Heteroduplex analysis for the three common HFE variants: methodology, reliability and analysis of over 5000 requests for testing

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Objective: To describe the analysis of over 5300 patient samples for the *HFE* genotype.

Methods: Blood samples received from hospitals in England, Wales and Ireland were analysed with a single, multiplex PCR using heteroduplex generators for the C282Y, H63D and S65C variants of the *HFE* gene. PCR products labelled with fluorescent dyes were analysed by capillary electrophoresis. Genotype frequencies were analysed according to the reasons given for testing.

Results: Analysis of 400 samples sent in duplicate revealed one error that was associated with reporting rather than the methodology. Of 5327 samples received, 1122 were for family testing, 2470 for diagnostic testing and in 1735 cases no reason was given. Overall, homozygosity for C282Y was found in 14% of samples received for family testing and in 16% of the remaining samples. Clinical indications such as "liver disease" were of little predictive value for homozygosity for C282Y, but this increased if a raised serum ferritin concentration or transferrin saturation was indicated. When the diagnosis was iron overload, 39% of subjects tested were homozygous for C282Y. Compound heterozygosity (C282Y/H63D) was more frequent than in the general population but the frequency was not further increased in subjects for whom there was a diagnosis of iron overload. The frequencies of heterozygosity for H63D or S65C and homozygosity for H63D were not significantly increased in any group compared with the general population frequency.

Conclusion: These results demonstrate the reliability of the methodology and confirm the difficulty of identifying genetic haemochromatosis purely on the basis of clinical suspicion that haemochromatosis may be responsible for liver disease, diabetes or arthritis.

Hereditary haemochromatosis (HH) is an autosomal recessive disorder in which there is iron accumulation as a result of increased dietary absorption. In the UK¹ over 90% of patients are homozygous for a single variant (C282Y) of the *HFE* gene.² Once diagnosed, HH is readily treatable by the means of venesection and, provided complications have not arisen, life expectancy is not reduced.^{3–5} The allele frequency of *HFE* C282Y in people of northern European origin in the UK is about 8% with about one in seven people being heterozygous, and one in 150 being homozygous for the C282Y allele.⁶ The frequency, availability of a genetic test and an effective treatment have led to pressure to implement population screening⁷ although there were always concerns about the clinical penetrance of *HFE* mutations.⁸ These concerns were justified as it is now clear that although most men, and about 50% of women, who are homozygous for C282Y will show evidence of iron accumulation (a raised transferrin saturation) the clinical penetrance of homozygosity for C282Y is low.^{9–11} Nevertheless, Cadet *et al*¹² found that 24% of adults from northern France had significant iron overload using a measure of "therapeutic penetrance" (removal of at least 5 g iron by venesection). This group may be considered to be at immediate risk of tissue damage from iron overload. Two other *HFE* variants are commonly found in European populations: H63D, with allele frequencies varying from 10% to 20%,¹³ and S65C with an allele frequency of 1–2%.¹⁴ These variants are not usually associated with significant iron loading in either the heterozygous or homozygous state but some compound heterozygotes with C282Y may accumulate iron.¹⁵ Detection of these variants, in addition to C282Y, is valuable in assessing their clinical significance. Here we describe a single, multiplex PCR using heteroduplex generators spanning the DNA

sequence encoding amino acids 63, 65 and 282 to detect the three common *HFE* variants and the application of the multiplex analysis to the testing of over 5000 samples sent for diagnostic or family testing.

METHODS

Blood samples received for testing

Samples were received, either for the investigation of possible iron overload or for family testing. One hospital in the Republic of Ireland, one in Northern Ireland, 19 hospitals in Wales and 37 hospitals in England have each sent samples on at least 20 occasions.

DNA preparation

DNA was prepared by rapid alkaline denaturation using 80 µl blood anticoagulated with EDTA.¹⁶

Construction of heteroduplex generators

The construction of the generator for amino acid 282 has been described.¹⁷ That for codon 63 was prepared in the same way using the mutagenic primer 63: CT GTT CGT GTT CTA * TCA TGA GAG TC to introduce the three nucleotide deletion (*) immediately 5' to the codon containing the mutation. The heteroduplex generator PCR product was cloned in *E.coli* strain JM109 using pGEM-T (Promega, Southampton, UK) and stored as a glycerol stock. The sequence of the generator was confirmed by DNA sequence analysis.

Recently, an alternative method that eliminates the cloning steps has been employed to construct the H63D heteroduplex

Abbreviations: HH, hereditary haemochromatosis; ?HH, suspected haemochromatosis

generator.¹⁸ A template of normal DNA (ie, lacking H63D, S65C and any other variants detected by heteroduplex analysis) was prepared using genomic DNA amplified with primers 3 and 4 (fig 1 and table 1), both at a final concentration of 0.67 µmol/l. Amplification was carried out in a heated-lid PCR machine (Eppendorf MasterCycler Gradient, Eppendorf AG, Hamburg) at 94°C for 30 s; 59.5°C for 30 s and 72°C for 30 s for 35 cycles, with a final incubation at 72°C for 3 min. The reaction mixture (15 µl in a 200 µl tube) also contained one × PCR buffer (Applied Biosystems), dNTPs, 200 µmol/l and Taq polymerase (Applied Biosystems), one unit. The product was electrophoresed on a 2.5% (w/v) agarose gel in 0.5 × Tris-borate-EDTA buffer, the band was excised and DNA released from the gel by diffusion in 100 µl water overnight at 4°C.

This template DNA (1 µl) was then amplified in two separate reactions using, in the first reaction, Deletion primer 5 and primer 4, and in the second reaction Primer 3 and Deletion primer 6 (table 1). The products were eluted into 100 µl water from a 2.5% (w/v) agarose gel and 1 µl of a one:one mixture of the two products amplified with primers 3 and 4. The two deletion products were also used as substrates in amplifications employing primers 3 and 4. It is necessary to demonstrate that no product is obtained from these reactions. If a product is obtained it would indicate contamination either from genomic DNA or from the original genomic PCR product.

The product of the reaction with both deletion PCR products was eluted from a 2.5% (w/v) agarose gel into 100 µl water and 1 µl was then re-amplified (primers 3 and 4) for 25 cycles. The product was eluted from a 2.5% (w/v) agarose gel, diluted to 1.0 ml with 5 mM Tris-HCl pH 8.0 and stored in 100 µl aliquots at -80°C.

PCR for C282Y and H63D/S65C

The labelled primers (table 1) were obtained from Applied Biosystems (Warrington, UK). The final reaction mixture (15 µl in a 200 µl tube) contained one × PCR buffer (Applied Biosystems), dNTPs 200 µmol/l, C282Y forward primer 1.0 µmol/l, reverse primer (FAM labelled) 0.13 µmol/l, H63D forward primer 0.46 µmol/l, reverse primer (TET labelled for ABI 310 and HEX labelled for ABI 3100) 0.13 µmol/l and Taq polymerase (Applied Biosystems) one unit. The optimum dilution of heteroduplex generators was determined by titration (approximately 1×10⁶ in the final reaction mixture). The reaction conditions in the heated-lid thermocycler were 94°C for 30 s, 59.5°C for 30 sec, 72°C for 30 s for 29 cycles. There was

a final incubation at 72°C for 3 min with the tubes cooled to 31°C to end the reaction. The PCRs may also be run separately and 1 µl of each PCR product added to the same sample well for detection on the GeneScan analyser (ABI GeneScan 310 or 3100, Applied Biosystems, Warrington, UK).

Analysis with the ABI GeneScan 310

The PCR product (1 µl) was diluted in 15 µl water containing 0.5 µl GS500 standard, TAMRA labelled. The capillary contained 7% (v/v) GeneScan polymer in one × GeneScan Buffer (all reagents from Applied Biosystems). The anode and cathode solutions were one × GeneScan buffer. Samples were taken into the capillary by electro-injection for 7 sec at 15 kV and were electrophoresed at 10 kV for 16 min at 29°C. The fluorescence readings for each dye in the selected channel were plotted against size (in base pairs) after analysis of the standard curve for each run using the least-squares method.

Analysis with the ABI GeneScan 3100

Samples were amplified using a microtitre plate and loaded on to the analyser in a microtitre plate (MicroAmp, Optical 96 well, Applied Biosystems). The PCR product (1 µl) was diluted in 15 µl water containing 0.5 µl GS500 standard (ROX labelled). The capillary contained POP4 in one × GeneScan Buffer (all reagents from Applied Biosystems). The anode and cathode solutions were one × GeneScan buffer. Samples were taken into the capillary by electro-injection for 6 s at 15 kV and were electrophoresed at 10 kV for 36 min at 30°C. The results were analysed as above.

RESULTS

The patterns obtained after capillary electrophoresis (ABI 310) of the multiplex PCR reactions are shown in fig 2a. For the ABI 3100 the POP4 polymer was used in order to be compatible with other mutation detection and microsatellite procedures carried out on the machine. Unique patterns for the various genotypes were obtained (fig 2b). Although samples were loaded in water the polymer contains denaturants and the patterns differ from those obtained with the ABI 310.

From September 1999 until March 2005 a total of 5605 samples were received for testing. Samples from subjects included in research projects and duplicate samples were excluded, leaving 5327 samples for the analysis. The frequencies of the genotypes for C282Y, H63D and S65C are shown in table 2 and compared with those for blood donors from South Wales.⁶ Genotype frequencies for this population do not differ significantly for those in several other regions of the UK¹⁹⁻²³ although the allele frequency for C282Y in Norfolk may be lower.²³

The heteroduplex analysis may detect polymorphisms, insertions and deletions within 50 bp of the nucleotides encoding C282Y and H63D, respectively. Only three changes have been detected by sequencing after finding an abnormal pattern: V272L, found in one patient and previously reported,²⁴ V295A found in four members of one family and one other patient, and a silent polymorphism at codon 63 (CAT/CAC). In the family with V295A, two members were compound heterozygotes for V295A/C282Y and one for V295A/H63D. There was no information to suggest that these rare variants were causing iron overload.

From January 1997 until April 2006 duplicate samples were sent for 400 patients (for 25 patients samples were sent on three occasions and for one patient on four occasions). During this time 8900 requests for testing were received and could be evaluated to identify duplicates (ie, identical name, date of birth and address) although coded diagnostic information was not available before September 1999. A review revealed one

Figure 1 Orientation and location of primers used to generate the heteroduplex generator for the detection of the H63D and S65C variants. The primer sequences are given in table 1. Exon 2 is underlined. The sequence is HSHLAH2, 874 bp DNA, H.sapiens HFE gene, exon 2 & 3. NCBI database ACCESSION Y09800, VERSION Y09800.1 GI:2370112. The mutated primers 5 and 6 lack the *tga* sequence shown in italics.

Table 1 Primer sequences for PCR reactions

Primer no.	Description	Sequence 5' to 3'
1	282 forward	ATG CCA AGG AGT TCG AAC AAG
2	282 reverse	CTC AGG CAC TCC TCT CAA CC (6 Fam labelled)
3	63 forward	ACA TGG TTA AGG CCT GTT GC
4	63 reverse	CAC ATC TGG CTT GAA ATT CTA CTG (TET or HEX labelled)
5	63 Deletion forward	GTT CGT GTT CTA TCA TGA GAG TC
6	63 Deletion reverse	CTC ATG ATA GAA CAC GAA CAG

discrepancy: a sample reported as heterozygous for S65C on the first occasion and heterozygous for H63D on the second occasion. The first result was correct and the different result on the second occasion was due to a reporting error (the GeneScan pattern was that for heterozygosity for S65C). The patient's consultant was informed immediately. The overall error rate is therefore one in 400 (0.25%), and was not associated with the methodology but with reporting.

About 20% of samples received were for testing of other family members after identification of a patient with haemochromatosis (table 3). Over half of these were carriers of C282Y, either simple heterozygotes or compound heterozygotes, mostly with H63D. This is slightly in excess of the predicted value of

Table 2 Genotype frequencies in samples received for testing from September 1999 to March 2005 compared with frequencies for blood donors.⁶

Genotype number	Genotype	Diagnostic samples n=5327	%	Blood donors n=10 556*	%
1	Wild-type	1916	36.0†	6152	58.3
2	Heterozygous for H63D	894	16.8†	2488	23.6
3	Homozygous for H63D	134	2.5	1342	2.4
4	Heterozygous for C282Y	1096	20.6†	249	12.7
5	Compound heterozygote C282Y/H63D	365	6.9†	253	2.4
6	Homozygous C282Y	805	15.1†	72	0.68
7	Heterozygous for S65C	73	1.4†	—	(3.9)
8	Compound heterozygote H63D/S65C	18	0.3	—	(0.4)
9	Compound heterozygote C282Y/S65C	24	0.5	—	(0.9)
10	Homozygous for S65C	2	0.04	—	(0)

*The 10 556 blood donors⁶ were not tested for S65C. The percentages refer to the genotypes 1–6. For genotypes including S65C the percentages in brackets (%) refer to 461 subjects tested in Cardiff. These were 179 blood donors and 282 patients presenting with myocardial infarct—C282Y and H63D. Genotype frequencies did not differ for the blood donors and patients with myocardial infarct (Carter K and Worwood M unpublished).

†Significantly different to blood donor frequency ($p < 0.001$).

A

B

Figure 2 Heteroduplex patterns after electrophoresis. a) The ABI 310 Genetic analyser. The genotypes are: 1. Homozygous C282Y. 2. Homozygous H63D. 3. Compound heterozygote C282Y/H63D. 4. Heterozygous for S65C. 5 and 6. Compound heterozygote H63D/S65C. b) The ABI 3100 Genetic analyser. The genotypes are: 1. Homozygous C282Y. 2. Homozygous H63D. 3. Compound heterozygote C282Y/H63D. 4. Heterozygous for S65C. 5. Homozygous for S65C. Size markers are in red, C282Y in blue and H63D/S65C in green.

50% if only siblings were tested. Fourteen per cent of subjects in this group were homozygous for C282Y, significantly less than the 25% expected if only siblings were being tested. These frequencies reflect a wide spectrum of family testing including parents, children and sometimes more distant relatives.

The samples received for diagnostic testing show a considerable and significant enrichment for homozygosity for C282Y, compound heterozygosity for C282Y and H63D and heterozygosity for C282Y. The "normal" genotype and heterozygosity for H63D or S65C were significantly less frequent than in the blood donor group and the frequency of homozygosity for H63D did not differ between the two groups.

In many cases physicians or laboratory staff have provided a clinical or biochemical reason for testing (table 3), although in one third of requests no reason for testing was given. In order to determine the predictive value for homozygosity for C282Y of the clinical or biochemical information this has been classified into eight major categories: clinical, biochemical, both, family history, suspected haemochromatosis (?HH), suspected HH with raised ferritin, known iron overload (information on liver iron concentration or iron removed by phlebotomy) or no reason given.

There were 2470 samples received for testing, accompanied by a reason for testing, but without any indication of a family history of iron overload. Of these 15.5% were homozygous for C282Y—a very similar proportion to that for the samples for which no indication for testing was provided (15.9%). For patients for whom only a clinical indication for testing was given (mostly liver disease) 3.7% were homozygous for C282Y. The predictive value increased if a clinical reason was coupled with a raised ferritin or transferrin saturation (10.3%). If a high serum ferritin concentration or transferrin saturation was reported as the reason for testing the frequency of C282Y homozygosity was 17.7%. In practice transferrin saturations were only infrequently reported. For 128 requests indicating a raised transferrin saturation 26% were homozygous for C282Y. Compound heterozygotes (C282Y/H63D) were also over-represented in the samples received for testing, but providing both a clinical reason for testing and a raised serum ferritin was not associated with any increase in frequency.

The frequency of homozygosity for C282Y was markedly increased if a suspicion of "genetic haemochromatosis" was recorded and was even higher if this information was coupled with the finding of a high ferritin (29.7%, $n = 118$). Where

evidence of iron overload was provided (raised iron on liver biopsy in most cases) the frequency of homozygosity was the highest found (39.4%).

Homozygosity for H63D was found in about 3% of cases overall although, as expected, the frequency was less in subjects with a family history of haemochromatosis than in the control population (0.84% and 2.5%, $p = 0.019$). This is because C282Y and H63D are very rarely found in the same *HFE* gene^{25, 26} and the high frequency of C282Y in the families with haemochromatosis is associated with a reduced frequency of H63D. In the group with ?HH the frequency of homozygosity for H63D was significantly higher than in the control group (3.9 and 2.5%, $p = 0.036$) but this frequency did not increase with additional evidence of iron overload.

DISCUSSION

Testing of over 8000 samples including 400 duplicate samples received demonstrates that the heteroduplex method is reliable and robust. With the methods described, an experienced operator can prepare DNA from up to 60 samples and controls, carry out the PCR and set up the capillary electrophoresis within 7 hours. Running the samples on the GeneScan 3100 overnight allows results to be available on the following morning (machine analysis of a 96-well plate takes approximately 5 hours). Positive and negative controls are included in each run. Any unusual patterns are investigated by sequencing. Several samples from the previous assay are also analysed to provide a check that samples are in the correct position on the microtitre plate. The DNA extraction is successful with over 95% of samples and extracts may be assayed successfully after storage for up to 1 year at 4°C or -20°C. Reagent costs are approximately £1.50 per sample for DNA extraction, PCR and GeneScan analysis and labour time is approximately 12 minutes per sample (including entering patient information and reporting time). Not included is the capital cost of the Capillary Electrophoresis analyser and its maintenance (in this case a proportion of the cost for multipurpose equipment at about 50p per sample). In principle, DNA extraction and PCR may be automated and after loading the microtitre plate, the GeneScan analysis is also automated.

Analysis of the pattern of testing for *HFE* variants in over 5000 samples at the University Hospital of Wales in Cardiff confirms the difficulty of identifying genetic haemochromatosis purely on the basis of clinical suspicion that haemochromatosis may be responsible for liver disease, diabetes or arthritis. Numerous case control and prospective studies have demonstrated that the frequency of homozygosity for C282Y is not significantly increased in patients attending gastroenterology, diabetes, arthritis and heart disease clinics.^{27, 28} Nevertheless, where clinicians have indicated "query haemochromatosis" as a possible diagnosis, nearly 25% of patients were found to be homozygous for C282Y. We do not know the diagnostic basis for these cases.

A high ferritin concentration was more powerful as a predictor of homozygosity for C282Y than the presence of liver disease, diabetes or arthritis. This is despite it being an acute phase protein and the resulting difficulties in its interpretation in hospital patients.²⁹ Transferrin saturation was relatively rarely given as an indicator of iron overload and for these samples, 26% were homozygous for C282Y. This again demonstrates the value of transferrin saturation as an indicator of iron overload but, like ferritin, transferrin saturation may be compromised in hospital patients by the presence of acute and chronic disease.²⁹

Detecting genetic haemochromatosis early has for many years been the aim of both hospital and community physicians. It is now clear that the clinical penetrance of homozygosity for

Table 3 Genotype frequencies (%) and reason for testing in 5327 samples received from September 1999–March 2005

Indication for testing	Number of samples	Genotype*									
		1	2	3	4	5	6	7	8	9	10
Family testing											
1. Family history of HH	1067	18.2	10.8	0.8	43.2	11.8	13.7	0.7	0.2	0.6	0
2. Spouse	55	58.2	27.2	1.8	12.7	0	0	0	0	0	0
Diagnostic testing											
3. Raised ferritin or TS (biochemical)	654	42.8	18.0	1.7	11.0	6.0	17.7	1.5	0.2	0.9	0.2
4. Clinical	700	48.7	24.9	2.6	12.0	4.6	3.7	2.0	0.9	0.7	0
5. Both biochemical and clinical	385	48.1	18.4	3.9	13.8	3.9	10.3	0.8	0.5	0.2	0
6. ?HH	509	30.8	16.1	3.9	15.9	7.1	24.6	1.2	0.2	0.2	0
7. ?HH+high ferritin	118	30.5	16.1	4.2	11.0	7.6	29.7	0.8	0	0	0
8. Diagnosis of HH	104	26.0	14.4	3.8	8.7	3.8	39.4	1.9	1.0	1.0	0
9. All diagnostic samples groups 3–8	2470	41.5	19.4	3.0	12.6	5.5	15.5	1.5	0.4	0.6	0.04
10. None given	1735	38.3	16.4	2.9	18.2	6.0	15.9	1.7	0.3	0.2	0.1

*The numbers indicate genotypes (see table 2).

Frequencies for homozygous C282Y genotype are shown in **bold type**.

Take-home messages

Evaluation of over 400 duplicate samples received for testing has demonstrated the reliability of the genetic analysis.

Homozygosity for HFE C282Y was most strongly associated with biochemical evidence of iron overload or a diagnosis of haemochromatosis. Compound heterozygotes (C282Y/H63D) were over-represented in the samples received for testing but homozygosity for H63D was not significantly more frequent in patients than in the blood donor controls.

Analysis of the indications for testing confirms the difficulty of identifying genetic haemochromatosis purely on the basis of clinical suspicion.

Careful evaluation of serum ferritin and transferrin saturation, along with indicators of the acute phase response and liver damage, should identify those with possible iron overload. Genetic testing will then rapidly identify the majority of patients with an inherited condition.

C282Y is lower than the "biochemical" penetrance. It is not yet possible to predict whether or not an individual homozygous for C282Y will continue to accumulate iron throughout life and become ill as a result. Until other genetic or environmental factors that predispose to the accumulation of major iron overload are identified, early identification of patients at risk will depend on finding biochemical evidence of iron overload. Careful evaluation of serum ferritin and transferrin saturation, along with indicators of the acute phase response and liver damage, should identify those with possible iron overload. Genetic testing will then rapidly identify the majority of patients with an inherited condition.

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REFERENCES

- 1 The UK Haemochromatosis Consortium, Worwood M. A simple genetic test identifies 90% of UK patients with haemochromatosis. *Gut* 1997;**41**:841-4.
- 2 Feder JN, Gnirke A, Thomas W, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nat Genet* 1996;**13**:399-408.
- 3 Niederau C, Fischer R, Sonnenberg A, et al. Survival and causes of death in cirrhotic and in noncirrhotic patients with primary hemochromatosis. *N Engl J Med* 1985;**313**:1256-62.
- 4 Niederau C, Fischer R, Purschel A, et al. Long-term survival in patients with hereditary hemochromatosis. *Gastroenterol* 1996;**110**:1107-19.
- 5 Adams PC, Speechley M, Kertesz AE. Long-term survival analysis in hereditary hemochromatosis. *Gastroenterol* 1991;**101**:368-72.
- 6 Jackson HA, Carter K, Darke C, et al. HFE mutations, iron deficiency and overload in 10500 blood donors. *Brit J Haematol* 2001;**114**:474-84.
- 7 Allen K, Williamson R. Screening for hereditary haemochromatosis should be implemented now. *Brit Med J* 2000;**320**:183-4.
- 8 Burke W, Thomson E, Khoury MJ, et al. Hereditary hemochromatosis: gene discovery and its implications for population-based screening. *JAMA* 1998;**280**:172-8.
- 9 Asberg A, Hveem K, Thorstensen K, et al. Screening for hemochromatosis: high prevalence and low morbidity in an unselected population of 65,238 persons. *Scand J Gastroenterol* 2001;**36**:1108-15.
- 10 Beutler E, Felitti VJ, Koziol JA, et al. Penetrance of 845G→A (C282Y) HFE hereditary hemochromatosis mutation in the USA. *Lancet* 2002;**359**:211-8.
- 11 McCune CA, Al Jader LN, May A, et al. Hereditary haemochromatosis: only 1% of adult HFE C282Y homozygotes in South Wales have a clinical diagnosis of iron overload. *Hum Genet* 2002;**111**:538-43.
- 12 Cadet E, Capron D, Gallet M, et al. Reverse cascade screening of newborns for hereditary haemochromatosis: a model for other late onset diseases? *J Med Genet* 2005;**42**:390-5.
- 13 Merryweather-Clarke AT, Pointon JJ, Jouanolle AM, et al. Geography of HFE C282Y and H63D mutations. *Genetic Testing* 2000;**4**:183-98.
- 14 Mura C, Raguenes O, Ferec C. HFE mutations in 711 hemochromatosis probands: evidence for S65C implication in mild form of hemochromatosis. *Blood* 1999;**93**:2202-5.
- 15 Worwood M. Inherited iron loading: genetic testing in diagnosis and management. *Blood Rev* 2005;**19**:69-88.
- 16 Bowen DJ, Standen GR, Granville S, et al. Genetic diagnosis of factor V Leiden using heteroduplex technology. *Thromb Haemost* 1997;**77**:119-22.
- 17 Jackson HA, Bowen DJ, Worwood M. Rapid genetic screening for haemochromatosis using heteroduplex technology. *Brit J Haematol* 1997;**98**:856-9.
- 18 Belli C, Bowen DJ, De Brasi C, et al. A single, multiplex analysis for all relevant activating NRAS gene mutations using heteroduplex generators. *Brit J Haematol* 2004;**126**:602-5.
- 19 Mullighan CG, Bunce M, Fanning GC, et al. A rapid method of haplotyping HFE mutations and linkage disequilibrium in a Caucasoid population. *Gut* 1998;**42**:566-9.
- 20 Murphy S, Curran MD, McDougall NCME, et al. High incidence of the Cys 282 Tyr mutation in the HFE gene in the Irish population-implications for haemochromatosis. *Tissue Antigens* 1998;**52**:484-8.
- 21 Merryweather-Clarke AT, Worwood M, Parkinson L, et al. The effect of HFE mutations on serum ferritin and transferrin saturation in the Jersey population. *Brit J Haematol* 1998;**101**:369-73.
- 22 Campbell S, George DK, Robb SD, et al. The prevalence of haemochromatosis gene mutations in the West of Scotland and their relation to ischaemic heart disease. *Heart* 2003;**89**:1023-6.
- 23 Willis G, Scott DGI, Jennings BA, et al. HFE mutations in an inflammatory arthritis population. *Rheumatology* 2002;**41**:176-9.
- 24 Worwood M, Jackson HA, Feeney GP, et al. A single tube heteroduplex PCR for the common HFE genotypes. *Blood* 1999;**94**(S1):405a.
- 25 Lucotte G, Champenois T, Semonin O. A rare case of a patient heterozygous for the hemochromatosis mutation C282Y and homozygous for H63D. *Blood Cells Mol Dis* 2001;**27**:892-3.
- 26 Best LG, Harris PE, Spriggs EL. Hemochromatosis mutations C282Y and H63D in 'cis' phase. *Clin Genet* 2001;**60**:68-72.
- 27 Worwood M. HFE Mutations as risk factors in disease. *Best Pract Res Clin Haematol* 2002;**15**:295-314.
- 28 Waalen J, Nordestgaard BG, Beutler E. Survival of homozygotes for the HFE C282Y hemochromatosis mutation: a meta-analysis. *Blood* 2003;**102**:2800.
- 29 Distant S. Phenotypic expression of the HFE gene mutation (C282Y) among the hospitalised population. *Gut* 2000;**47**:575-9.