Iron loading and morbidity among relatives of *HFE* C282Y homozygotes identified either by population genetic testing or presenting as patients

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**Abbreviations:** Hb haemoglobin, HH haemochromatosis, MCH mean cell haemoglobin, MCV mean corpuscular volume, PCP physical component score, QOL quality of life survey, sFe serum iron concentration, sFn serum ferritin concentration, TIBC total iron binding capacity, UIBC unsaturated iron binding capacity, TS transferrin saturation.
ABSTRACT

Background and aims: Although most cases of hereditary haemochromatosis are associated with homozygosity for the C282Y mutation of the HFE gene, the clinical penetrance varies and other genes may modify disease expression. If so, relatives from clinically affected families, by inheriting such genes, may accumulate more iron. To seek evidence for this, we compared iron status and morbidity in unselected first-degree relatives of two groups of index cases from South Wales, namely asymptomatic C282Y homozygotes identified by genetic screening of blood donors (n=56) and C282Y homozygous haemochromatosis patients presenting clinically (n=60).

Methods: All participating relatives had a structured interview, clinical assessment and laboratory investigations. Health-related quality of life was measured (SF-36 v2).

Results: 92% of 180 eligible first-degree relatives were interviewed in the “screened” family group and 85% of 143 eligible relatives in the “patient” group. Of 59 relatives homozygous for C282Y, 76% of the men and 32% of the women had the “iron phenotype” (raised transferrin saturation and serum ferritin). Logistic regression modelling of the iron phenotype risk showed that 42% of the initial model deviance could be explained by homozygosity for C282Y, another 6% by life style factors and 6% by being male. Family group membership was not a significant risk factor. Morbidity and SF-36 scores did not differ significantly either between C282Y homozygotes and relatives lacking C282Y, or between C282Y homozygotes from the “screened” and “patient” groups. Serious morbidity (including cirrhosis) was low in both groups of relatives.

Conclusions: HFE C282Y homozygosity has a high penetrance for iron accumulation but a low clinical penetrance. The lack of excess morbidity among C282Y homozygous relatives of index cases who presented clinically suggests that residual unknown genetic or environmental factors do not greatly influence clinical outcome among C282Y homozygotes.
Hereditary haemochromatosis (HH) is an autosomal recessive condition in which too much iron is absorbed from the diet. Undetected, progressive iron accumulation may have serious clinical consequences including arthritis, diabetes, heart disease and cirrhosis. Premature illness and death from the disease can be avoided by phlebotomy, provided treatment begins before cirrhosis has developed.[1] In the UK, over 90% of HH patients are homozygous for the Cys282Tyr (C282Y) mutation of the HFE gene on chromosome 6p.[2][3] Another 4% are compound heterozygotes, with C282Y on one chromosome and a second HFE mutation (mostly His63Asp, H63D) on the other. Rarer causes of HH include mutations within the HAMP [4][5], TFR2 [6], SLC11A3 [7][8][9] and HIV [10] genes.

The availability of effective treatment, the high frequency of the C282Y mutation among individuals of northern European descent [11], and the introduction of a diagnostic test for HFE gene mutations has led some to advocate HH population screening.[12][13] However, recent research-based population screening has revealed that the clinical penetrance of homozygous HFE gene mutations is lower than previously thought. [14][15][16] There is therefore uncertainty and controversy about the overall clinical significance of HFE C282Y homozygosity [17][18][19][20] and the overall cost-effectiveness of genetic screening for HH.

In contrast to C282Y homozygotes identified by population screening, the prevalence of additional disease-predisposing factors may be higher in first-degree relatives of C282Y homozygotes who have presented with clinical symptoms and signs of haemochromatosis. Studies of twins [21] and of several strains of HFE knock-out mice indeed suggest that genetic factors other than HFE play a role in HH aetiology. [22][23] Most C282Y homozygous relatives of HH patients have biochemical evidence of iron accumulation (i.e. a raised level of transferrin saturation), and about 50% of men and a lower proportion of women were reported to manifest at least one clinical feature of the disease. [24][25] Relatives in these families who lacked HFE mutations were not investigated which limits the conclusions that can be drawn. As well as endogenous genetic factors, some exogenous exposures also contribute to the development of serious disease in some patients. These include alcohol [26] and chronic hepatitis C infection.[27]

The aim of the present study was to define further the relative contributions of the HFE gene, genetic influences other than HFE and some exogenous factors upon the iron storage phenotype. To test the possibility that the ancestral haplotype for HFE is associated with more severe iron overload, [28][29][30] microsatellite markers around the HFE gene were analysed in family members. To minimise the possibility of ascertainment bias, we studied first-degree relatives of two groups of index cases, namely C282Y homozygotes ascertained through a genetic screening programme among healthy blood donors, [31] and C282Y homozygous patients who presented with clinical HH. We hypothesised that, if genes other than HFE were of significance in determining the phenotype, then iron overload (i.e. high transferrin saturation and serum ferritin) would be displayed by a higher proportion of relatives of clinically affected index cases than of cases detected by genetic screening.

METHODS

Ascertainment of index cases

The identification of 72 C282Y homozygotes among 10,556 blood donors has been described elsewhere.[31][32] To define the incidence of HH in South Wales, a rigorous search for cases was undertaken in the Bro Taf and Gwent Health Authorities, which serve a population of 1.3 million.[16] Extension of the study to Iechyd Morgannwg Health Authority allowed identification of a total of 69 HH patients who were homozygous for HFE C282Y, and who had been treated by venesection. The study ran from January 1998 to December 1999. Patients
selected as index cases for the current study were those who had presented clinically (rather than by family or population screening) and were confirmed to be C282Y homozygous. All were under 70 years of age, the same age range for the C282Y homozygotes detected by blood donor screening.

**Contacting first-degree relatives**

All prospective index cases were informed by mail about the current project and asked to consider discussing the study with their first-degree relatives (parents and siblings over the age of 18 years), even if they had already been tested for haemochromatosis. Once written consent to contact a family member had been obtained, a letter was sent to the relative in question, outlining the study and inviting their participation. Those agreeing to participate were then interviewed personally by one of us (A.McC.) between July 2001 and December 2002, after written consent was obtained.

**Questionnaire**

All participants answered a structured interview to record their demographic details, family history (including details of deceased first degree relatives), ancestry, medical history and current health status. Enquiry was made into all factors known to affect body iron stores, including details of past and current blood donation or bleeding disorder, iron/vitamin C supplementation and non-steroidal anti-inflammatory drug use. Additional information was sought from females about menstrual history, contraceptive pill use, number of pregnancies, breast-feeding and fertility. Detailed enquiry was made into diet, current and past alcohol consumption, tea and coffee intake, together with full details of their medication use (both prescribed and over the counter).

All participating relatives were invited to complete a validated quality of life (QOL) questionnaire. The SF-36 (v2) health survey, which has been used extensively to compare health status of different groups,[33] was selected because it provides a generic measure of health status and yields quantitative scores, as well as summary scores of physical and mental well-being.

The interview usually took place in the individual’s home, and, in most cases, neither physician nor participant were aware of any genotyping or iron test results. Among 286 first degree relatives, only 13 had been previously diagnosed as having HH. Test results recorded for these cases were the ones taken at presentation (i.e. before venesection). They were encouraged to respond to questions as best they could recall from the periods before or after the diagnosis, as appropriate. The clinical notes for these cases were also scrutinised for inaccuracies arising from recall bias. Any discrepancies were further clarified with the respondent.

**Laboratory analysis**

Serum iron (sFe) and unsaturated iron binding capacity (UIBC) were determined by a microtitre plate assay.[34] Total iron binding capacity (TIBC) and transferrin saturation (TS) were calculated from sFe and UIBC values.[34] Serum ferritin (sFn) levels were measured on an Elecsys 2010 immunoanalyser (Roche). Full blood count and reticulocyte count (Advia 2120) and liver function tests (Aeroset analyser, Abbott Diagnostics) were also arranged.

DNA was extracted from whole blood. *HFE* alleles (wild type, C282Y and H63D) were identified by heteroduplex analysis performed using a capillary electrophoresis system (GeneScan 310, Applied Biosystems, Warrington, UK).[35][36] The “ancestral” haplotype was identified by microsatellite markers around the *HFE* gene.[37] One PCR primer of each reaction was labelled with a fluorescent dye in a multiplex reaction and DNA fragment sizes were measured on the Applied Biosystems GeneScan 310.
Medical follow-up of relatives
First-degree relatives found to be C282Y homozygous, but with normal iron indices, were given their results, offered counseling, and provided with a written summary of the findings. Arrangements were made for annual follow-up review. Those with iron abnormalities were sent an explanatory letter. Arrangements were made for a follow-up appointment so that the findings could be discussed and also for any additional clinical assessments that were required. In accordance with UK Guidelines [38] no separate provision was made to arrange liver biopsy for C282Y homozygotes provided they had normal LFTs, no hepatomegaly and sFn <1000 μg/l, as the risk of significant fibrosis/cirrhosis is low. Therapeutic intervention, when needed, was applied in accordance with UK National Guidelines.[38] The degree of iron overload was evaluated retrospectively by quantitative phlebotomy.

Statistical analysis
The data set had 80% power at the 5% significance level to unravel a decrease in iron overload prevalence from 50% among the homozygous relatives of patients to 20% among the homozygous relatives of blood donors. Measurements of sFe, TIBC and TS were expressed as mean ± SD. Data that were not normally distributed, such as age and sFn, were summarized by median and range. Comparisons between groups were made using a two-sample t-test or a Mann-Whitney U-test, as appropriate. Frequency differences between groups were assessed for statistical significance by means of a Chi-squared or Fisher’s exact test and the results expressed as odds ratio with 95% confidence intervals. Logistic regression was used to assess the association between HFE genotype, iron phenotype and the recorded environmental and physiological parameters. Where appropriate, environmental and medical measurements were combined in a propensity function for use in the logistic regression analysis. Model construction was performed using the “Akaike” procedure of the statistical programme R [39] which allows minimization of the model deviance by stepwise inclusion or exclusion of variables, beginning with a null model. The presence of the iron phenotype was defined as TS > 50% and sFn > 300μg/l (sFn > 200μg/l for premenopausal women), and entered into the procedure as a dependent variable. Multiple linear regression analysis was used to predict the SF-36 Physical Component Score (PCS) from genetic, demographic and environmental covariates. All statistical analyses were performed using SPSS version 10 and Statistica 5.1 customised programmes.

Ethical Approval
Ethical approval for the study was obtained from Bro Taf, Gwent and Iechyd Morgannwg regional ethics committees.

RESULTS
Recruitment of first-degree relatives of C282Y homozygous probands
The iron and health status of the C282Y homozygous blood donors has been described elsewhere.[31] Family details were available for 66 of the 72 originally identified probands. A total of 56 probands (median age at screening: 43 years) were finally available for inclusion in the study. These told us about 180 eligible relatives of whom 165 (92%) agreed to take part (96 siblings, 69 parents).

The iron and health status of most of the 69, C282Y homozygous patients with haemochromatosis identified in Bro Taf and Gwent have been described elsewhere.[16] For the 60 probands the median age at diagnosis was 51 years for males and 52 years for females. The median time interval since diagnosis was 2.7 years (range <1 to 27 years). Some 121 (85%) of the 143 identified first-degree relatives (95 siblings, 26 parents) were included in the study.

All the blood donor siblings took part in the survey (or had died). However, from the somewhat older families of patients, 20 siblings did not reply or did not wish to take part.
**Demographics**

Parents and siblings in the clinical group were significantly older than their respective counterparts in the screened group (Table 1). The latter were significantly more likely to have a history of blood donation, although the mean number of units of blood donated in a lifetime was not significantly different. Siblings reported consuming more units of alcohol per week than their parents, however the differences were not statistically significant when analysed within proband groups.

**Table 1. Demographic characteristics of first-degree relatives (n=286) of C282Y homozygous probands.**

<table>
<thead>
<tr>
<th>Relatedness</th>
<th>First-degree relatives of C282Y homozygotes detected by genetic screening (n = 165)</th>
<th>First-degree relatives of C282Y homozygotes presenting with clinical haemochromatosis (n = 121)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Parents  69</td>
<td>Siblings 96</td>
</tr>
<tr>
<td>Age in years*</td>
<td>66 (44-89)</td>
<td>43 (14-73)</td>
</tr>
<tr>
<td>Sex ratio F:M</td>
<td>35:34</td>
<td>49:47</td>
</tr>
<tr>
<td>History of blood donation (%)</td>
<td>21 (30.4)</td>
<td>42 (43.8)</td>
</tr>
<tr>
<td>Units of blood donated in lifetime, all relatives*</td>
<td>5.3±12.6</td>
<td>5.2±8.4</td>
</tr>
<tr>
<td>Units of blood donated in lifetime, blood donors only*</td>
<td>0.0 (0-60)</td>
<td>0.0 (0-30)</td>
</tr>
<tr>
<td>Units of alcohol consumed per week</td>
<td>17.3±17.8</td>
<td>11.4±9.2</td>
</tr>
<tr>
<td>Units of alcohol consumed per week</td>
<td>10.0 (1-60)</td>
<td>10.0 (1-30)</td>
</tr>
</tbody>
</table>

Measurements are given as mean ± SD except *: median (range). Note: one unit of alcohol contains 8 g alcohol. For each variable, comparisons were made separately between the two parent and the two sibling groups, respectively. ṅ: p≤0.05, †: p≤0.01, λ: p≤0.0001.

A total of 277 relatives (97%) provided data on the ancestry of all four grandparents. All except two families described themselves as being of Northern-European descent and 244 (85%) claimed some “Celtic” descent, defined as Welsh, Scottish or Irish. A total of 169 relatives (59%) had at least 75% Celtic ancestry, 44% had four Celtic grandparents and 35% had four Welsh grandparents.

**Causes of death among relatives**

The observed number of C282Y homozygous siblings of probands (n=50, 26.4%) was close to the number expected from the population frequency of *HFE* C282Y. This suggests that there is no major excess of sibling deaths arising from haemochromatosis, an assertion that is further corroborated indirectly by the assessment of the causes and circumstances of death among deceased first-degree relatives. Four deaths (all male) had occurred among the 51 male and 49
female siblings from the blood donor families, three of which occurred neonatally or during infancy (stillbirth, “convulsions” and pneumonia). The fourth had died at the age of 38 years of carcinoma of the pancreas. Of the 69 male and 77 female siblings of the clinical cases, 22 and 9 had died, respectively. There were 8 neonatal/infant deaths, 4 accidental deaths, 8 deaths due to cancer, 3 from myocardial infarction, 3 from respiratory disease, 3 from unknown causes and 2 from liver disease. Twelve were aged 60 or over.

Genotypes and haematology
The observed genotype frequencies for siblings of C282Y homozygous probands (Table 2) did not differ from those expected from the population frequencies [31] of the three most common HFE variants, namely wild type, C282Y and H63D. [40] The mean corpuscular volume (MCV) and mean cell haemoglobin (MCH) were significantly higher in C282Y homozygous compared to “wild type” homozygous relatives (Table 2). The MCH, but not the MCV, was also significantly higher in compound heterozygotes for C282Y and H63D. No significant differences were observed for haemoglobin, red blood cell count and haematocrit (data not shown) between individuals grouped according to HFE genotype.

Table 2. HFE genotype and haematology for 282 first-degree relatives of C282Y homozygous probands

<table>
<thead>
<tr>
<th>Genotype (C282Y/H63D)</th>
<th>Parents n = 92 (%)</th>
<th>Siblings N = 189 (%)</th>
<th>Hb (g/dl)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>--/--</td>
<td>0</td>
<td>38 (20.1)</td>
<td>14.0±1.00</td>
<td>90±5</td>
<td>30.0±2.0</td>
</tr>
<tr>
<td>--/+</td>
<td>0</td>
<td>20 (10.6)</td>
<td>14.2±1.52</td>
<td>89±7</td>
<td>29.9±2.6</td>
</tr>
<tr>
<td>--/+</td>
<td>0</td>
<td>2 (1.1)</td>
<td>13.8±0.85</td>
<td>88±1</td>
<td>29.7±0.3</td>
</tr>
<tr>
<td>++/--</td>
<td>68 (73.9)</td>
<td>65 (34.4)</td>
<td>14.1±1.42</td>
<td>91±5</td>
<td>30.3±2.0</td>
</tr>
<tr>
<td>+/--</td>
<td>15 (16.3)</td>
<td>14 (7.4)</td>
<td>14.6±1.50</td>
<td>93±6</td>
<td>31.3±2.3φ</td>
</tr>
<tr>
<td>++/--</td>
<td>9 (9.8)</td>
<td>50 (26.4)</td>
<td>14.3±1.59</td>
<td>94±5¶</td>
<td>31.5±1.8¶</td>
</tr>
</tbody>
</table>

Measurements are given as mean ± SD. Comparisons of blood parameters were made between individual genotypes and wild type genotype (--/--). φ: p≤0.05, ¶: p≤0.001. Note: the data of one parent had to be omitted since DNA analysis revealed non-paternity.

Genotypes and iron status
Compared with the relatives lacking mutations (Table 3) a statistically significant increase in TS and sFn was observed only in C282Y homozygotes. Thirty-four (12%) of the first-degree relatives were discovered to have the “iron phenotype”, defined as both raised TS and sFn. Thirty of these were C282Y homozygous (11 females, 19 males), one lacked C282Y and H63D, one was heterozygous for C282Y and two were compound heterozygotes. Thus, 32% of female homozygotes and 76% of male homozygotes showed biochemical evidence of iron overload (Fisher’s exact test for sex difference: p = 0.001). Serum ferritin was significantly higher for men than women in all except the group lacking C282Y and H63D (data not shown). A total of 25 C282Y homozygotes (median age: 50 years) were identified among relatives of screened cases. Their mean TS was 51% and the median sFn was 251µg/l. Transferrin saturation above 50% was found in five women (35%) and six men (60%). Three women (20%) and six men (60%) had the biochemical iron phenotype (p = 0.03). Among first degree relatives of clinical cases, we identified 34 C282Y homozygotes (median age: 53 years) with a mean TS of 60% and a median
sFt of 427µg/l. Transferrin saturation above 50% was found in 12 women (63%) and 12 men (80%). Eight women (42%) and 12 men (80%) had the iron phenotype (p = 0.04).

Table 3. Iron status and HFE genotype of first-degree relatives of C282Y homozygous probands

<table>
<thead>
<tr>
<th>Genotype (C282Y/H63D)</th>
<th>Proband group</th>
<th>Age in years*</th>
<th>Sex ratio F:M</th>
<th>sFe (µmol/l)</th>
<th>TIBC (µmol/l)</th>
<th>TS (%)</th>
<th>sFn* (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>--/--</td>
<td>S</td>
<td>39 (16-73)</td>
<td>9:13</td>
<td>16±9</td>
<td>62±8</td>
<td>27±15</td>
<td>57 (4-381)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>57 (39-80)</td>
<td>9:8</td>
<td>12±7</td>
<td>59±8</td>
<td>21±12</td>
<td>79 (26-175)</td>
</tr>
<tr>
<td>--/+</td>
<td>S</td>
<td>50 (35-69)</td>
<td>6:4</td>
<td>16±9</td>
<td>62±6</td>
<td>26±14</td>
<td>46 (6-1368)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>46 (38-68)</td>
<td>5:5</td>
<td>15±6</td>
<td>61±7</td>
<td>25±10</td>
<td>47 (10-396)</td>
</tr>
<tr>
<td>--/++</td>
<td>S</td>
<td>41 (39-42)</td>
<td>1:1</td>
<td>13±5</td>
<td>57±5</td>
<td>25±11</td>
<td>40 (22-57)</td>
</tr>
<tr>
<td>+/--</td>
<td>S</td>
<td>57 (17-89)</td>
<td>45:43</td>
<td>16±7</td>
<td>59±8</td>
<td>27±11</td>
<td>64 (5-451)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>65 (35-88)</td>
<td>32:13</td>
<td>15±9</td>
<td>59±6</td>
<td>26±15</td>
<td>67 (6-714)</td>
</tr>
<tr>
<td>+/-+</td>
<td>S</td>
<td>68 (43-82)</td>
<td>7:7</td>
<td>21±8</td>
<td>58±8</td>
<td>36±13</td>
<td>138 (6-490)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>60 (28-76)</td>
<td>7:8</td>
<td>19±10</td>
<td>59±6</td>
<td>34±18</td>
<td>106 (11-723)</td>
</tr>
<tr>
<td>++/--</td>
<td>S</td>
<td>50 (14-73)</td>
<td>15:10</td>
<td>24±11</td>
<td>50±5</td>
<td>51±27</td>
<td>251 (6-2607)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>53 (20-86)</td>
<td>19:15</td>
<td>29±11</td>
<td>50±8</td>
<td>61±24</td>
<td>427 (12-4000)</td>
</tr>
</tbody>
</table>

Proband group S: screened, C: clinical presentation. Measurements are given as mean ± SD except *: median (range). Comparisons of iron parameters were made between individual genotypes and wild type genotype (--/--). φ: p≤0.05, †: p≤0.01, ¶: p≤0.001, λ: p≤0.0001,Ψ: p≤10⁻⁷.

The median age of all 286 relatives combined was 55 years (range: 14-89 years) and varied with genotype (Table 3). Compared to relatives lacking mutations (all of whom were siblings), C282Y heterozygotes and compound heterozygotes were significantly older. Moreover, a greater proportion in the latter two groups were parents. A small increase in sFt values was noted with increasing age for all genotypes although this trend reached statistical significance only for the relatives lacking mutations (coefficient of determination R² = 0.171, p = 0.009) and C282Y heterozygotes (R² = 0.088, p = 0.005). Relatives lacking mutations were significantly older in the clinical group (56.6 years) than in the screened group (38.7 years, p ≤ 0.0001). The same was true of HHCY heterozygotes. Despite these age differences, there were no significant differences in iron status between the two sets of relatives in these genotype groups. By contrast, the median sFt concentrations in the two C282Y homozygous sibling groups differed significantly despite absence of a significant age difference between the two groups (clinical: 427 µg/l, screened: 251 µg/l, p = 0.03).

Iron deficiency
Iron deficiency, defined as sFt <15 µg/l, was observed in 18 women (age range: 34 - 78 years) and two men (46 and 54 years). For further analysis see Appendix.
Quantitative phlebotomy
To date, 26 of the 59 C282Y homozygous relatives have completed a venesection program, 24 have been assessed as currently not requiring venesection, and assessment is ongoing in six. Two homozygotes have defaulted on their follow-up appointments and no further details are available for them. One C282Y homozygote (a 67-year-old male sibling, TS: 69%, sFn: 596 µg/l) from the clinical group died during the study period of an unrelated pneumonia complicating fibrosing alveolitis.

Among the 26 venesected homozygotes, 12 (5 female, 7 male) were from the screened family group and 14 (4 female, 10 male) were from the clinical group. The median amount of iron requiring removal for sFn and TS to fall to recommended levels was 2.3 g (range: 0.4 - 5.2) for the screened group members and 3.5 g (range 1–8) for the relatives of clinical patients (p = 0.09). More iron needed to be removed from men (3.0 g, range 1–8) than from women (2.3 g, range 0.4–4), although the difference was also not statistically significant (p = 0.11). Only 10 homozygotes required removal of more than 4 grams of iron (7 from the clinical group, 3 from screened group).

Risk factors for iron overload
Univariate analysis revealed that C282Y homozygosity was the greatest risk factor when compared to all other genotypes (Table 4, odds ratio: 56.6, 95% CI: 17.3-206). First-degree homozygous relatives of clinical cases were at greater risk of iron overload than relatives of blood donors (OR 2.76; 95% CI: 1.24-6.23). The possession of at least one H63D mutation appeared to be protective ( OR: 0.25, 95% CI: 0.04-1.14) probably because the two mutations hardly ever occur on the same chromosome.(41;42) Gender was also identified as a significant cofactor, with males having a significantly greater risk for iron overload (OR=2.50: 95%, CI 1.12-5.63). That age was not identified as a significant risk factor (p>0.5), despite the observed age-dependence of sFn, is explicable by the latter effect being confined mainly to the non-pathological sFn range for relatives lacking mutations or heterozygous for C282Y. From the data collected at interview about medical history, dietary history and lifestyle, seven potential parameters modifying iron accumulation were identified in the univariate analysis as being either significant (p ≤ 0.05) or of borderline significance (0.05 < p ≤ 0.10), namely H63D carriershship, history of liver disease, current blood donorship, having been previously deferred as a blood donor, fresh fruit consumption, alcohol consumption, and regular aspirin intake. For the purpose of estimating the iron overload risks, corrected for life-style and health status, associated with homozygosity for C282Y, gender and proband group, the seven parameters listed above were collapsed into a single propensity score and the outcome used as a single explanatory variable in a multivariate logistic regression model (Table 5). The resulting model showed that, while homozygosity for C282Y explains 42% of the model deviance, and gender and the propensity score each explain 6%, proband group was no longer a significant risk factor for iron overload. No significant interaction was observed between any factors. In total, 57% of the initial model deviance could be explained by the selected variables.
Table 4. Univariate analysis of potential risk factors for iron overload

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Iron overload (yes:no)</th>
<th>p*</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE genotype:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous C282Y vs. other</td>
<td>30.3 vs. 4:2</td>
<td>≤0.001</td>
<td>56.6</td>
<td>17.3-205.6</td>
</tr>
<tr>
<td>proband group</td>
<td>23.0 vs. 12:1</td>
<td>0.009</td>
<td>2.76</td>
<td>1.24-6.23</td>
</tr>
<tr>
<td>clinical vs. screened</td>
<td>22:1 vs. 12:1</td>
<td>0.017</td>
<td>2.50</td>
<td>1.12-5.63</td>
</tr>
<tr>
<td>gender</td>
<td>29:2 vs. 4:8</td>
<td>0.024</td>
<td>3.28</td>
<td>1.05-11.42</td>
</tr>
<tr>
<td>fresh fruit per week</td>
<td>22:1 vs. 11:1</td>
<td>0.040</td>
<td>2.30</td>
<td>1.01-5.31</td>
</tr>
<tr>
<td>units of alcohol per week</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤7 vs. &gt;7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5 vs. ≤5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR: odds ratio, CI: confidence interval. a: only risk factors with a two-tailed Fisher p-value ≤ 0.05 are listed. All other recorded risk factors were either of borderline significance, including H63D carriership, liver disease, current blood donorship, previously deferred blood donorship, Aspirin intake (all 0.05 < p ≤ 0.10), or not significant at all, including age, diabetes, thyroid disease, heart disease, history of bleeding disorder or anaemia, iron/vitamin C consumption, red meat intake, coffee, tea, other dietary/mineral supplements, smoking and all female-specific factors such as menorrhagia and pregnancy (all p > 0.10).

Table 5. Logistic regression modelling of iron phenotype risk

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Standard error</th>
<th>Deviance explained (%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>-5.17</td>
<td>0.89</td>
<td>-</td>
</tr>
<tr>
<td>Homozygous C282Y</td>
<td>4.86</td>
<td>0.79</td>
<td>84.15 (41.7%)</td>
</tr>
<tr>
<td>gender</td>
<td>-1.79</td>
<td>0.61</td>
<td>12.72 (6.3%)</td>
</tr>
<tr>
<td>propensity score</td>
<td>7.31</td>
<td>2.33</td>
<td>11.38 (5.6%)</td>
</tr>
<tr>
<td>Proband group</td>
<td>0.51</td>
<td>0.57</td>
<td>6.66 (3.3%)</td>
</tr>
</tbody>
</table>

a: p value from a z-approximation of the maximum likelihood estimate of each regression coefficient. All p values are two-sided except (b). Use of a one-sided test for the proband group was justified by the prior expectation that relatives in the clinical group should be at a higher risk than relatives in the screened group.

Iron status and ancestral haplotype
The “ancestral” haplotype was defined as D6S265-1, D6S105-8, D6S1260-4 (37). For the 54 C282Y homozygotes that could be typed, nine (16.7%) had two copies of the ancestral haplotype, 30 (55.5%) carried alleles suggesting the presence of one copy, and 15 (27.8%) lacked marker alleles from the ancestral haplotype. There were no significant differences in age, serum ferritin or transferrin saturation between the three groups (data not shown).
Morbidity in relatives
Compound heterozygotes (C282Y/H63D) had significantly higher rates of diabetes, hypertension and heart disease than relatives lacking the C282Y mutation (Table 6).

Table 6. Morbidity and HFE genotype in first-degree relatives (n=282) of C282Y homozygous probands.

<table>
<thead>
<tr>
<th>Genotype (C282Y/H63D)</th>
<th>--/--</th>
<th>--/+</th>
<th>--/+</th>
<th>++/--</th>
<th>++/+</th>
<th>++/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>39 (13.8)</td>
<td>20 (7.1)</td>
<td>2 (0.7)</td>
<td>133 (47.2)</td>
<td>29 (10.3)</td>
<td>59 (20.9)</td>
</tr>
<tr>
<td>Sex ratio F:M</td>
<td>18:21</td>
<td>11:9</td>
<td>1:1</td>
<td>77:56</td>
<td>14:15</td>
<td>34:25</td>
</tr>
<tr>
<td>Age in years*</td>
<td>(16-80)</td>
<td>(35-69)</td>
<td>(39-42)</td>
<td>(17-89)</td>
<td>(29-82)</td>
<td>(14-86)</td>
</tr>
<tr>
<td>Iron phenotype</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0</td>
<td>1 (5.0)</td>
<td>0</td>
<td>10 (7.5)</td>
<td>4 (13.8)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Raised AST</td>
<td>0</td>
<td>1 (5.0)</td>
<td>0</td>
<td>2 (1.5)</td>
<td>1 (3.5)</td>
<td>5 (8.5)</td>
</tr>
<tr>
<td>Liver Disease</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (1.5)</td>
<td>0</td>
<td>2 (3.4)</td>
</tr>
<tr>
<td>Any arthralgia</td>
<td>23 (59.0)</td>
<td>13 (65.0)</td>
<td>2 (100.0)</td>
<td>83 (62.4)</td>
<td>22 (75.9)</td>
<td>36 (61.0)</td>
</tr>
<tr>
<td>Distal arthralgia</td>
<td>14 (35.9)</td>
<td>7 (35.0)</td>
<td>2 (100.0)</td>
<td>38 (28.6)</td>
<td>14 (48.3)</td>
<td>23 (39.0)</td>
</tr>
<tr>
<td>Severe arthralgia</td>
<td>5 (12.8)</td>
<td>5 (25.0)</td>
<td>1 (50.0)</td>
<td>23 (17.3)</td>
<td>8 (27.6)</td>
<td>9 (15.3)</td>
</tr>
<tr>
<td>Joint investigation (X-ray)</td>
<td>15 (38.5)</td>
<td>10 (50.0)</td>
<td>0</td>
<td>43 (32.3)</td>
<td>9 (31.0)</td>
<td>18 (30.5)</td>
</tr>
<tr>
<td>Lethargy</td>
<td>14 (35.9)</td>
<td>9 (45.0)</td>
<td>1 (50.0)</td>
<td>41 (30.8)</td>
<td>12 (41.4)</td>
<td>25 (42.3)</td>
</tr>
<tr>
<td>Thyroid disease</td>
<td>3 (7.7)</td>
<td>2 (10.0)</td>
<td>0</td>
<td>10 (7.5)</td>
<td>3 (10.3)</td>
<td>8 (13.6)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4 (10.3)</td>
<td>5 (25.0)</td>
<td>0</td>
<td>30 (22.6)</td>
<td>10 (34.5)</td>
<td>7 (11.9)</td>
</tr>
<tr>
<td>Heart disease</td>
<td>1 (2.6)</td>
<td>2 (10.0)</td>
<td>0</td>
<td>18 (13.5)</td>
<td>7 (24.1)</td>
<td>4 (6.8)</td>
</tr>
<tr>
<td>Coeliac disease</td>
<td>1 (2.6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper abdominal pain</td>
<td>4 (10.3)</td>
<td>0</td>
<td>0</td>
<td>11 (8.3)</td>
<td>1 (3.5)</td>
<td>3 (5.1)</td>
</tr>
</tbody>
</table>

* Age is given as median (range). AST reference range 5 - 45 IU /L. Comparisons of measurements were made between relatives of a given genotype and those lacking the C282Y mutation (ie genotypes --/--, --/+ and --/++) except for age where a comparison was made with wild type (--/--). $p \leq 0.05$, $\lambda: p \leq 0.01$. 

$\phi$: $p \leq 0.05$, $\lambda$: $p \leq 0.01$. 

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No significant differences were detected among C282Y carriers lacking H63D, both heterozygous and homozygous. The higher morbidity in HDCY compound heterozygotes did not relate to the degree of iron accumulation (Table 3). Instead, the difference in morbidity may have resulted from a substantial difference in age since the compound heterozygotes were significantly older than the wild-type relatives (median: 63 years vs. 44 years), reflecting the high proportion of parents in this group.

Among C282Y homozygotes, when thirteen morbidity comparisons were stratified by the presence or absence of the iron phenotype, a significant difference was detected only for the proportions with elevated aspartate aminotransferase (AST) levels. Of the C282Y homozygotes with iron overload, five (17%) had raised AST levels, compared to none among those without the iron phenotype. All those with raised levels had histories of regular alcohol intake. When morbidity among C282Y homozygotes were stratified by proband group (screened vs. clinical), the only significant difference to emerge was a higher prevalence of hypertension among relatives in the clinical group (7/34 in the clinical group and 0/25 in the screened group, p < 0.05).

Quality of life survey among relatives
The SF-36 QOL questionnaire was completed in full by 152 relatives (94%) from the screened and 109 (90%) from the clinical group. No significant score differences were observed between C282Y homozygotes and those lacking mutations (Figure 1). However, the HDCY compound heterozygotes had significantly lower scores than relatives lacking mutations. No significant difference in the quality of life profiles was observed between previously diagnosed C282Y homozygotes and newly identified C282Y homozygotes and relatives lacking mutations. Furthermore, there were no significant differences between C282Y homozygotes from the screened group and the clinical group. C282Y homozygotes with or without the iron phenotype also showed no significant difference in QOL scores (data not shown).

Relatives with raised aspartate aminotransferase (AST) levels
Nine relatives had elevated AST levels. The median amount of alcohol consumed per week by those with raised AST levels was 16 units (range 1 - 190) compared with 5 units (0 - 80) for those with normal AST levels (p < 0.018). Five were C282Y homozygous of whom two had AST values higher than twice the upper normal limit (45 IU/L). All five C282Y homozygotes also had raised iron indices, and in all but one, the liver function tests returned to normal following lifestyle advice and venesection. Details of the remaining subjects are given in the Appendix.

Relatives with a previous history of liver disease and liver biopsy results for newly identified C282Y homozygotes
Four relatives had a history of liver disease, of whom three had a history of heavy alcohol consumption. The two C282Y homozygotes had clinically evident cirrhosis. For further details see Appendix. As a result of our investigations a further three C282Y homozygous cases (2F,1M) had a liver biopsy. All had raised transferrin saturation and serum ferritin concentrations but normal AST levels. The histology in all three showed grade 2 iron, but no fibrosis or cirrhosis. There were another three homozygotes (all male) in whom the sFn level was above 1500 µg/l. They either did not wish to have a biopsy (one case) or their clinician did not deem it necessary. None had obviou clinical evidence of liver disease. Their clinical examination, synthetic liver function (albumin and prothrombin time), haematology (platelet count) and liver ultrasound scans were all normal.
DISCUSSION
The potential public health impact of iron overload and the availability of effective treatment, make it essential to assess more accurately the penetrance of HFE gene mutations. In order to identify evidence for possible genetic modifiers of the associated haemochromatosis disease phenotype, we have examined the relative contribution of genetic, environmental and physiological factors to both iron phenotype and morbidity in two groups of first-degree relatives of probands, genetically predisposed to haemochromatosis.

Studying relatives, rather than index cases, limits ascertainment bias, a drawback of many previous studies. Studies of blood donors [31] have been criticised [18] since they represent a population preselected to be healthy and relatively young. In addition, blood donation may modify the iron phenotype although donation is usually followed by partial compensation through increased iron absorption.[43] Our present study would also be rightly criticised if the relatives of blood donors had given more blood than those of clinical cases. This was not, however, the case. A mean of 5.2 and 3.6 units was donated in adult life by the screened and clinical siblings respectively. In the United Kingdom, 6% of the general population are current blood donors (www.blooddonor.org.uk), as were 9.7% of the screened and 5.0% of the clinical group. Although a univariate analysis of our data suggested a protective effect against iron overload of blood donation, this did not reach statistical significance (p=0.087).

Using genetic, environmental and physiological factors recorded in the relatives of genetically predisposed probands, we have shown that by far the highest risk for iron overload is associated with C282Y homozygosity. Of the 34 relatives with both raised TS and sFn 30 were C282Y homozygous. Two, male compound heterozygotes (7%) showed biochemical evidence of iron overload confirming the much lower biochemical penetrance of this genotype [31][44][45][46][47] in comparison to C282Y homozygosity. Only two life-style parameters were found to be significant risk factors for the iron phenotype, namely high alcohol and low fresh fruit consumption. Whilst the effect of alcohol on indicators of iron status appears straightforward, the protective effect of high fruit consumption is more difficult to explain. People who eat more fruit may also eat more vegetables and less red meat, but further examination of the data did not confirm this supposition.

The only HFE genotype associated with significantly higher morbidity was compound heterozygosity (C282Y/H63D). Diabetes, hypertension and heart disease increase in frequency with age and the compound heterozygotes, being mostly parents, were indeed the oldest group. That age was the major determinant of morbidity for all relatives was also confirmed by a stepwise multiple regression analysis to predict the SF-36 physical component score from demographic, genetic and environmental covariates. In addition to the observed effect of compound heterozygosity, only age and (not surprisingly) aspirin intake were highly predictive (p ≤ 0.0001).

Only one difference in morbidity was observed when the C282Y homozygous relatives were stratified by the presence or absence of the iron phenotype. Five homozygotes with the iron phenotype also had raised AST activity, but none of the others (p ≤ 0.05). This limited difference in morbidity supports a study from Ireland where the frequency of fatigue, arthropathy and impotence in C282Y homozygous relatives was not found to be related to iron overload.[48] When morbidity was compared in our study between C282Y homozygotes from the screened and clinical group, only the frequency of hypertension was found to differ significantly. Neither stratification revealed a significant age difference.
Cirrhosis of the liver and hepatoma represent significant, albeit less common, complications associated with homozygosity for \textit{HFE} C282Y.[49][50] Only two of 59 C282Y homozygotes were found to have cirrhosis, and although we could not confidently exclude significant fibrosis in a further three homozygotes, none of the latter had clinical evidence of cirrhosis. The prevalence of cirrhosis is therefore low, as observed in the largest screening study of a general population to date [14] and in Irish C282Y homozygotes identified through family screening.[48] The two C282Y homozygotes with cirrhosis both had long-standing histories of excessive alcohol intake, consistent with an earlier report of a substantially higher risk of cirrhosis among HH patients who drink alcohol excessively.[26]

Our family-based observations are consistent with large-scale surveys of primary care patients [15][47] where the incidence of arthritis, diabetes and heart disease was no higher than in subjects lacking C282Y or H63D. However, in both surveys, there was an increased frequency of self-reported liver disease in C282Y homozygotes. Previous observational studies have also revealed that patients with diabetes, heart disease and arthritis do not have significantly increased frequencies of homozygosity or heterozygosity for C282Y.[51] Furthermore a study among subjects recruited prospectively from general practices in Oxfordshire, in which genetic testing was prompted by symptoms commonly associated with HH, failed to identify a single case with an iron-loading susceptibility genotype.[52]

Our finding that C282Y homozygosity has a poor positive predictive value for morbidity, would seem to weaken further the case for HH population screening based on \textit{HFE} genotyping. As was advocated by some before the discovery of the \textit{HFE} gene, [53] as well as others more recently [54], screening based on biochemical testing of iron indices offers the benefit of detecting only those who are already accumulating iron, as well as avoiding the problem of identifying large numbers of people with C282Y homozygosity who may be at low risk of developing clinical complications from iron overload. Screening healthy subjects is desirable as inflammation and infection increase ferritin concentration and decrease transferrin saturation.[55] Cases with iron overload detected in this way and found not to be homozygous for C282Y or compound heterozygous may then be tested for mutations in genes other than \textit{HFE}, including \textit{HAMP} and \textit{HJV}. [56]

The main aim of the present study was to compare the relatives of index cases detected by a genetic screening programme with relatives of clinical cases. We hypothesised that biochemical expression of the iron phenotype would be higher in relatives of clinical cases and speculated that the presence of additional familial modifiers (most likely genetic) would explain any differences in expression found. Indeed, biochemical expression was found in 40% of C282Y homozygous relatives from the screening group and in 59% from the clinical group. The difference was statistically significant upon univariate analysis. In a multivariate logistic regression analysis, however, only C282Y homozygosity, gender and a collapsed propensity score remained important, but not the proband group. Its univariate effect may therefore be explicable mainly through confounding by the other factors, although a trend towards a higher risk in relatives of clinical than screened probands remained discernible.

Since only 57% of the variability in iron phenotype was explicable by the genetic, environmental and physiological factors considered here, there must be other yet unidentified influential factors. These are however unlikely to be of great clinical importance since we have shown that significant morbidity is rare in both groups of relatives. Results of the microsatellite analysis to identify the haemochromatosis “ancestral” haplotype also suggest that modifier genes close to the \textit{HFE} gene do not play an important role in altering biochemical expression in these families confirming a recent report by Barton et al.[57]
The clinical and biochemical observations among a cohort of C282Y homozygotes ascertained through family history reveal that HFE C282Y homozygosity has a high penetrance in terms of iron accumulation but has poor predictive value for morbidity. These observations further weaken the cases for HH population screening based primarily on HFE genotyping.

Acknowledgments
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Ethical Approval
Ethical approval for the study was obtained from Bro Taf, Gwent and Iechyd Morgannwg regional ethics committees.

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Competing interests
The authors have declared no competing interests.
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APPENDIX

Iron deficiency
Iron deficiency, defined as sFn <15 µg/l, was observed in 18 women (age range: 34 - 78 years) and two men (46 and 54 years). This sex difference was statistically significant (Fisher’s exact test: p=0.008). The proportions with iron deficiency were similar in the two groups of relatives (screened group, 8.7%; clinical group, 5.0%). Fifteen of the 18 iron deficient women (83%) were premenopausal. The proportion of relatives with iron deficiency varied with HFE genotype, from 5% (n = 3) among C282Y homozygotes to 15% (n = 3) among individuals heterozygous for H63D, but none of the differences reached statistical significance. One of the iron deficient women had a recent diagnosis of coeliac disease, eleven had a history of menorrhagia, and 16 had one or more children. None was vegetarian, but nine had a history of blood donation (five were current donors). These women had donated a mean of 15.8 units of blood during adult life (two women had donated 30 units). Nine relatives (8 females, 1 male) had iron deficiency and anaemia.

Relatives with raised aspartate aminotransferase (AST) levels
Nine relatives had elevated AST levels. Five were C282Y homozygous. The remaining subjects included two C282Y heterozygotes (1M, 1F) with normal iron indices and minor derangement of AST (57.53 IU/l) of no obvious cause, a 50-year-old obese female compound heterozygote (normal iron indices, AST: 152 IU/l), subsequently diagnosed with non-alcoholic fatty liver disease and a 57-year-old male H63D heterozygote (TS: 41%, sFn: 1368 µg/l, AST: 115 IU/l, MCV: 100fl) with a history of heavy alcohol consumption. Our investigations also revealed type II diabetes. After management of his diabetes and a reduced alcohol intake the AST level returned to the normal range and his sFn fell to 425µg/l.

Relatives with a previous history of liver disease
Four relatives had a history of liver disease. Two were C282Y heterozygous and two were C282Y homozygous. One heterozygote was the 60 year-old father of a blood donor index case. He had finished a course of chemotherapy for carcinoma of the colon with liver metastases prior to interview. The other C282Y heterozygote was a 53 year-old female sibling from the clinical group with normal iron indices. She had a history of heavy alcohol consumption and was previously diagnosed with alcoholic hepatitis (no liver biopsy). Her liver function tests returned to normal with abstinence and remained normal at interview. Her brother is currently being treated for haemochromatosis and another brother died, possibly of liver cirrhosis, at the age of 31 years. Both C282Y homozygotes are siblings of clinical cases and were detected through family screening. One, a 47 year-old male with a history of heavy alcohol consumption was found at liver biopsy to have grade 4 haemosiderosis with fibrosis and possible early cirrhosis. He had 5 g iron removed at venesection and is currently well on a maintenance venesection programme. The other, a 48 year-old female, had a clinical diagnosis of chronic liver disease and a history of post-operative acute hepatic failure (TS: 91%, sFn: 4000 µg/l at review). She was a heavy drinker (>190 units alcohol per week) and had clinical signs of chronic liver disease. Her liver function tests were abnormal at presentation (AST: 125 IU/l, alkaline phosphatase: 273 IU/l). She was an inconsistent attendee and did not have liver biopsy.
Legend to figure

1. HFE genotype and QOL profile of first-degree relatives of C282Y homozygous probands. Numbers relate to those who completed QOL survey (261 of 282 genotyped, 92.6%). Norm-based scoring: higher scores represent better health. For the US population: mean = 50, standard deviation = 10.

Key: PF- physical functioning; RP- role physical; BP- bodily pain; GH- general health; VT- vitality; SF- social functioning; RE- role emotional; MH- mental health; PCS- physical component summary; MCS- mental component summary.

Significance of difference from wild type (−/−); *p<0.05, **p<0.01, ***p=0.001