# Changes in erythropoiesis in hereditary hemochromatosis are not mediated by HFE expression in nucleated red cells

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Background and Objectives. The HFE protein interacts with the transferrin receptor (TfR) to regulate cellular iron uptake. Nucleated erythroid cells have the highest number of TfR and the greatest iron uptake. The aim of this study was to investigate whether erythroid iron uptake is directly affected by *HFE* mutations.

Design and Methods. Iron status and erythropoiesis was investigated in sixty, asymptomatic *HFE C282Y* homozygotes. Reverse transcription-polymerase chain reaction, flow cytometry and immunocytochemistry were employed to investigate the *HFE* expression profile of normal peripheral blood, nucleated erythroid cells and several cultured cell lines.

Results. The *HFE C282Y* homozygous subjects showed subtle erythropoietic changes with raised transferrin saturation and reticulocyte counts and low-normal serum transferrin receptor levels, but normal erythrocyte count and mean cell volume. HFE mRNA was detected in macrophages and monocytes and HFE protein was detected in granulocytes and at low levels in monocytes. Cultured primary human erythroid colonies did not express HFE mRNA or protein.

Interpretation and Conclusions. There is evidence that *HFE C282Y* homozygotes display increased plasma iron turnover and increased erythropoiesis, despite there being no evidence that HFE is expressed in erythroid colonies with a normal *HFE* genotype. It is likely that *HFE* mutations do not directly alter erythroid iron handling, but alter the supply of iron to the erythroid tissues.

Key words: iron, hemochromatosis, erythropoiesis, HFE, transferrin receptor.

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Hereditary hemochromatosis is an autosomal recessive disorder characterized by increased iron absorption and storage of iron in parenchymal tissues.[1] In 1996, Feder *et al.*[2] cloned the *HFE* gene and found that 85% of US hemochromatosis patients were homozygous for the mutation *C282Y*, whilst many of the remainder were compound heterozygotes for *C282Y* and a common second variant, *H63D*. In the UK over 90% of patients are homozygous for *C282Y*.[3] The mutant *HFE* alleles are common in the UK, with allelic frequencies of about 8% for *C282Y* and 15% for *H63D*.[4] Thus around 1 in 150 of the general UK population are homozygous for *HFE C282Y*.

The HFE protein encodes an MHC class I molecule that forms a functional heterodimer with $\beta_2$-microglobulin.[5] C282Y, but not the H63D variant, disrupts both the association with $\beta_2$-microglobulin and the subcellular distribution of HFE.[5] Normal HFE has been co-immunoprecipitated with the transferrin receptor from placenta[6] and from human duodenum,[7] suggesting that the function of HFE is to alter the kinetics of transferrin receptor mediated iron uptake. Further evidence that HFE alters transferrin iron uptake comes from studies in which HFE has been over-expressed in cell lines. Feder *et al.*[8] found that HFE lowered the affinity of transferrin receptor for diferric transferrin, whilst several studies have demonstrated reduced iron uptake from transferrin by cells over-expressing HFE.[9-12] One of the key problems in understanding how HFE mutations may lead to the development of iron overload is to determine how HFE protein expression alters iron turnover in the body. The HFE protein is strongly expressed in placenta,[6] the duodenal crypts[7] and in Küpffer cells of the liver.[13] Thus major sites of HFE expression coincide with principal sites of iron handling. It might be supposed that if HFE expression prevents the uptake of excess iron then in genetic hemochromatosis, in which HFE function is compromised, patients would accumulate iron at these sites. However, villus epithelial cells and the Küpffer cells of the liver contain little

iron in patients with genetic hemochromatosis.[1] Similar findings have been reported for the, $\beta_2$-microglobulin knock-out mouse[14] and the *HFE* knockout model.[15] In addition, the transfection of *wildtype* HFE into monocytes from hemochromatosis (*C282Y* homozygous) patients resulted in increased iron and ferritin accumulation.[16] Townsend and Drakesmith[17] reported a similar finding in the reticuloendothelial cell line THP-1 and hypothesized that HFE may act, in some cells, by inhibiting the export of iron from cells, perhaps by interaction with ferroportin. There is evidence of some disturbance of erythropoiesis in patients with genetic hemochromatosis, including a raised red cell hemoglobin concentration and increased mean cell volume (MCV).[18] Ferrokinetic comparisons of hemochromatosis patients and normal subjects have revealed that there is a 40% increase in plasma iron turnover in hemochromatosis patients.[19] Cavill *et al.*[20] demonstrated that this was attributable to increased erythropoietic turnover. Furthermore, erythroid iron uptake occurs via transferrin receptor mediated endocytosis, the process that has been shown to be altered by HFE expression (see earlier). Until now, there have been no reports of the pattern of HFE expression in the erythroblast marrow, the major site of iron deployment. Investigation of the pattern of HFE expression in erythroid precursors is therefore of particular importance to the understanding of the role of HFE protein in iron handling and the effects of the *HFE* mutations for hemochromatosis patients.

The implications of altered erythroid iron handling in hemochromatosis may be far reaching as it has been proposed that increased erythroid iron turnover alone could drive an increased plasma iron turnover, leading to the iron overload seen in hemochromatosis patients.[21] In this study, we further investigated the erythroid iron demands of subjects homozygous for *HFE C282Y* and the distribution of HFE mRNA and protein expression among cultured primary human erythroid cells.

## Design and Methods

### Subjects

In a survey of 10,500 blood donors[4] 72 were found to be homozygous for *C282Y*. Sixty-five of these were later interviewed and a further blood sample was taken to confirm the genetic test result, to carry out a blood count and to determine serum ferritin, transferrin saturation and serum transferrin receptor concentration. Of these, 60 had neither been treated for iron overload nor given blood within one month of the interview. This study was approved by the Local Research Ethics Committee. Peripheral blood samples for HFE expression studies were obtained from control subjects who had been tested for *HFE C282Y* and *H63D* mutations (laboratory staff members who gave informed consent).

### Laboratory investigations

Blood counts were made using the Advia 120. Serum ferritin concentrations were determined with the Roche 2010 immunoanalyzer and serum iron, unsaturated iron binding capacity and transferrin saturation were determined as described by Worwood.[22] Serum transferrin receptor concentrations were determined with the Orion IdeA kit (Orion Comporitu, Espoo, Finland).

### Cells

#### Human cell lines

Human cell lines CCF-STTG1 (astrocytoma), CACO-2 (colon adenocarcinoma), Hep G2 (hepatocyte carcinoma) and V937 (histiocytic lymphoma) were obtained from the European Collection of Animal Cell Lines (Porton Down, Salisbury, UK) and cultured under the recommended conditions. The human B-lymphocyte line SUPT13 was kindly provided by Mr. S. Austin (Hematology Department, University Hospital of Wales).

#### Mononuclear cells

Mononuclear cells were purified from peripheral blood by density gradient separation with Lymphoprep (Life Technologies International). Monocytes and lymphocytes were separated according to their scatter characteristics using a FACSIII cell sorter (Beckton Dickinson). Granulocytes were separated from mononuclear cells and erythrocytes on Mono-Poly resolving medium (ICN). Purity was established by staining cytospin preparations with hematoxylin and eosin followed by examination under the light microscope. In terms of nucleated cells, each preparation was >95% pure. Granulocyte preparations contained some erythrocytes.

#### Macrophages

Macrophages were obtained by culturing normal peripheral blood mononuclear cells in RPM1-1640 medium containing 10% fetal calf serum in 25 $cm^2$ flasks for one week. Macrophages were harvested by discarding the culture medium and non-adherent cells followed by release of the macrophages by adding 3 mL trypsin-EDTA solution, incubating for 10 minutes at 37°C and scraping. The macrophages were washed in medium, and the samples split to prepare both cytospin slides and RNA.

### Culture of erythroid colonies

Platelet-depleted, non-adherent mononuclear cells

from normal peripheral blood were cultured at $4x10^5$/mL in semi-solid conditions using 0.9% methylcellulose (A4 Premium, Dow Chemicals, Orpington, UK) in serum-free medium.[23] Briefly, this medium was based on Iscove's modified Dulbecco's medium (Gibco BRL, Paisley, UK) and contained deionized bovine serum albumin, cholesterol, bovine insulin and α-monothioglycerol (all from Sigma, Poole, UK) and human, 30%-iron saturated transferrin (Boehringer Mannheim, Bracknell, UK). Recombinant human interleukin-3 (Sandoz, Bordon, Hants, UK) was added at 2.5 ng/mL and recombinant human erythropoietin (Roche, Lewes, UK) at 2 U/mL. Six 0.5 mL aliquots were cultured in each of two 24-well tissue culture plates (Nunc, Fisher, Loughborough, UK) with sterile distilled water placed in the outer wells to maintain humidity. The cells were incubated for 11 days at 37°C in a humidified incubator with 5% $CO_2$ in air. Burst-forming units erythroid (BFU-E) were sufficiently hemoglobinized by 10 days to allow easy visual identification using a Leitz Diavert inverted microscope. Individual BFU-E were extracted using a Gilson P20 micropipette. BFU-E from each well were pooled in 200 μL sterile PBS with 0.1% bovine serum albumin (Sigma). The study was repeated with cells harvested at 10 and 14 days.

### *Preparation of RNA*

Cell pellets (approx. $5x10^6$ cells) were lysed in 0.5-1 mL Rnazol B (Biogenesis), followed by vigorous vortexing of the suspension and storage at –70°C. RNA was extracted by the acid-guanidium isothiocyanate, phenol chloroform method designed by Chomczynski and Sacchi.[24]

### *Reverse transcription polymerase chain reaction (RT-PCR)*

Reverse transcription reactions were performed using the PE Applied Biosystems RNA, PCR Core Kit. HFE expression was detected with primers designed to amplify reverse transcribed HFE mRNA to produce a cDNA PCR product of 354bp and to amplify HFE genomic DNA to produce a PCR product of 563bp. $\beta_2$ microglobulin mRNA was amplified to produce a PCR product of 306bp. There was no amplification of a $\beta_2$ microglobulin PCR product from genomic DNA using the RT-PCR primers because of the long intronic sequence between the primers. RT-PCR to detect HFE expression was performed using the upstream primer 5' cag gac ctt ggt ctt tcc ttg ttt g 3' at 1 μM and the downstream primer 5' tgc tct cca atc cag tgt gtc agg 3' at 1 μM. The cycling conditions were: 1 cycle at 95°C for 120s then 38 cycles of 95°C for 40s, 62°C for 40s and 72°C for 60s. The Taq polymerase was Amplitaq (Applied Biosystems, Warrington, UK). The assay was controlled by amplification of $\beta_2$ microglobulin from the same cDNA samples using the primers 5' ctt agc tgt gct cgc gct ct 3' and 5' gac aaa gtc aca tgg ttc aca cgg 3', both at 0.25 μM final concentration. The cycling conditions for the PCR were identical to those for the HFE amplification. The PCR products were analyzed by agarose gel electrophoresis.

### *HFE antiserum*

The rabbit antiserum to a C-terminal peptide from HFE[7] ('CT-16 antibody') was kindly supplied by Dr. W. Sly, St. Louis University.

### *Flow cytometry using the CT-16 antibody*

Peripheral blood cells (whole blood) were fixed (Leukoperm Kit, Serotec, Oxford,UK) and washed in phosphate-buffered saline (PBS). Two aliquots were incubated with 5 μL anti-CD68:FITC antibody (Dako, Ely, UK), and another two were stained with 5 μL control anti-rabbit IgG:FITC antibody (Dako). After 15 min incubation at room temperature, two aliquots, one with and one without the FITC-labeled antibody marker, were incubated with CT-16 antiserum, whilst the other two samples were incubated with non-immune rabbit serum (final dilutions 1:40). Finally cells were incubated with goat anti-rabbit:PE antibody (Sigma, 1 in 8 dilution). The samples were washed, resuspended in 1 mL PBS, and analyzed immediately using a FACSIII flow cytometer (Beckton Dickinson).

The flow cytometry data were analyzed using Win MDI software. Monocytes were gated according to CD68 staining. Lymphocytes and granulocytes were gated according to their forward and side scatter characteristics. Histograms of each gated cell population stained with control non-immune serum and CT16 serum were compared in order to recognize HFE staining.

### *Fluorescent staining of cytospin preparations*

Cytospin preparations of peripheral blood leukocytes and pooled BFU-E were air dried, fixed in methanol for 5 minutes, dried, then incubated with 0.5% BSA in 50 mM Tris buffered saline (TBS) pH 7.5 for 30 minutes. The leukocyte slides were washed twice in TBS and stained with 1/100 dilution of CT-16 in 0.5% BSA in TBS for 1 hour at room temperature. A control slide was incubated with a 1/100 dilution of normal rabbit serum. The slides were washed three times in TBS, then stained with goat anti-rabbit immunoglobulins conjugated with PE (1 in 40 dilution, P-8172 Sigma) for one hour. The slides were washed three times in TBS, incubated with mouse anti-actin monoclonal antibody (1/20, A4700 Sigma), washed three times in TBS and incubated with goat anti-mouse IgG Fab specific, FITC conjugate (1 in 40

dilution, F4018 Sigma) to detect all of the cells present. For BFU-E the cells were incubated with a 1 in 100 dilution of CT16 primary antibody (control cells with 1 in 100 dilution of normal rabbit serum) followed by a 1 in 40 dilution of goat anti-rabbit IgG FITC conjugate (F-1262 Sigma). Cells were then stained with a 1 in 50 dilution of RPE-conjugated mouse anti-glycophorin A (Dako). Control cells were stained with RPE-conjugated mouse IgG1 (Dako). The slides were washed in TBS, in sterile water, mounted using glycergel aqueous mountant (Sigma) and inspected using a fluorescent microscope (Zeiss Axiophot). Images were captured with a digital camera, using equivalent exposure settings for each sample in order to allow a direct comparison of CT16 antiserum staining.

## Results

### *Erythropoiesis in asymptomatic subjects homozygous for HFE C282Y*

The median interval between the last blood donation and testing was 217 days. Over the previous 3 years the men had donated 3.9±1.4 (SD) units of whole blood and the women 2.8±1.6 units. Hematology and iron status are summarized in Table 1. None of the subjects was anemic, the red cell count and MCH were normal but the mean reticulocyte count was raised. This did not correlate with frequency of blood donation, time elapsed since last donation, or transferrin saturation. The percentage reticulocyte count (mean 2.0%) was also above the reference range (0.5-1.6%). The transferrin saturation was > 50% in 10 of 36 women (28%) and 144 of 24 men (58%). Most subjects had normal levels of serum ferritin (male, 15-300 μg/L and female 15-200 μg/L). One man had serum ferritin >1,000 μg/L and three women had serum ferritin <15 μg/L. Median levels of serum transferrin receptor were at the lower border of the reference range. Stepwise regression analysis of sTfR concentration against serum iron, TIBC, transferrin saturation, ferritin, Hb, reticulocyte count and frequency of blood donation revealed only a correlation with transferrin saturation (Figure 1).

**Table 1. Hematologic parameters and iron status in 60 subjects homozygous for *HFE C282Y* identified by genetic testing of 10,500 blood donors.**

| *Variable* | *Reference range* | *Mean ± SD* *Female (n=36)* | *Male (n=24)* |
|---|---|---|---|
| Hb (g/dL) | 11.5–15.5 F<br>13.0–16.5 M | 13.7±0.9 | 15.3±1.0 |
| RBC ($\times 10^{12}$/L) | 3.4–5.2 F<br>3.8– 5.6 M | 4.4±0.4 | 4.8±0.3 |
| MCH (pg) | 27.0–34.0 | 31.4±1.5 | 31.7±1.4 |
| Reticulocyte count ($\times 10^{9}$/L) | 20–70 | 89±32 | 96±34 |
| % reticulocytes | 0.5–1.6 | 2.0±0.7 | 2.0±0.7 |
| Transferrin saturation (%) | <50% | 44±22 | 58±20 |
| Serum ferritin (μg/L)* | 15–200F<br>15–300 M | 55 (30–101) | 236 (109–393) |
| Transferrin receptor (mg/L)* | 1.3–3.3 | 1.3 (0.9–1.8) | 1.1 (0.8–2.2) |

** median (1st and 3rd quartiles).*

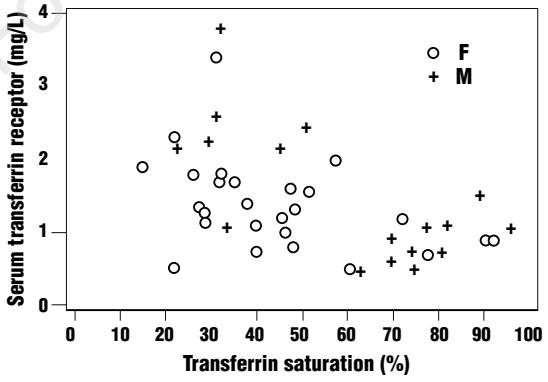


**Figure 1. Correlation between sTfR concentration and transferrin saturation in the 44 subjects for whom sTfR concentrations were available. The regression equation is y: 2.25 - 0.0163x, $R^2$: 0.248, *p*=0.001.**

### *HFE expression in blood cells*

#### *HFE mRNA expression by RT-PCR*

Lymphoid cell lines and all granulocyte and lymphocyte preparations examined were negative for HFE expression (Table 2). The expression of the control gene, $\beta_2$-microglobulin, was detected in all. Expression was detected in 1 of 3 monocyte preparations, and in macrophages derived from cultured monocytes. Erythroid colonies were grown from normal, peripheral blood mononuclear cells and in the first preparation mRNA was detected by RT-PCR. However the colonies contained some macrophages (derived from monocytes present in the mononuclear cell preparation). When erythroid colonies (harvested at 10, 12 and 14 days) were grown from mononuclear cells from peripheral blood depleted of monocytes, mRNA was not detectable by RT-PCR whilst the control $\beta_2$-microglobulin mRNA was detected.

### *HFE protein expression*

In order to characterize the expression profile of HFE within peripheral blood leukocytes further, the

**Table 2. HFE mRNA expression in various tissues, cultured primary cells and cell lines.**

| *Cell type* | *HFE expression (++, +, or none)* |
|---|---|
| CCF-STTG1 (human astrocytoma) | ++ |
| CACO-2 (human colon adenocarcinoma) | ++ |
| HepG2 (human hepatocyte carcinoma) | ++ |
| U937 (human histiocytic lymphoma) | Negative |
| SUPT13 (human B-lymphocyte line) | Negative |
| Human duodenal biopsy | ++ |
| Undifferentiated K562 cells (human CML) | ++ |
| Cultured vascular endothelial cells | + |
| Peripheral blood monocytes | 1/3 + |
| Peripheral blood lymphocytes | Negative |
| Macrophages derived from PB monocytes | ++ |
| Peripheral blood reticulocytes | Negative |
| Peripheral blood granulocytes | Negative |
| Erythroid colonies (from monocyte-depleted mononuclear cells) harvested at day 10 and 11 | Negative |

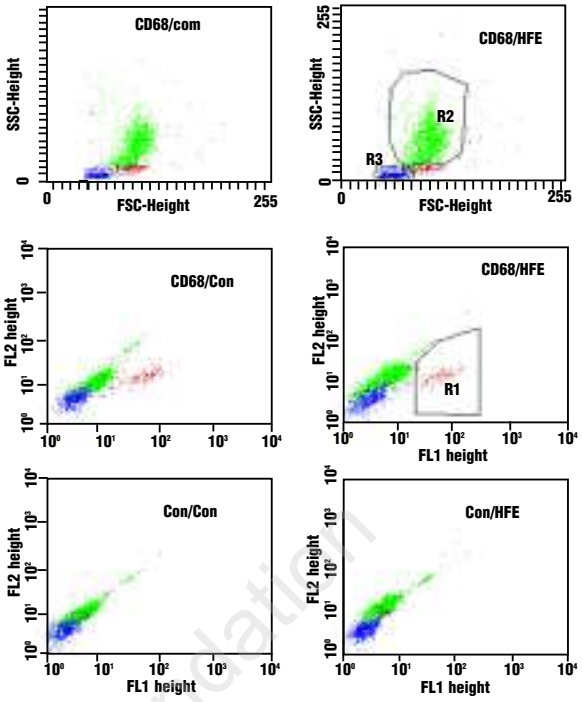


**Figure 2A.** Flow cytometry analysis of HFE expression in normal peripheral blood. Dot plots of peripheral blood flow cytometry data showing the gating used for analysis. The top two dot plots in each column show the scatter characteristics of the peripheral blood cells stained with CD68 antibody and non-immune serum (CD68/Con) and of the peripheral blood cells stained with CD68 antibody and with CT16 HFE antibody (CD68/HFE). Below these are the dot plots of fluorescence for the same samples: FL-1 represents FITC fluorescence (CD68 staining), whilst FL-2 represents phycoerythrin fluorescence (HFE staining). The HFE staining of the granulocytes (green) can be seen clearly. The bottom dot plots shows the fluorescence of cells stained with control-FITC antibody and for HFE with CT16 antibody (Con/HFE) together with the dual control stain (Con/Con). The logic gates R1 for monocytes, R2 for granulocytes and R3 for lymphocytes are shown.

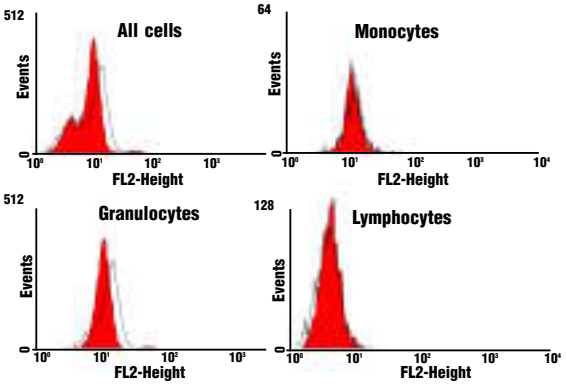


**Figure 2B.** Flow cytometry analysis of HFE expression in normal peripheral blood. The pattern of HFE expression in normal peripheral blood is illustrated as histograms of phycoerythrin fluorescence for the different peripheral blood cell subsets which are distinguished according to the gating shown in Figure 2A. The red histograms represent fluorescence for cells stained with control non-immune rabbit serum, whilst the overlaid unshaded histograms represent staining for HFE using the CT16 polyclonal antibody. The *all cells* histogram shows that a subset of peripheral blood cells stained for HFE, whilst many did not. Monocytes and lymphocytes did not stain for HFE, whilst the granulocytes did. Fluorescence (FL-2 height) is shown on the horizontal axes and is in logarithimic scale, the numbers of cells (events) are shown on the vertical axes. Note that the events axes are to different scales.

expression of HFE protein was investigated using the CT16 antiserum to HFE (Figure 2). The most efficient strategy for distiguishing lymphocytes, monocytes and granulocytes for HFE expression analysis is by flow cytometry. Peripheral blood granulocytes contain multi-globular nuclei and have a highly granular cytoplasm; these gross morphological features alter the light scatter characteristics of cells such that they can be readily identified, using flow cytometry, in non-malignant peripheral blood samples solely on the basis of their increased side scatter (Figure 2A) compared to that of lymphocytes and monocytes (granulocytes are gated as R2 and colored green in the dot plots). The lymphocytes and monocytes can also be distinguished by subtle differences in their scatter characteristics, although it is more reliable to use an antigenic marker unique to one cell type. Expression of CD68 (detected on the FL-2 channel) was used in this instance to identify monocytes positively (gated R1 and colored red in dot plots, Figure 2A). The remaining cells with low forward and side scatter characteristics were regarded as being lymphocytes (gated R3 and colored blue in the dot plots). Having identified the respective cell populations, comparisons of HFE staining to control non-immune

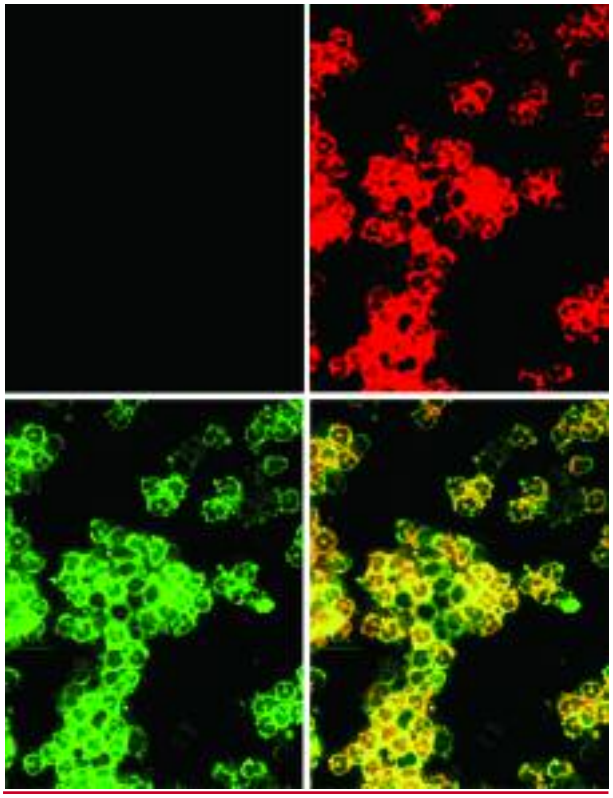

**Figure 3. An example of the immunofluorescent staining for HFE protein in peripheral blood leukocytes obtained using the CT16 antibody. Top left: The fluoresence obtained from a control sample subjected to staining with the non-immune serum and secondary antibody alone. A test sample triple stained for β-actin and HFE is shown with its two color components, bottom left, β-actin staining alone (green), top right, HFE CT-16 antibody staining alone (red) and bottom right, merged demonstrating that a subset of peripheral blood leukocytes stained positively for HFE.**

serum staining, both detected with a secondary antibody conjugated to the fluor FITC were performed for each of the gated cell populations and, for completeness, all cells analyzed. In these comparisons (Figure 2B), an increase in a given population's fluorescence is indicative of HFE CT16 antiserum staining. A subset of the 'all cells' population was weakly stained by the CT16 antiserum and when the individual cell populations were examined, this subset was shown to be granulocytes, whilst neither lymphocytes nor monocytes were stained more by the antibody than by an an equivalent concentration of non-immune serum.

In order to both confirm and visualize the flow cytometry results, leukocytes prepared from peripheral blood were attached to a microscope slide by cytospin and subjected to immunofluorescent staining with either the CT16 antiserum or with an equivalent concentration of non-immune serum. In order to stain 'all cells' and to aid subcellular localization of the staining by the CT16 antiserum, this was detected using a secondary antibody conjugated to phycoerythrin. Actin was detected using an anti-actin antibody and a FITC-conjugated secondary antibody.

Figure 3A shows the staining achieved using non-immune rabbit serum and the secondary goat anti-rabbit immunoglobulins conjugated with PE. This control slide demonstrated that the *red* phycoerythrin fluorescence observed in *test* samples was due to the staining from the CT16 antiserum. Figure 3B-D demonstrates the fluorescence obtained from a sample stained using the CT16 antiserum and anti-B actin antibodies. There was a clear subset of peripheral blood cells with complex multi-globular nuclei (the dark holes), consistent with granulocyte cells, which stained for HFE (HFE CT16 antibody), whilst the remaining cells (almost exclusively lymphocytes in these types of preparation) were not stained for HFE. BFU-E (harvested at 10 and 14 days) were negative on staining cytospin preparations with CT16 anti-serum (*data not shown*).

## Discussion

It has been shown by the study of transfected cells and knock-out mice that the HFE protein modifies transferrin iron uptake by the transferrin receptor; however, these observations do not provide a direct link between loss of HFE function and enhanced iron absorption in subjects with hereditary hemochromatosis and do not provide a convincing explanation as to how the C282Y mutated protein might causes enhanced iron absorption.[17] Recently evidence has been presented to suggest that HFE protein is required for the regulation of hepcidin synthesis in the liver.[25] Hepcidin levels in the plasma normally correlate with levels of storage iron and regulate iron uptake at the apex of the intestinal mucosa by regulating the synthesis of iron transport proteins including DcytB[26] and DMT1.[27] The degree of regulation of iron uptake by hepcidin in erythroid bone marrow is not known. However, hemoglobin synthesis does not appear to be limited in hemochromatosis as patients can usually tolerate weekly or even twice weekly phlebotomy of 450 mL blood.

An alternative proposal concerns the regulation of iron supply to nucleated red cells in the bone marrow. If the HFE protein is expressed in erythroid precursors and HFE normally reduces iron uptake from transferrin it is likely that the C282Y mutated protein will permit increased erythroid iron uptake. The resulting increase in plasma iron turnover would lead to an increase in intestinal iron absorption and the

deposition of iron throughout the body.[21]

In this study, we found that asymptomatic hemochromatosis subjects had normal hemoglobin and red cell hemoglobin concentrations and red cell counts, but slightly raised mean reticulocyte counts, indicating increased erythropoeisis. This might be expected to lead to an increase in serum transferrin receptor concentration but serum transferrin receptor concentrations were low when compared to the reference range. This probably reflects the effect of the increased iron stores as the transferrin receptor concentration correlated inversely with transferrin saturation. Thus the current study provides evidence for an increased rate of erythropoeisis among subjects with the hemochromatosis *HFE C282Y* homozygous genotype.

Given that loss of HFE function due to the *C282Y* mutation may be associated with changes in erythroid iron handling, we investigated the expression of HFE in nucleated red cells and in white cells. In peripheral blood, although granulocytes expressed HFE protein, they no longer contained mRNA indicating that HFE expression had been down-regulated. Monocytes showed little expression but macrophages derived from peripheral blood monocytes expressed HFE mRNA. No HFE expression was detected in lymphoid cells. Among maturing erythroid cells, cultures of primary differentiated cells were found not to express HFE mRNA or protein. Currently, there is agreement that monocytes and macrophages express HFE protein;[13,16] but there are conflicting reports about expression of HFE in lymphoid cells. Feder *et al.*[2] reported weak expression of HFE on Northern blotting and Chitamber *et al.*[28] reported expression for lymphoblastoid cell lines derived from normal lymphocytes. Parkilla *et al.*[29] did not detect expression of mRNA in lymphocytes and neither did we.

There have, to our knowledge, been no previous reports about HFE expression in human erythroid cells, where most iron is utilized. During erythroid development iron uptake is maximal during the early normoblast stage and declines during maturation to the reticulocyte[30]. Cultured erythroid colonies (mostly intermediate and late normoblasts) did not express HFE protein. Therefore, it is likely that HFE does not play a direct role in the regulation of transferrin iron uptake by erythroid bone marrow cells. Thus transferrin receptor mediated erythroid iron uptake would proceed without the hurdle of HFE intercession. Furthermore, within the bone marrow, erythroid cells surround macrophages and may obtain some iron directly from the macrophages via ferritin – a process called *rhopheocytosis* by Policard and Bessis;[31] this is another process independent of the direct effects of HFE. Erythroblasts also have ferritin receptors that can scavenge extracellular ferritin.[32] However, macrophages express HFE protein, and the C282Y mutation may modify HFE function and thus iron transport within the macrophage and so influence red cell iron uptake.[16] The quantitative significance of these pathways of iron supply is not well understood. Interestingly, investigations into the effects of *HFE C282Y* mutations on the levels of circulating non-transferrin bound iron have shown that significantly higher levels of non-transferrin bound iron, which can catalyze lipid peroxidation, are present in both *HFE C282Y* homozygotes (1.79 μmol/L) and in *C282Y* heterozygotes (0.51 μmol/L) than in control subjects (-0.3 μmol/L).[33] These results indicate that greater than normal levels of non-transferrin bound iron are present in the circulation of subjects with *HFE* C282Y mutations before substantial iron loading develops. These increased levels of non-transferrin bound iron, which continue to increase as hereditary hemochromatosis develops,[34-37] perhaps combined with increases in intracellular erythrocyte non-heme iron,[38] may lead to weakening of red cell plasma membranes,[39,40,] generalized oxidative damage,[39] and shortening of the red cell life-span. The necessary increase in the rate of erythropoeisis, and therefore increase in plasma iron turnover, may be one contributor to the gradual, progressive iron loading observed in hereditary hemochromatosis patients.

In summary, we present new evidence of altered erythropoeisis in *HFE C282Y* homozygotes and describe the distribution of HFE mRNA and protein expression in normal, peripheral blood cells and BFU-E derived from peripheral blood cells. Despite the changes in erythropoeisis, HFE expression was not found in the cells that are most active in iron uptake and hemoglobin production.

*GPF: carried out the cellular RNA and immunological studies and co-wrote the first draft of the paper; KC: carried out the laboratory work on the blood donor samples; GSM: carried out the BFU cultures; HAJ: clinician who undertook the study of blood donors and provided relevant data; IC: responsible for the data and interpretation of the studies of erythropoiesis; MW: planned the study, co-wrote the first draft. All authors were involved in revising the manuscript and approved the final version. The authors declare that they have no potential conflicts of interest.*

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