SMALL INTESTINE

Duodenal expression of iron transport molecules in untreated haemochromatosis subjects

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Background and aims: In HFE associated hereditary haemochromatosis, the duodenal enterocyte behaves as if iron deficient and previous reports have shown increased duodenal expression of divalent metal transporter 1 (*DMT1*) and iron regulated gene 1 (*Ireg1*) in affected subjects. In those studies, many patients had undergone venesection, which is a potent stimulus of iron absorption. Our study investigated duodenal expression of *DMT1* (IRE and non-IRE), *Ireg1*, *hephaestin*, and duodenal cytochrome-b (*Dcytb*) in untreated C282Y homozygous haemochromatosis patients, iron deficient patients, and iron replete subjects.

Methods: Total RNA was extracted from duodenal biopsies and expression of the iron transport genes was assessed by ribonuclease protection assay.

Results: Expression of *DMT1* (IRE) and *Ireg1* was increased 3–5-fold in iron deficient subjects compared with iron replete subjects. Duodenal expression of *DMT1* (IRE) and *Ireg1* was similar in haemochromatosis patients and iron replete subjects but in haemochromatosis patients with elevated serum ferritin concentrations, both *DMT1* (IRE) and *Ireg1* expression were inappropriately increased relative to serum ferritin concentration. *Hephaestin* and *Dcytb* levels were not upregulated in haemochromatosis. *DMT1* (IRE) and *Ireg1* levels showed significant inverse correlations with serum ferritin concentration in each group of patients.

Conclusions: These findings are consistent with *DMT1* (IRE) and *Ireg1* playing primary roles in the adaptive response to iron deficiency. Untreated haemochromatosis patients showed inappropriate increases in *DMT1* (IRE) and *Ireg1* expression for a given level of serum ferritin concentration, although the actual level of expression of these iron transport genes was not significantly different from that of normal subjects.

Hereditary haemochromatosis (HHC) is a common autosomal recessive disorder of iron metabolism characterised by progressive accumulation of iron, primarily in the liver, pancreas, and heart, which may ultimately result in organ failure.¹ The majority (80–90%) of patients with HHC are homozygous for a missense mutation that results in substitution of tyrosine for cysteine at position 282 (C282Y) in the haemochromatosis protein, HFE.^{2–4} A major pathophysiological feature of HHC is a rate of intestinal iron absorption that is inappropriately elevated for a given level of body iron stores. The precise mechanism by which HFE regulates intestinal iron absorption is unknown but the available evidence indicates that in HHC the duodenal enterocyte behaves as if it is iron deficient. This conclusion is supported by studies showing that in patients with HHC, the duodenal mucosal content of ferritin mRNA and protein is relatively low while the corresponding levels of transferrin receptor gene and protein expression are relatively high, and the binding affinity of iron regulatory protein is increased.^{5–8}

Iron absorption involves the uptake of iron from the lumen of the small intestine into the mature duodenal enterocyte, followed by its basolateral transfer into the portal circulation.⁹ Kinetic studies suggest that the transfer of iron across the basolateral membrane is the main regulatory step in normal subjects and HHC patients.^{10–12} Each iron transport step involves a transmembrane transporter that is likely to be coupled to an enzyme that changes the oxidation state of iron. Our understanding of intestinal iron absorption has improved in recent years following identification of several key proteins involved in the trafficking of iron into and out of cells. These include divalent metal transporter 1 (*DMT1*), iron regulated

gene 1 (*Ireg1* also known as SCL11A3, ferroportin1, or MTP1), hephaestin, and duodenal cytochrome-b (*Dcytb*).

DMT1 is a brush border transmembrane transporter for several divalent metals, including iron, copper, and zinc. It pumps iron into the enterocyte via an active proton coupled transport mechanism.^{13–14} At least two alternatively spliced transcripts of *DMT1* exist, encoding alternative carboxy termini and 3' untranslated regions (UTRs). The 3' UTR of one variant contains a consensus iron responsive element (IRE) sequence that binds to iron regulatory proteins. *DMT1* transports ferrous iron, indicating that dietary iron (which is mainly in the ferric form) must first be reduced before it can be utilised. A strong candidate for this ferrireductase activity is *Dcytb*, an enzyme that was recently identified as upregulated in the duodenal mucosa of the *hpx* mouse.¹⁵ *Dcytb* localises to the brush border membranes of mature enterocytes of the upper villous region, and both protein and mRNA levels are regulated by hypoxia, iron deficiency, and anaemia.¹⁵

Ireg1 is predicted to function as an iron transporter on the basolateral membrane of the mature villous enterocyte and elevated duodenal *Ireg1* mRNA levels are observed in situations associated with increased intestinal iron absorption.^{16–18} *Ireg1* is postulated to associate with hephaestin, a membrane bound ceruloplasmin homologue with ferroxidase activity.¹⁹

Abbreviations: *Dcytb*, duodenal cytochrome-b; *DMT1*, divalent metal transporter 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HHC, hereditary haemochromatosis; IRE, iron responsive element; *Ireg1*, iron regulated gene 1; RPA, ribonuclease protection assay; SF, serum ferritin concentration; TS, transferrin saturation; UTR, untranslated region.

Recently, Zoller *et al* reported 100-fold increase in duodenal *DMT1* expression in 20 C282Y homozygous HHC subjects who were undergoing, or who had recently completed, therapeutic phlebotomy, compared with 10 subjects with normal body iron stores.²⁰ Subsequently, the same authors (using a different technique) reported much lower increases, 8–10-fold and 2–5-fold in duodenal *DMT1* and *Ireg1* expression, respectively, in eight untreated C282Y homozygous HHC subjects compared with normal subjects, and reported that expression of these genes was similar to that observed in 19 subjects with iron deficiency.²¹ Rolfs *et al* have reported similar findings of increased *DMT1* and *Ireg1* expression in nine C282Y homozygous HHC subjects, all of whom had been treated to a state of iron deficiency or were undergoing venesection at the time of duodenal biopsy.²² Prior studies indicate that venesection per se causes a significant increase in iron absorption in HHC patients^{10 23 24} and inclusion of phlebotomised subjects may have had a confounding effect on the results of these studies. Thus despite recent advances, the relationship between body iron stores and expression of these iron transporters has not been clearly defined in untreated HHC patients. To address this, duodenal expression of *DMT1*, *Ireg1*, *hephaestin*, and *Dcytb* was investigated in untreated C282Y homozygous HHC patients, subjects with iron deficiency, and subjects with normal biochemical iron indices. Expression of these iron transport genes was related to serum iron indices and haematological parameters.

METHODS

Study population

Twelve C282Y homozygous HHC patients, 11 subjects with iron deficiency, and 13 iron replete subjects (that is, normal serum iron indices) were studied. The 12 HHC subjects were diagnosed in a tertiary hospital phenotypic screening study²⁵ whereby subjects with a corrected total iron binding capacity $\geq 40\%$ were genotyped for the common mutations in the HFE gene. All subjects homozygous for the C282Y mutation were contacted and reviewed in a dedicated hepatology clinic. Percutaneous liver biopsy was performed at the time of diagnosis in six HHC subjects, all of whom had (i) a serum ferritin concentration (SF) ≥ 1000 $\mu\text{g/L}$, (ii) elevated serum alanine or aspartate transaminase levels, (iii) hepatomegaly (assessed either clinically or ultrasonographically)²⁶ and/or (iv) coexistent cause for liver disease. Hepatic iron stores were assessed and graded following Perls' Prussian blue stain by a single hepatopathologist according to the method described by Searle and colleagues.²⁷ Quantitative hepatic iron measurements were performed on fresh liver tissue using a modification of the colorimetric method of Torrance and Bothwell.²⁸

All study subjects underwent gastroscopy for investigation of a range of clinical indications, including dyspepsia, iron deficiency, anaemia, heartburn, or dysphagia. For the purposes of this study, iron deficiency was defined as a transferrin saturation (TS) of $<15\%$ and an SF of <30 $\mu\text{g/L}$, or documented absence of iron stores on bone marrow biopsy. Subjects in the iron replete group had serum iron indices within the laboratory reference ranges (that is, TS 15–45%; SF 30–300 $\mu\text{g/L}$). HFE genotyping was determined in non-HHC subjects, as described elsewhere.⁴

Fasting serum iron indices and haematological parameters were measured in all 36 subjects within two weeks of gastroscopy. Patient records were reviewed for a history of red blood cell or iron transfusions, or upper gastrointestinal bleeding during the weeks preceding gastroscopy. None of the subjects was a regular blood donor or taking oral iron during the study. Written consent was obtained from all subjects and the study was approved by the Princess Alexandra Hospital Research Ethics Committee.

Tissue collection

All duodenal biopsy specimens were obtained before phlebotomy was initiated in HHC subjects with significant iron

overload who required treatment. Biopsies were collected from the second part of the duodenum, snap frozen in liquid nitrogen, and stored at -70°C for total RNA extraction at a later date.

RNA preparation and ribonuclease protection assays

Total RNA was extracted from two pooled duodenal biopsies from each individual using TRIzol reagent (Invitrogen, Melbourne, Australia) according to the manufacturer's instructions. Ribonuclease protection assays (RPAs) were performed on total RNA using riboprobes corresponding to the cDNA sequences (table 1). Riboprobe synthesis was performed using the appropriate linearised vector and the Riboprobe Combination System (SP6/T7 polymerase kit; Promega, Madison, Wisconsin, USA) with minor modifications. Each transcription was carried out in a 20 μL reaction containing 40 mM Tris HCl (pH 7.9), 10 mM NaCl, 6 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM each of rATP, rGTP, and rCTP, 17.5 μM cold rUTP, 1 μg vector DNA, 50 μCi [α -³²P]UTP (3000 Ci/mmol; NEN, Adelaide, Australia), 20 U RNasin, and 15–20 U T7 or SP6 RNA polymerase, as appropriate. The transcription reaction was incubated at 37°C for one hour before digestion with 1 U RQ1 RNase-Free DNaseI (Promega) for a further 15 minutes at 37°C . The riboprobes were electrophoresed on a 6% polyacrylamide, 50% urea gel for three hours at 200 V. Riboprobes visualised using autoradiography were excised and eluted for three hours at 37°C in 400 μL elution buffer (2 M ammonium acetate, 1% sodium dodecyl sulphate, 25 $\mu\text{g/ml}$ yeast tRNA) before ethanol precipitation and resuspension in 50 μL of hybridisation buffer (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide).

Human duodenal RNA samples (2 μg) were hybridised overnight at 45°C with 10^5 cpm of all riboprobes. Following hybridisation, unprotected RNA was digested by addition of RNase digestion buffer (10 mM Tris HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA) containing 80 $\mu\text{g/ml}$ of RNase A and 80 U/ml of RNase T1 (Roche Molecular Biochemicals, Castle Hill, Australia), and incubated at 37°C for 60 minutes. The reaction was extracted with 400 μL of phenol/chloroform/isoamyl alcohol (25:24:1) and the RNA-riboprobe hybrid precipitated with ethanol. Hybridised riboprobe samples were resolved on a 6% polyacrylamide, 50% urea gel for five hours at 200 V and detected after exposure for up to 96 hours at -70°C . The intensity of specific bands was determined by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, California, USA). All RPAs were examined at different exposures and only those bands that lay within the linear range of the film were analysed. Expression of each of the iron transport genes was reported as a ratio of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to correct for differences in total mRNA loading.

Table 1 cDNA sequences of riboprobes

	Size (nt)	cDNA sequence	GenBank accession No.
<i>DMT1</i> (non-IRE)	273	nt 1501–1773	AF064484*
<i>DMT1</i> (IRE)	224		
<i>Ireg1</i>	173	nt 1190–1362	AF231121
<i>Hephaestin</i>	190	nt 1207–1396	AF148860
<i>Dcytb</i>	242	nt 272–513	XM015916
<i>GAPDH</i>	155	nt 580–734	M33197

*This riboprobe is based on the non-IRE splice variant but can also detect the iron responsive element (IRE) form as it crosses the splice site that distinguishes the two transcripts.
Dcytb, duodenal cytochrome-b; *DMT1*, divalent metal transporter 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *Ireg1*, iron regulated gene 1.

Table 2 Clinical and laboratory characteristics of the study population

	Haemochromatosis (n=12)	Iron deficient (n=11)	Iron replete (n=13)	p Value
Sex (M:F)	9:3	4:7	7:6	0.174
Age (y)	44.8 (15.9)* [17–69]	60.1 (19.6) [19–80]	57.4 (11.6) [39–77]	0.05
Hb (g/l)	148.8 (14.3)	106.2 (17.2)†††	142.1 (11.6)	<0.001
Hct (%)	43.9 (3.9)	33.6 (4.4)†††	42.9 (2.9)	<0.001
TS (%)†††	63.0 (16.0)	6.7 (3.1)	25.5 (8.6)	<0.001
SF (µg/l)†††	900 (39–1806)	13 (2–65)	137 (31–244)	<0.001

Hb, haemoglobin; Hct, haematocrit; TS, transferrin saturation; SF, serum ferritin concentration.

Laboratory reference ranges for TS, SF, Hb, and Hct are 15–45%, 30–300 µg/l, 135–180 g/l, and 39–52%, respectively.

Data expressed as mean (SD) [range] or median (range).

Pearson's χ^2 analysis was used to determine significant differences between groups for sex distribution. For all other variables, ANOVA was used to compare data between the three groups (right hand column) and the Student's *t* test to compare data between two specific groups.

* $p < 0.05$ for subjects with hereditary haemochromatosis (HHC) compared with iron deficient or iron replete subjects; ††† $p < 0.001$ for iron deficient subjects compared with HHC patients or iron replete subjects;

††† $p < 0.001$ for HHC subjects compared with both other groups and iron deficient subjects compared with iron replete subjects.

Statistical analysis

All patient demographic and laboratory data are expressed as mean (SD), except for SF which is presented as median (range). Duodenal levels of the iron transport genes were expressed as a ratio of *GAPDH* and reported as mean (SEM). SF and iron transport gene ratios showed a skewed distribution and were converted to logarithms before further statistical analysis. All statistical analyses, including the Student's *t* test, analysis of variance, Pearson's correlation, and regression analysis were performed on logarithmically transformed data. Comparisons between proportions were made by Pearson's χ^2 analysis. A *p* value of ≤ 0.05 was regarded as statistically significant.

RESULTS

Demographic and laboratory characteristics of the study population

Age and sex distributions, and laboratory characteristics of the 36 patients included in the study are shown in table 2. Sixteen of 36 patients were female. While the majority of iron deficient subjects were female, most HHC patients were male. Mean age of the HHC patients was significantly lower than subjects in the iron deficient and iron replete groups ($p = 0.05$). There were significant differences in mean haemoglobin concentration, haematocrit, TS, and median SF between the three groups ($p < 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively).

All HHC patients had TS levels $> 40\%$ but three subjects, all female, had normal SF values (39, 59, 65 µg/l). Two of these women were of child bearing age and still menstruating, and the third was aged 56 years with a history of eight pregnancies. The remaining nine subjects were male and all had SF values > 504 µg/l. Of the six HHC patients who underwent liver biopsy, five subjects had minimal or no fibrosis and one subject had evolving cirrhosis. Three HHC subjects, all male, had a history of heavy alcohol consumption (> 60 g/day) but only one had mild steatosis on liver biopsy. Another patient with a body mass index of greater than 30 kg/m² but no history of alcohol consumption had severe steatosis. All six patients had grade 3 or 4 stainable liver iron and a hepatic iron concentration between 47 and 160 µmol/g dry weight.

Expression of *DMT*, *Ireg1*, *hephaestin*, and *Dcytb*

mRNA levels of the iron transport molecules were examined by RPA and assays of three representative patients from each group are shown in fig 1A. Figure 1B shows mean levels for *DMT1* (*IRE*), *Ireg1*, *hephaestin*, and *Dcytb* expressed as a percentage of the relative expression of *GAPDH*. There were significant differences in mean levels of *DMT1* (*IRE*), *Ireg1*, and

hephaestin between the three groups ($p < 0.001$, $p < 0.001$, and $p = 0.015$, respectively) but there was no significant difference between the groups in *Dcytb* expression ($p = 0.12$). Duodenal expression of *DMT* (*IRE*) and *Ireg1* was similar in HHC patients and iron replete subjects (0.54 (0.07) v 0.59 (0.11), $p = 0.95$;

Figure 1 Duodenal expression of iron transport genes. (A) Ribonuclease protection assays of three representative patients from each of the hereditary haemochromatosis (HHC) (n=12), iron deficient (n=11), and iron replete (n=13) groups of patient. Total RNA was extracted from duodenal biopsies and gene expression determined by RPA using 2 µg of RNA. Gels were autoradiographed for varying times due to variations in the level of expression between the iron transport genes, and exposure times are indicated on the right. *DMT1*, divalent metal transporter 1; *Dcytb*, duodenal cytochrome-b; *Ireg1*, iron regulated gene 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. (B) Relative gene intensities were estimated from densitometric scanning of autoradiographs. Values are expressed relative to *GAPDH* expression and are presented as mean (SEM). *** $p < 0.001$ for *DMT1* (*IRE*) and *Ireg1* expression in iron deficient subjects compared with HHC patients or iron replete subjects; †† $p < 0.01$ for *hephaestin* expression in HHC patients compared with iron deficient or iron replete subjects; ‡ $p < 0.05$ for *Dcytb* expression in HHC subjects compared with iron deficient patients.

Table 3 Correlation between biochemical iron indices, haematological parameters, and expression of *DMT1 (IRE)* and *Ireg1* in non-HHC subjects and patients with HFE related haemochromatosis

	Hb	Hct	Log SF	TS
Non-HHC subjects (n=23)				
Log <i>DMT1 (IRE)</i>				
<i>r</i>	-0.680	-0.677	-0.569	-0.677
<i>p</i>	0.002	0.003	0.004	0.003
Log <i>Ireg1</i>				
<i>r</i>	-0.589	-0.574	-0.541	-0.667
<i>p</i>	0.002	0.003	0.007	0.004
HHC subjects (n=12)				
Log <i>DMT1 (IRE)</i>				
<i>r</i>	0.069	-0.244	-0.798	0.049
<i>p</i>	0.820	0.450	0.002	0.881
Log <i>Ireg1</i>				
<i>r</i>	-0.270	-0.432	-0.620	-0.102
<i>p</i>	0.390	0.171	0.030	0.760

Hb, haemoglobin concentration; Hct, haematocrit; TS, transferrin saturation; SF, serum ferritin concentration; HHC, hereditary haemochromatosis *DMT1*, divalent metal transporter 1; *Ireg1*, iron regulated gene 1.
Pearson's correlation coefficient was used to determine whether there was a linear relationship between gene expression of each iron transporter and TS, SF, Hb, or Hct.

0.78 (0.10) ν 0.61 (0.07), $p=0.25$; respectively). In contrast, *hephaestin* expression in HHC subjects was approximately 60% of values in iron replete subjects and iron deficient subjects (0.53 (0.05) ν 0.90 (0.09), $p=0.02$; ν 0.96 (0.14), $p=0.01$; respectively). Similarly, expression of *Dcytb* was decreased in HHC patients compared with iron deficient patients (0.62 (0.08) ν 1.32 (0.23); $p=0.02$) but not compared with iron replete subjects (1.12 (0.19); $p=0.22$).

Expression of *DMT1 (IRE)* and *Ireg1* was increased 4–5-fold and 3–4-fold, respectively, in patients with iron deficiency compared with iron replete subjects (2.96 (0.38) ν 0.59 (0.11), $p<0.0001$; 2.26 (0.18) ν 0.61 (0.07), $p<0.0001$) (fig 1B). In contrast, there were no significant differences in *hephaestin* or *Dcytb* expression in subjects with iron deficiency compared with iron replete subjects (0.96 (0.14) ν 0.90 (0.09), $p=0.76$; 1.32 (0.23) ν 1.12 (0.19), $p=0.44$, respectively). Expression of *DMT1 (non-IRE)* was extremely low in duodenal biopsies from all patients and did not contribute significantly to the level of total *DMT1* present (data not shown).

Expression of *DMT1 (IRE)*, *Ireg1*, *hephaestin*, and *Dcytb* in relation to serum iron indices and haematological parameters

In this series of untreated HHC patients, a strong inverse relationship between log SF and *DMT1 (IRE)* expression ($r=-0.789$, $p=0.002$) was found and a similar association was observed for *Ireg1* ($r=-0.621$, $p=0.03$) but there was no significant correlation between TS, haemoglobin concentration, or haematocrit and duodenal iron transport gene expression (table 3). In non-HHC subjects, strong inverse correlations were found between *DMT1 (IRE)* expression and haemoglobin concentration ($r=-0.680$, $p=0.0002$), haematocrit ($r=-0.677$, $p=0.003$), log SF ($r=-0.569$, $p=0.004$), and TS ($r=-0.677$, $p=0.003$) (table 3). Similar results were observed for *Ireg1* (table 3). There were no significant correlations between expression of *hephaestin* or *Dcytb* and serum iron indices or haematological parameters in any of the three groups.

In order to determine if *DMT1 (IRE)* and *Ireg1* expression were inappropriately increased in relation to body iron stores in HHC patients, the relationship between gene expression of these iron transporters and log SF was examined based on correlations observed in non-HHC patients (fig 2). Nine of 12

Figure 2 Association between serum ferritin concentration (SF) and gene expression of (A) divalent metal transporter 1 (*DMT1 (IRE)*) and (B) iron regulated gene 1 (*Ireg1*) in hereditary haemochromatosis (HHC) and non-HHC patients. Correlation between log SF and *DMT1 (IRE)* and *Ireg1* is shown for all study subjects (all single values). Regression lines (with 95% confidence intervals) are based on data from non-HHC patients only.

HHC patients lay outside and above the predicted linear extension of the regression lines for *DMT1 (IRE)* and *Ireg1* based on non-HHC patients, suggesting that for a given SF, *DMT1 (IRE)* and *Ireg1* levels were higher than predicted in HHC subjects with elevated SF. The three female HHC patients had appropriate levels of *DMT1 (IRE)* and *Ireg1* expression for their SF.

When all 36 subjects were analysed together, a significant positive correlation was found between expression of *DMT1 (IRE)* and *Ireg1* ($r=0.826$, $p<0.0001$). This relationship was still present when HHC patients ($r=0.754$, $p=0.005$) and non-HHC patients were analysed separately ($r=0.841$, $p<0.0001$).

DISCUSSION

Recent identification of several molecules that play critical roles in iron transport across the duodenal enterocyte has greatly advanced our understanding of intestinal iron absorption. The potential roles of *DMT1*, *Ireg1*, *hephaestin*, and *Dcytb* in this pathway have been examined in animal models of disturbed iron metabolism but in relatively few human studies.

In this study, duodenal expression of *DMT1 (IRE)* and *Ireg1* was similar in untreated HHC patients and iron replete subjects but in most of the HHC patients there appeared to be an increase in *DMT1 (IRE)* and *Ireg1* expression relative to SF. Furthermore, *hephaestin* and *Dcytb* were not upregulated in HHC and appear unlikely to play primary roles in the relative increase in iron absorption observed in HHC. As predicted, *DMT1 (IRE)* and *Ireg1* expression was increased in iron deficient patients compared with iron replete subjects, thus acting as internal controls and confirming that the laboratory methods used in this study were appropriate. Significant positive correlations were present between *DMT1 (IRE)* and *Ireg1*

expression in all patient groups. There were significant inverse correlations between *DMT1* (*IRE*) and *Ireg1* and SF in HHC patients, suggesting that body iron stores still play a role in the regulation of duodenal expression of these iron transport genes. Expression of these iron transporters was assessed by RPAs only as recent studies indicate that protein levels parallel changes observed in gene expression of these molecules.^{15 16 21 29}

Total body iron absorption may be normal^{12 23 24 30} or elevated only 2–3-fold in untreated HHC subjects^{10 31} compared with normal controls and this appears to be predominantly due to an increase in mucosal transfer and not to an increase in mucosal uptake of iron.^{10 12} Despite increased body iron stores, phlebotomy in HHC patients results in a further marked increase in total body iron absorption, indicating the importance of increased erythropoiesis and plasma iron turnover in the control of iron uptake and absorption.^{10 23 24 32} Iron absorption frequently remains elevated for a prolonged period after completion of venesection in HHC.^{10 23 24} In the original study of intestinal gene expression performed by Zoller *et al*, the 20 HHC subjects were a heterogeneous group.²⁰ The majority had completed treatment or were undergoing venesection at the time of the study and *DMT1* expression was reported to be increased 100-fold.²⁰ Similarly, in a recent study by Rolfs *et al*, five of nine subjects with HHC were fully treated and the remaining four subjects were being venesected.²² Venesection is likely to have contributed to the increase in *DMT1* expression reported by Zoller²⁰ and Rolfs²² and evidence for such an effect was provided in a subsequent study by Zoller *et al* who reported only a 2–10-fold increase in *DMT1* and *Ireg1* expression in eight untreated HHC patients with biochemical iron overload compared with normal controls.²¹ This marked variation in the reported increase in *DMT1* expression in HHC patients and conflicting data on iron transporter expression in animal models of iron overload mandates the need for further studies of iron transporters in untreated human HHC patients.

In contrast with previous reports, an increase in the absolute level of *DMT1* (*IRE*) or *Ireg1* expression in untreated HHC patients was not demonstrated in this study. However, whether the level of expression of these iron transport genes was inappropriately high for the degree of iron loading warranted further consideration. SF, a laboratory indicator of body iron stores,^{33 34} was significantly associated with expression of both *DMT1* (*IRE*) and *Ireg1* in non-HHC subjects. Figure 2A and 2B show that all but three of the HHC patients lay beyond and above the regression lines relating SF and *DMT1* (*IRE*) and *Ireg1* levels in non-HHC subjects, suggesting that for a given SF, *DMT1* (*IRE*) and *Ireg1* levels were higher than predicted in most HHC subjects. For this comparison, we have assumed a linear relationship between SF and expression of these iron transport genes over all values of SF. These results support early HHC studies showing that although the absolute value of intestinal iron absorption is frequently not increased compared with normal controls, it is inappropriately increased in relation to their SF.^{12 30 31} Similarly, these findings are relevant in the context of previous studies investigating markers of intracellular iron status in duodenal tissue from patients with HHC. These studies showed that although the duodenal mucosal content of ferritin mRNA and protein is relatively low and the corresponding levels of transferrin receptor gene and protein expression are relatively high in patients with HHC,^{5–8} absolute levels were similar to those observed in normal control subjects.

Furthermore, in HHC patients there were significant linear relationships between SF and *DMT1* (*IRE*) and *Ireg1* expression. In addition, *DMT1* (*IRE*) expression showed a positive correlation with *Ireg1* expression in HHC and non-HHC patients. Iron absorption studies have shown a similar inverse relationship between SF and whole body iron absorption in subjects with HHC.^{12 30 31} Collectively, these results suggest that in HHC patients, changes in duodenal transport gene expres-

sion and consequently intestinal iron absorption are still subject to some regulation by body iron stores. In the current study, patient numbers were too small to examine the relationship between hepatic iron stores and duodenal expression of the iron transport genes.

Interestingly, three HHC patients had appropriate levels of *DMT1* and *Ireg1* for their SF. All three subjects were female with normal SF. It is interesting to speculate whether phenotypic non-expression in these three patients was a result of environmental factors or whether they have constitutively lower levels of expression of iron transporters. Further studies of intestinal iron transporters in non-expressing C282Y homozygous HHC patients would be of particular interest in determining the contributors to their attenuated phenotype.

Dietary factors may account in part for the difference in results between this study and that of Zoller and colleagues.²¹ Iron absorption is markedly influenced by recent dietary iron intake and studies conducted in rodents support a rapid regulatory effect of increased dietary non-haeme iron content resulting in decreased duodenal expression of iron transport genes.^{35 36} There are significant differences in the haem iron content of diets of different populations around the world. Australians and Americans consume a significantly greater amount of red meat in their diet than Europeans.^{37–40} It is well documented that red meat stimulates non-haem iron uptake but the mechanism is unknown.^{41–43} Ultimately it may be expected that this would result in a further increase in body iron stores and a relative downregulation of iron transport gene expression.

Human and animal studies support the existence of genes other than *HFE* that modify the HHC phenotype by influencing expression of iron transport molecules, and such genetic factors may provide a possible explanation for the differences in findings between different populations. In humans, for instance, the severity of iron loading and clinical disease is quite variable in patients with identical *HFE* genotypes, and in an Australian study examining disease expression in a large series of patients (and their relatives) referred for evaluation of iron overload, approximately one third of women and up to 10% of men homozygous for the C282Y mutation were non-expressers.⁴⁴ Despite such variation, affected siblings have similar iron indices and several studies suggest that the ancestral haplotype is associated with more severe iron loading in HHC.^{45–47} There have been several recent population studies examining disease penetrance in *HFE* related HHC with marked variability in reported clinical expression of 1–50%.^{48–51} This is a controversial area and interpretation of the findings of these studies is difficult as they differ considerably in population selection and the use of controls. Furthermore, two groups have reported significant differences in duodenal expression of *DMT1* in *Hfe*^{−/−} mice of different genetic backgrounds.^{52 53} More recently, Dupic *et al* showed that differences in the severity of hepatic iron loading between *Hfe*^{−/−} mice of two different strains were probably accounted for by variations in expression of *DMT1*, *Ireg1*, and *Dcytb*.⁵⁴

There are no prior data on *Dcytb* protein or gene levels in human subjects with iron deficiency or HHC. In this study, *Dcytb* levels were similar in iron deficient patients and iron replete subjects. However, in *hpx*^{−/−} mice, duodenal *Dcytb* mRNA and protein levels increased in response to iron deficiency and anaemia.¹⁵ Studies from our group showed regulation of *Dcytb* mRNA levels by changes in dietary iron content and body iron status in rodents.²⁹ However, the mechanism by which iron regulates *Dcytb* expression is still unclear as *Dcytb* is reported not to possess an IRE. Expression of *Dcytb* was not increased in HHC subjects, suggesting that it does not play a primary role in the increased iron absorption characteristic of HHC. In vitro studies examining mucosal surface ferric iron reducing activity in duodenal fragments taken from untreated and treated patients with HHC have shown a significant increase in duodenal ferric iron reduction and

increased iron uptake compared with normal controls.⁵⁵ However, in *Hfe*^{-/-} mice, Griffith *et al* showed no difference in duodenal ferriredutase activity between *Hfe*^{-/-} and wild-type animals.⁵⁶ More recently, Dupic *et al* showed that *Dcytb* follows a similar expression pattern to that of *DMT1* and *Ireg1*, with marked differences observed between *Hfe* knockout mice of different genetic backgrounds.⁵⁴ The role of *Dcytb* in the adaptive response to iron deficiency in humans remains to be clarified and further studies are needed to determine the precise function of *Dcytb* in iron absorption and the mechanism of its regulation by iron.

Expression of *hephaestin* was decreased in subjects with HHC compared with iron replete and iron deficient subjects but levels of *hephaestin* were similar in iron deficient and iron replete subjects. It is unlikely that the decrease in *hephaestin* levels is of physiological significance as the difference was less than twofold in magnitude. These results, together with those of Rolfs and colleagues,²² suggest that changes in *hephaestin* expression cannot account for the increased iron absorption characteristic of HHC. Duodenal expression of *DMT1* (non-*IRE*) was negligible and there were no observable differences in expression between the three groups. From these results and those of others,²² it appears unlikely that *DMT1* (non-*IRE*) plays a major role in the changes observed in duodenal *DMT1* gene levels.

In summary, this study supports the primary roles of *DMT1* and *Ireg1* in mediating increased iron absorption in human subjects with iron deficiency. Duodenal expression of these iron transport genes was inversely regulated by body iron stores in iron deficient and iron replete subjects. Although we did not find an absolute increase in expression of *DMT1* (*IRE*) or *Ireg1* in a group of untreated C282Y homozygous HHC patients, there appears to be a relative increase in the levels of these iron transport genes for any given serum ferritin concentration in most HHC patients. In these patients, *DMT1* (*IRE*) and *Ireg1* levels were inversely proportional to serum ferritin concentration, suggesting that body iron stores still have some regulatory effect on expression of these iron transport genes in HHC.

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