5470 from women and 5086 from men. The C282Y mutation frequency was 8·23% and the H63D mutation frequency 15·2%. Genotype frequencies for all donors are given in Table I. The observed distribution of genotypes did not differ from the expected distribution (Hardy–Weinberg equilibrium). Frequencies were not significantly different for men and women. When donors were divided into age-groups (< 31, 31–50, > 50 years), there were no significant differences in genotype frequencies for men or women. When genotype frequencies were calculated according to postcode (Newport, NP; Cardiff, CF; Swansea, SA), there were no differences in frequency.

Age, blood donation, iron status and HFE genotype

For 10 379 of these donors an adequate serum sample was available for analysis. The mean age of the 5005 men was greater than for the 5374 women tested (37·8 ± 11·5 years and 35·7 ± 11·5 years, mean ± SD, $P < 0·0001$). There were no significant differences between the mean ages for any genotype group and the mean for all donors. Men had given a mean of 2·7 ± 2·0 units of whole blood in the 3 years prior to testing and women 2·1 ± 1·8 units ($P < 0·0001$). There were no significant differences in donation frequency between the genotypes except for the men homozygous for C282Y who had given a mean of 3·7 ± 1·9 units in the previous 3 years (compared with all men, $P < 0·01$). This is partly explained by the observation that only one of these (3%) was a first-time donor compared with 12% of all male donors. However, even after excluding first-time donors, the difference remains significant (all men, 3·1 ± 1·9; C282Y homozygotes 3·8 ± 1·8 units/3 years; $P < 0·05$). Serum iron, TS and sFn concentrations were

Table I. *HFE* genotypes in blood donors ($n = 10556$).

Genotype*	Number of donors	% of donors
HHCC	6152	58·3
HDCC	2488	23·6
HHCY	1342	12·7
DDCC	249	2·4
HDCY	253	2·4
HHYY	72	0·68

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc.

Table II. Iron status and genotype in male blood donors (mean ± SD).

Genotype* Number of donors (%)	SFe (μmol/l)	UIBC (μmol/l)	TIBC (μmol/l)	TS (%)	sFn (μg/l)†
All donors: 4952 (100)	16·7 ± 6·4	39·2 ± 11·2	55·8 ± 10·6	30·0 ± 12·1	81 (24–196)
HHCC: 2896 (58·5)	16·0 ± 6·1	40·8 ± 11·0	56·8 ± 10·6	28·3 ± 11·0	79 (23–193)
HDCC: 1167 (23·6)	17·4 ± 6·5‡	38·2 ± 10·5‡	55·6 ± 10·4§	31·8 ± 11·7‡	81 (23–197)
HHCY: 644 (13·0)	17·3 ± 6·0‡	35·8 ± 10·0‡	53·1 ± 10·0¶	33·1 ± 11·5‡	82 (29–201)¶
DDCC: 105 (2·1)	19·1 ± 6·9‡	34·4 ± 10·5‡	53·5 ± 10·1§	36·4 ± 13·1‡	99 (33–210)‡
HDCY: 111 (2·2)	20·2 ± 6·9‡	31·2 ± 12·1‡	51·4 ± 11·6‡	40·5 ± 15·5‡	107 (24–260)‡
HHYY: 29 (0·59)	27·5 ± 9·9‡	15·6 ± 8·4‡	43·1 ± 8·3‡	63·6 ± 17·8‡	154 (50–410)‡

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc.
†Median (95% range). Mann–Whitney test for significance.
Significance of difference from wild-type donors ‡$P < 0{\cdot}0001$, §$P < 0{\cdot}001$, ¶$P < 0{\cdot}01$.

significantly higher in men than in women (Tables II and III) and the UIBC was lower ($P < 0{\cdot}0001$). For men there was a positive correlation between TS and sFn ($r = 0{\cdot}119$, $P < 0{\cdot}0001$) and a negative correlation between UIBC and sFn ($r = 0{\cdot}60$, $P < 0{\cdot}0001$). For women the corresponding values were 0·237 ($P < 0{\cdot}0001$) and 0·258 ($P < 0{\cdot}0001$).

Tables II and III also summarize measures of iron status for donors of each genotype. There were significant differences for sFe, UIBC, TIBC and TS between mean values for donors lacking mutations and all other genotype groups. sFn also differed except that the median value for H63D heterozygotes was not significantly different from donors lacking mutations and C282Y heterozygous women did not have a higher, median sFn than women lacking mutations. Serum ferritin concentrations increased with age and decreased with donation frequency in all genotype groups except C282Y homozygotes and female H63D homozygotes (Tables IV and V). The decrease in sFn with donation frequency is shown in Fig 1 for men lacking mutations.

The percentage of donors failing the copper sulphate screening test for anaemia during the 3 years preceding testing or with a sFn less than 15 μg/l at the time of testing was calculated. Only donors who had given at least 2 units of blood were included. Overall 16·3% of women failed the screening test and 4·5% of men. At the time of testing, 8% of women and 0·9% of men had a sFn less than 15 μg/l. There were no significant differences in frequencies between donors lacking *HFE* mutations and any other genotype groups for both men and women.

First-time donors

First-time donors comprised 12% of all male donors and 16% of women. They were younger than the whole group. For men, the mean age was 28·3 ± 10·5 years, compared with 37·8 ± 11·5 years for all men ($P < 0{\cdot}0001$). For women, the mean age was 26·8 ± 10·5 years years compared with 35·7 ± 11·5 ($P < 0{\cdot}0001$). Among both men and women sFe, UIBC, TIBC, TS and sFn did not differ between C282Y heterozygotes, H63D heterozygotes and donors lacking mutations (Tables VI and VII). In men, the mean UIBC was significantly lower than in donors lacking mutations for homozygotes for H63D and compound heterozygotes. Mean TS was also significantly higher in the latter group. In women, mean values for UIBC were significantly lower in homozygotes for H63D and all values were significantly different from donors lacking mutations for compound heterozygotes and homozygotes for C282Y.

Variation with age for the measures of iron status was

Table III. Iron status and genotype in female blood donors (mean ± SD).

Genotype* Number of donors (%)	SFe (μmol/l)	UIBC (μmol/l)	TIBC (μmol/l)	TS (%)	sFn (μg/l)†
All donors: 5373 (100)	14·3 ± 6·3	42·9 ± 12·9	56·8 ± 11·2	25·4 ± 12·4	44 (9–130)
HHCC: 3100 (57·7)	13·8 ± 6·1	44·2 ± 12·1	58·1 ± 11·4	24·6 ± 10·9	44 (9–126)
HDCC: 1277 (23·8)	14·7 ± 6·2§	41·5 ± 12·2‡	56·2 ± 10·9‡	27·0 ± 12·0‡	43 (10–127)
HHCY: 682(12·7)	14·6 ± 6·1¶	40·7 ± 12·5§	55·1 ± 11·5‡	27·3 ± 12·1‡	44 (8–116)
DDCC: 135 (2·5)	15·6 ± 7·1§	38·4 ± 11·8‡	54·0 ± 11·1‡	29·5 ± 13·8‡	46·5 (11–140)
HDCY: 138 (2·6)	16·6 ± 6·8‡	34·2 ± 10·5‡	50·8 ± 8·6‡	33·2 ± 14·0‡	50 (10–180)¶
HHYY: 41 (0·76)	21·4 ± 8·4§	23·4 ± 12·6‡	44·3 ± 10·4‡	49·9 ± 20·5‡	65 (8–238)§

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc.
†Median (95% range). Mann–Whitney test for significance.
Significance of difference from wild-type donors ‡$P < 0{\cdot}0001$, §$P < 0{\cdot}001$, ¶$P < 0{\cdot}01$.

Table IV. Multiple regression analysis, sFn with age and donation frequency in males.

Genotype*	Regression equation	*R*	Significance (*P*)	
			Age (years)	Number of donations in 3 years
HHCC	Fn = 85·4 + 0·662 age−8·80D	0·371	< 0·0001	< 0·0001
HDCC	Fn = 87·4 + 0·673 age−8·57D	0·351	< 0·0001	< 0·0001
HHCY	Fn = 90·5 + 0·682 age−8·87D	0·371	< 0·0001	< 0·0001
DDCC	Fn = 109 + 0·543 age−9·93D	0·313	< 0·0001	< 0·0001
HDCY	Fn = 83·5 + 1·80 age−13·2D	0·470	< 0·0001	< 0·0001
HHYY	Fn = 116 + 3·82 age−16·1D	0·110	0·153	0·297

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc; D, units of blood donated in the 3 years prior to testing.

examined in first-time donors. For male, first-time donors there was a small but significant increase in sFn with age ($r = 0{\cdot}379$, $P < 0{\cdot}0001$). There were no significant changes with age for sFe, UIBC, TIBC and TS. The correlation between sFn and age is shown for donors lacking mutations in Fig 2. There were similar correlations for C282Y heterozygotes and H63D heterozygotes. Neither the intercept on the *y*-axis nor the slope of the regression line differed between these three groups. Numbers were small in the other genotype groups but there was a significant increase in TS with age in compound heterozygotes ($r = 0{\cdot}642$, $P = 0{\cdot}018$).

Among first-time, female donors, sFn increased with age ($r = 0{\cdot}255$, $P < 0{\cdot}0001$) and sFe decreased ($r = 0{\cdot}112$, $P = 0{\cdot}0009$). There were also small decreases for UIBC ($r = 0{\cdot}115$, $P = 0{\cdot}0006$) and TIBC ($r = 0{\cdot}186$, $P < 0{\cdot}0001$), but not for TS. Serum ferritin concentrations increased with age for donors lacking mutations (sFn = 30·6 + 0·834 age, $r = 0{\cdot}262$, $P < 0{\cdot}001$), and in C282Y heterozygous and H63D heterozygous women. There were no significant differences in intercept on the *y*-axis or slope of the regression line. We did not find the expected correlations between age and sFn for compound heterozygotes, H63D homozygotes and C282Y homozygotes, possibly because of the small numbers in these groups.

HFE genotype and iron accumulation

In order to determine reference ranges for the various measures of iron status in the absence of any influence from blood donation and *HFE* mutations, mean values were calculated for first-time donors lacking mutations. Although iron and ferritin concentrations are expected to increase in women after the menopause, only 19 of the 540 women were over the age of 50 years and these donors were not excluded. Upper limits (or in the case of the UIBC, lower limits) have been determined for the various measures of iron status. For sFe, UIBC and TS this value was 1·96SD from the mean. For sFn, the 97·5 percentile is given. The limits were sFe > 28 μmol/l, UIBC < 20 μmol/l, TS > 50% and sFn > 210 μg/l (men) or 130 μg/l (women).

In Tables VIII and IX, the number of donors with sFe, TS and sFn values above the reference range, or UIBC values below the reference range, are shown. There were only 14 men (eight homozygous for C282Y) and 16 women (seven homozygous for C282Y) with both high TS and sFn. The age distribution among C282Y homozygous donors was the same as that for all donors, but there was no correlation between sFn and age or donation frequency in C282Y homozygotes in contrast to other donors (Tables IV and V). TS declined with age for the 10 female, first-time donors (linear regression analysis: TS = 80·5–0·943 age, $r = 0{\cdot}61$, $P = 0{\cdot}035$).

Table V. Multiple regression analysis, sFn with age and donation frequency in women.

Genotype*	Regression equation	*R*	Significance (*P*)	
			Age (years)	Number of donations in 3 years
HHCC	Fn = 36·7 + 0·534 age−2·95D	0·202	< 0·0001	< 0·0001
HDCC	Fn = 36·1 + 0·541 age−2·74D	0·205	< 0·0001	< 0·0001
HHCY	Fn = 36·4 + 0·526 age−2·70D	0·197	< 0·0001	< 0·0001
DDCC	Fn = 35·8 + 0·602 age−1·18D	0·055	0·127	0·636
HDCY	Fn = 33·5 + 1·20 age−7·62D	0·311	< 0·0001	< 0·002
HHYY	Fn = 38·3 + 1·93 age−91D	0·176	0·093	0·226

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc; D, units of blood donated in the 3 years prior to testing.

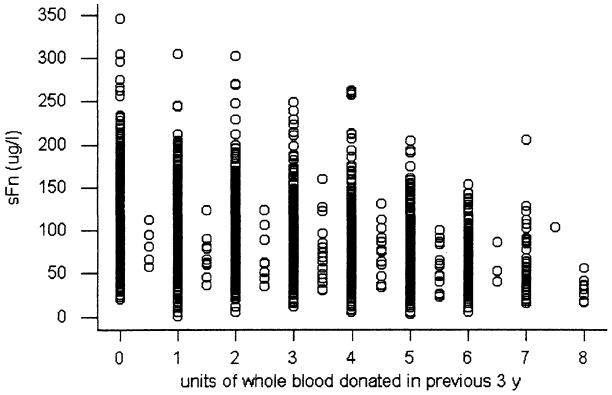

Fig 1. sFn concentration and blood donation frequency in 2944 male donors lacking *HFE* mutations. sFn = 106–7·33D ($r = 0{\cdot}336$, $P < 0{\cdot}001$), in which D = number of units of blood donated in the previous 3 years.

Males who were homozygous for C282Y had donated a total of 15 ± 13 units of blood (mean ± SD). If it was assumed that blood was donated regularly since the age of 18 years, they had given 0·8 ± 0·5 units of blood/year. Female donors homozygous for C282Y had given 6·4 ± 7·5 units in total (0·4 ± 0·5 units/year). Only 2 of the 63 homozygous donors interviewed to date had possible, early symptoms of iron overload. Both were female: one with arthralgia and one with abdominal pain. None showed physical signs of overload and none were aware of relatives with haemochromatosis. There were three female donors homozygous for C282Y with sFn < 15 μg/l. There was only one donor with evidence of iron overload (TS > 50% and sFn > 300/200 μg/l, men/women) who was not homozygous for *HFE* C282Y (a woman homozygous for H63D with TS 63% and sFn 442 μg/l). On repeat testing, her iron status was normal.

DISCUSSION

About 1 in 7 donors carried the C282Y mutation and 1 in 4 the H63D mutation. These frequencies are among the highest in Europe. While donors were not asked about ethnic origin, the ethnic communities are known to be under-represented. Within the area included in the survey, about 3·0% belong to ethnic minorities and, of these, nearly 67% are Asian, Black or of Mediterranean origin (Hogg & Modell, 1998; personal communication Dr A. May). The C282Y mutation of the *HFE* gene is rarely found in non-European populations and is uncommon in Southern Italy and Greece. The H63D allele is found in Mediterranean populations at a similar frequency to that among northern Europeans, but elsewhere its frequency is lower although variable (Merryweather-Clarke *et al*, 2000). As less than 3% of blood donors were from the ethnic minorities, the frequencies given above relate to the population of Northern European origin.

Serum iron and ferritin concentrations are influenced by age, sex, diet and disease, as well as by biological variation within an individual and by methodological variation (Worwood, 1994b). In the case of blood donors, the frequency of blood donation also needs to be considered (Milman & Sondergaard, 1984). Although there is diurnal variation of serum iron concentration and blood was taken between 10·00 and 18·00 h, samples were assayed in order of collection and not according to sex or genotype, so methodological and biological variation should be similar for all genotypes.

There were no differences in age or donation frequency between the genotype groups except among the male, C282Y homozygotes who had given significantly more blood than other donors. Generally sFe, TS and sFn increased, and UIBC and TIBC decreased, in the order: wild type, H63D heterozygotes, C282Y heterozygotes, H63D homozygotes, compound heterozygotes and C282Y homozygotes. Despite the statistical significance of these differences in iron status, they are small and only the mean UIBC and TS in the male C282Y homozygotes lie outside the normal range established for first-time blood donors.

For female donors, sFn did not differ between wild type, C282Y or H63D heterozygotes and donors homozygous for H63D. Although this may indicate that there is little change in total body iron stores, it may also reflect a failure of sFn to respond to an increase in iron concentration in hepatic

Table VI. Iron status in first-time, male blood donors (mean ± SD).

Genotype* Number of donors	SFe (μmol/l)	UIBC (μmol/l)	TIBC (μmol/l)	TS (%)	sFn (μg/l)†
All donors:612	16·7 ± 6·0	37·7 ± 9·9	54·2 ± 10·0	31·1 ± 10·9	101 (35–220)
HHCC: 376	16·2 ± 5·9	38·6 ± 9·6	54·8 ± 11·1	29·7 ± 10·2	101 (36–212)
HDCC: 135	17·5 ± 6·0	37·8 ± 11·4	55·3 ± 10·1	31·6 ± 11·1	98 (35–216)
HHCY: 74	16·7 ± 6·0	35·0 ± 10·4	51·8 ± 9·6	32·1 ± 12·2	117 (47–217)
DDCC: 12	20·4 ± 8·0	31·8 ± 11·0§	52·1 ± 9·0	39·6 ± 15·9	109 (41–177)
HDCY: 14	19·3 ± 3·8	28·4 ± 9·1‡	47·6 ± 7·9	41·6 ± 12·8‡	130 (63–230)
HHYY: 1	10·0	25·3	35·3	28·4	149

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc.
†Median (95% range). Mann–Whitney test for significance.
‡$P < 0{\cdot}001$, §$P < 0{\cdot}01$ compared with wild-type (HHCC) genotype.

Table VII. Iron status in first time, female blood donors (mean ± SD).

Genotype* Number of donors	SFe (μmol/l)	UIBC (μmol/l)	TIBC (μmol/l)	TS (%)	sFn (μg/l)†
All donors: 890	14·3 ± 6·7	42·7 ± 13·2	56·7 ± 12·1	25·5 ± 12·9	47 (9–136)
HHCC: 540	13·9 ± 6·8	43·9 ± 12·2	57·9 ± 11·9	24·2 ± 12·4	48 (9–136)
HDCC: 200	14·6 ± 6·1	41·7 ± 12·9	56·3 ± 11·7	26·7 ± 12·2	45 (9–123)
HHCY: 100	14·7 ± 6·6	42·2 ± 13·1	57·2 ± 13·2	25·6 ± 12·1	45 (7–123)
DDCC: 15	13·4 ± 6·5	33·9 ± 10·3¶	52·1 ± 9·0	28·7 ± 13·6	53 (15–106)
HDCY: 26	17·2 ± 5·6§	31·6 ± 9·6§	47·6 ± 7·9‡	34·5 ± 14·7§	66¶ (22–199)
HHYY: 10	24·7 ± 8·6§	18·8 ± 7·1§	43·5 ± 7·0‡	56·4 ± 15·1§	101¶ (41–202)

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc.
†Median (95% range). Mann–Whitney test for significance.
‡$P < 0{\cdot}0001$, §$P < 0{\cdot}001$, ¶$P < 0{\cdot}01$ compared with wild-type (HHCC) genotype.

parenchymal cells in the early stages of iron accumulation, when iron concentrations in reticuloendothelial cells are not increased (Worwood, 1990). Edwards *et al* (2000) summarized reports of liver iron concentration in subjects heterozygous for GH. Despite increases in liver iron concentrations to about the upper limit of normal, serum ferritin levels remained within the normal range.

Storage iron levels are influenced more by the frequency of donation rather than by the total number of units given (Milman & Søndergaard, 1984) and, in both men and women, sFn decreased with the number of units of blood given in the 3 years prior to testing. Storage iron levels were calculated by assuming that 1 μg of sFn is equivalent to 8 mg of storage iron in the body (Walters *et al*, 1973). Median iron stores for all donors were 0·65 g for men and 0·35 g for women. Among men with only wild-type alleles, giving 3 units of blood, equivalent to 720 mg of Fe, in the previous 3 years caused a reduction in the level of storage iron of about 200 mg compared with those who had not donated during the period. This is a result of the increased iron absorption that is stimulated by blood donation (Garry *et al*, 1995). The effect of blood donation on sFn concentration was similar in donors lacking *HFE* mutations and heterozygous donors.

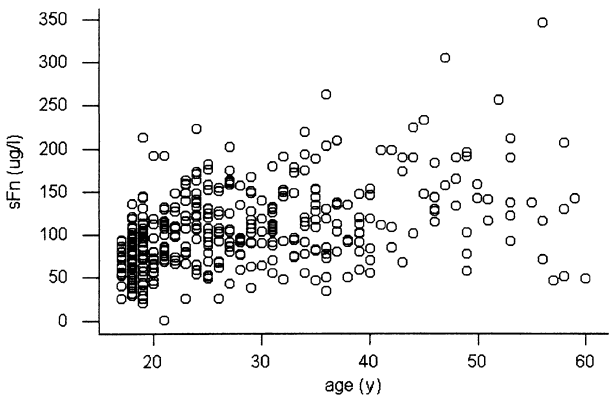

Fig 2. sFn concentration and age in 376 male, first-time donors lacking *HFE* mutations. sFn = 52·7 + 1·90 (age). $r = 0{\cdot}414$, $P < 0{\cdot}0001$.

If heterozygosity for C282Y provides young women with protection against iron deficiency anaemia then it was not apparent in our study. Serum ferritin concentrations did not vary between heterozygotes and wild-type subjects and the proportion of donors with sFn concentrations < 15 μg/l did not differ. The proportion of donors failing the copper sulphate screening test for anaemia was not decreased by heterozygosity for C282Y. Thus, there was little indication that heterozygotes for C282Y will be able to give blood more frequently than donors not carrying this mutation. However, this finding is not in agreement with Beutler *et al* (2000), who noted a small but significant reduction in the prevalence of anaemia among subjects carrying *HFE* mutations compared with those lacking mutations.

If storage iron levels are not increased in C282Y heterozygotes, any association between heterozygosity for *HFE* C282Y and disease (Dorak *et al*, 1999; Maclean *et al*, 1999; Roest *et al*, 1999; Tuomainen *et al*, 1999) may be related to the subtle changes in plasma iron and iron binding capacity. These changes may also explain the strong association between both heterozygosity and homozygosity for H63D in Italian patients with sporadic porphyria cutanea tarda (Sampietro *et al*, 1998) in which the C282Y mutation is rare. They may also be related to the increase in non-transferrin-bound iron recently reported in subjects heterozygous for C282Y (de Valk *et al*, 2000).

Approximately 1 in 1200 (90% CI 1 in 690 to 1 in 4545) donors lacking mutations and H63D heterozygotes had both a raised TS and sFn (based on the thresholds established for first-time donors lacking mutations). For C282Y heterozygotes, 1 in 437 (90% CI 1 in 225 to 1 in 7692) had raised levels. For donors homozygous for H63D, 1 in 200 (1 in 90 to > 1 in 10 000) had raised values for TS and sFn. These donors are not at significant risk of iron overload compared with donors lacking mutations (Fisher's exact test, $P > 0{\cdot}05$). For compound heterozygotes, about 1 in 80 (90% CI 1 in 42 to 1429) had raised levels ($P = 0{\cdot}003$). For C282Y homozygosity, about 1 in 4 male donors and 1 in 5 female donors had raised levels (1 in 4·6 90% CI 3·3 to 7·4

Table VIII. Number of male blood donors (%) with abnormal sFe and sFn levels.

Genotype*	Number of donors**	sFe (> 28 μmol/l)	UIBC (< 20 μmol/l)	TS (> 50%)	sFn (> 210 μg/l)	TS + sFn
HHCC	2896	119 (4·1)	43 (1·5)	103 (3·6)	38 (1·3)	2
HDCC	1152	72 (6·3)	34 (3·0)	70 (6·1)	15 (1·3)	1
HHCY	639	28 (4·4)	29 (4·5)	44 (6·9)	13 (2·0)	1
DDCC	104	8 (7·7)	9 (8·7)	12 (11·5)	3 (2·9)	0
HDCY	108	13 (12)	15 (14)	24 (22)	5 (4·5)	2
HHYY	29	9 (31)	22 (76)	25 (86)	10 (34)	8

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC, etc.
**Numbers differ slightly from Table II as donors were only included if all assay values were available.

$P < 0{\cdot}0001$). It should also be noted that the threshold for sFn was not the usual upper limit of normal taken for diagnostic purposes and even fewer donors exceeded 300 μg/l (male) and 200 μg/l (female). Heterozygotes and donors homozygous for H63D appear to be at little risk from iron overload and, for compound heterozygotes, the risk is an order of magnitude less than for C282Y homozygotes.

The UIBC has been proposed as a cheap and rapid screening test for iron overload (Pintar *et al*, 1981; Adams *et al*, 2000). In this survey the UIBC appears to have a similar sensitivity and specificity to TS.

The mean age of the C282Y homozygous men was 38 years, reflecting that for all donors, only 8 years younger than the mean age of 46 years at diagnosis for 251 patients (90% men) reported by (Niederau *et al*, 1996). In a group of 200 patients (10% of whom were women), responding to a questionnaire, the mean age of onset of symptoms was only 41 years, even though a diagnosis of GH was not usually made for several more years (McDonnell *et al*, 1999). Bassett *et al* (1984) studied 34 relatives of patients with GH who shared the same HLA haplotypes. All were under 35 years of age and were classified by HLA typing as homozygous for haemochromatosis. For men and women the median TS was 87% and 67%, respectively, and was 450 μg/l and 320 μg/l for sFn. A median of 7 g of iron was removed by phlebotomy. Initial sFn correlated with iron stores determined by phlebotomy ($r = 0{\cdot}92$) with the same relationship between storage iron (determined by quantitative phlebotomy) and sFn as that found in normal subjects by Walters *et al* (1973). At the time of diagnosis, the patients studied by Niederau *et al* (1996) had accumulated $21{\cdot}2 \pm 1{\cdot}1$ g (SEM) of iron. If storage iron is estimated by assuming that 1 μg of serum ferritin is equivalent to 8 mg of storage iron (Walters *et al*, 1973), the median level of storage iron in our study was 1·23 g for male blood donors homozygous for C282Y and 0·52 g for female donors. Median iron stores, calculated in the same way for all donors, were 0·65 and 0·35 g respectively. There was thus an order of magnitude difference in the level of iron stores between blood donors homozygous for C282Y and patients presenting with clinical symptoms of GH or homozygotes found by screening the families of relatives. This is unlikely to be solely as a result of the age difference.

Are the low iron stores a result of blood donation? While regular blood donation reduces the level of storage iron, there is partial compensation because of the increase in iron absorption (Garry *et al*, 1995). Among men lacking *HFE* mutations, donating 3 units of blood in 3 years caused a mean reduction of 200 mg in iron stores, although donation losses were about 700 mg. However, among those homozygous for C282Y, there was no relationship between donation frequency and sFn. An enhanced rate of iron absorption in C282Y homozygotes may ensure that iron losses as a result of donation are more fully

Table IX. Number of female blood donors (%) with abnormal sFe and sFn levels.

Genotype*	Number of donors**	sFe (> 28 μmol/l)	UIBC (< 20 μmol/l)	TS (> 50%)	sFn (> 130 μg/l)	TS + sFn
HHCC	3057	68 (2·2)	35 (1·1)	68 (2·2)	43 (1·4)	3
HDCC	1262	37 (2·9)	32 (2·5)	54 (4·3)	19 (1·5)	2
HHCY	673	21 (3·1)	18 (2·7)	31 (4·6)	11 (1·6)	2
DDCC	134	5 (3·7)	6 (44)	11 (8·2)	4 (3·0)	1
HDCY	137	5 (3·6)	4 (2·9)	16 (11·7)	7 (5·1)	1
HHYY	40	8 (20)	20 (50)	18 (45)	9 (22)	7

*Wild type, HHCC; homozygous C282Y, HHYY; homozygous H63D, DDCC; etc.
**Numbers differ slightly from Table III as donors were only included if all assays were available.

compensated (Lynch *et al*, 1989), thus explaining this lack of relationship. An intriguing finding of ours is that male donors homozygous for C282Y had donated significantly more blood in the 3 years before testing than all donors (3·7 units and 2·8 units). While this observation is of interest, particularly because there may be an underlying biological explanation, it should be noted that Barton *et al* (1999) found that voluntary blood donation before diagnosis did not decrease the severity of iron overload.

Subjects homozygous for haemochromatosis might be excluded as blood donors either because they do not feel well or because they are receiving medical treatment. There is no evidence to support exclusion of C282Y homozygotes. Based on overall allele frequencies for men and women, we expected to find 34 men and 42 women homozygous for C282Y. There were 30 men and 42 women discovered by genetic testing. Furthermore, the age distribution was the same as that for all donors.

The C282Y genotype is present in 90% of patients with haemochromatosis and is strongly associated with iron accumulation in blood donors. Among first-degree relatives of patients with clinical haemochromatosis, approximately 50% of subjects homozygous for *HFE* C282Y eventually develop clinical complications of haemochromatosis (Bradley *et al*, 1996). A similar penetrance was reported by Olynyk *et al* (1999) among randomly selected subjects discovered by genetic testing of the adult population of Busselton, Australia. In other surveys, however, in which subjects have been identified as homozygous for C282Y by genetic testing, the penetrance has been lower (Adams *et al*, 2000; Beutler *et al*, 2000). Within the region served by the Welsh Blood Service, about 140 000 people give blood each year, of whom about 950 will be homozygous for C282Y. Our data suggest that the great majority of these are probably healthy subjects unaware of any family history of iron overload. Locally, the clinical burden appears to be low. Cardiff and the Vale of Glamorgan has a population of 434 000 and is part of the region covered by our survey. The data from the current survey reveals that 1 in 150 people are homozygous for C282Y. If all men over 50 years of age have clinical manifestations of iron overload, there should be 480 men with GH. We used the ICD10 classification (haemochromatosis E83·1) to identify patients from hospital records and validated the list of patients from local knowledge and laboratory records. There were 31 patients (23 men) resident in the area and being treated in the two district general hospitals for the years 1998–2000 (unpublished observations).

Prior to the discovery of the *HFE* gene, Edwards *et al* (1988) surveyed 11 065 blood donors from Utah, USA. The Utah donors were of similar mean age to the donors from South Wales and they had similar mean values for non-fasting TS (6·2% of Utah donors with TS $>$ 50% compared with 4·2% for South Wales). Within this Utah population of donors, 18 men and eight women were classified as homozygous for GH on the basis of a fasting TS ($>$ 62%), liver biopsy and family studies. As women were clearly under-represented, the male data were used to calculate the frequency of the GH genotype to be 0·0045 (1 in 222). Unfortunately, data for the frequency of *HFE* mutations are not yet available for the Utah population. In South Wales, the frequency of homozygosity for *HFE* C282Y was 0·0068. At the time of testing, 25/29 men and 18/41 women had a TS $>$ 50%. This confirms that most men homozygous for C282Y would have been identified in the Utah study and that less than 50% of the women would have been detected. Measuring TS would fail to detect 20% of young men and over 50% of women. In female, first-time donors homozygous for C282Y we found that TS decreased with age, and in people presenting at a health appraisal clinic, average age 58 years, Beutler *et al* (2000) found that only a minority of those homozygous for C282Y had a raised TS. This suggests that measuring TS may be less effective for detecting GH in older people than in people aged 30–50 years, but numbers in both studies were small.

While an alternative detection strategy for haemochromatosis is population screening by genetic testing, it is apparent from our data and that of others that this will identify large numbers of healthy people who are at risk from iron accumulation, yet who may never suffer from the consequences of iron overload. All those detected in this way will need to be counselled and offered a family investigation. Those not requiring immediate treatment require regular testing to detect the onset of iron overload. There is an urgent need to identify the cofactors that convert a relatively benign process of iron accumulation into iron overload, tissue damage and disease in a proportion of those homozygous for *HFE* C282Y. Until these additional risk factors are identified, it is likely that the costs to the individual and community will outweigh the benefits. Population screening for haemochromatosis has been strongly advocated, as the condition is common and preventable by regular blood donation (Allen & Williamson, 2000). However, our study lends support to those advocating a more cautious approach (Haddow & Bradley, 1999).

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