The role of iron and haemochromatosis gene mutations in the progression of liver disease in chronic hepatitis C

D Thorburn, G Curry, R Spooner, E Spence, K Oien, D Halls, R Fox, E A B McCudden, R N M MacSween, P R Mills

Background: Chronic hepatitis C virus (HCV) infection is frequently associated with elevated markers of iron stores. Recessively inherited mutations in the HFE gene are responsible for iron accumulation in most cases of hereditary haemochromatosis and may have a role in HCV infection. They may also be associated with progressive liver fibrosis although this remains controversial.

Aims: To assess the prevalence of HFE mutations in Scottish HCV infected patients and to explore the effect of the carrier state on serum and liver iron stores, and the severity of liver disease.

Patients: A total of 164 patients with antibodies to HCV who underwent liver biopsy were assessed prospectively.

Methods: Each patient was screened for HFE mutations (Cys282Tyr and His63Asp). Iron markers were assessed in serum (ferritin, transferrin saturation) and on liver biopsy (stainable iron, liver iron concentration [LIC] and hepatic iron index).

Results: There were 67 (41%, 26 Cys282Tyr, 33 His63Asp, eight compound) heterozygotes. Forty four (28%) patients had elevated serum iron markers, 24 (15%) had stainable liver iron, and five (3%) had elevated LICs. Carriage of HFE mutations was not associated with any clinical, biochemical, virological, or pathological features, including accumulation of liver iron. Elevated serum iron markers were associated with male sex, increased alcohol consumption, and increased liver inflammation and fibrosis. Patients with elevated LICs were older, acquired HCV infection earlier, and had more liver inflammation.

Conclusions: Patients with chronic HCV infection frequently have elevated serum iron markers although elevated LICs are uncommon. Elevated serum iron studies and LICs occur in patients with more severe liver disease. Carriage of HFE mutations, although frequently observed in these HCV infected patients, does not have a role in the accumulation of iron or the progression of liver disease in HCV infection.

Abbreviations: HCV, hepatitis C virus; LIC, liver iron concentration; RT-PCR reverse transcription-polymerase chain reaction.
(three patients), human bite (one patient), and 32 (19.5%) patients had no identifiable risk factors for the acquisition of HCV infection. Twenty five patients had received previous antiviral therapy with interferon. The proportion of interferon treated patients was not significantly different among the four groups. Duration of infection at the time of liver biopsy was recorded for those patients who had an identifiable year of infection. For those patients who acquired HCV through intravenous drug use, the year of infection was assumed to be the year that injecting commenced. Local data indicate that approximately half of those intravenous drug users who acquire HCV infection are infected within one year of commencing injecting and almost all are infected within 3–5 years (personal communication, Avril Taylor, Scottish Centre for Infection and Environmental Health).

HCV determination

Anti-HCV was tested by third generation enzyme linked immunosorbent assay (Ortho Diagnostics, Raritan, New Jersey, USA). Reactive samples were confirmed positive for anti-HCV by the RIBA-3 testing assay (Ortho Diagnostics). Reverse transcription-polymerase chain reaction (RT-PCR) was performed on patient sera using an inhouse method.\(^\text{14}\) Genotyping of HCV was performed by restriction fragment length polymorphism on RT-PCR positive samples.\(^\text{15}\)

Iron parameters

Iron status was assessed for each patient using biochemical tests, histological grading of liver iron content, and by measurement of liver iron concentration (LIC). Serum iron (normal for males 10–40 µmol/l, females 8–40 µmol/l) was measured by the ferrozine method, ferritin (normal 15–300 ng/ml) was measured by turbidimetry using a Chiron ACS180 automatic analyser (Bayer, USA), and transferrin (normal 2–4 g/l) was measured by nephelometry (Beckmann Array Analyser, USA). Transferrin saturation was calculated as follows: serum iron×70.9/serum transferrin (normal 16–45%).

Histological evaluation

All liver biopsies were performed under ultrasound guidance. As is our practice, two cores of liver tissue were obtained from each patient: one formalin fixed for histological analysis and one stored immediately at 20°C for LIC estimation. Formalin fixed specimens were paraffin embedded and sections stained with haematoxylin and eosin and Perl's Prussian blue stain. Slides were then evaluated by two experienced pathologists (RMacS and KO) blinded to the clinical and laboratory information.

All biopsy specimens were graded for the degree of necro-inflammatory activity and staged for the extent of fibrosis using the criteria of Ishak and colleagues.\(^\text{16}\)

Determination of hepatic iron

Histological assessment of hepatocyte and macrophage iron stores was graded on a scale of 0–4 on Perl's Prussian blue stained liver sections, as previously described.\(^\text{17}\) In addition, 120 patients had LICs measured by atomic absorption spectrometry with results expressed as µg/g of dry weight of liver tissue (normal range 170–1400 µg/g dry weight liver tissue). The hepatic iron index was calculated by dividing LIC (in µmol/g dry wt) by the age of the patient (in years).\(^\text{18}\) Forty four patients did not have a second liver biopsy specimen for hepatic iron assay for the following reasons: lack of patient consent or reluctance of the radiologist to take a second biopsy specimen for clinical reasons, inadequacy of specimens obtained for LIC estimation, and occasional omissions at the time of biopsy.

HFE mutation analysis

The presence of HFE gene mutations was verified by means of restriction fragment length polymorphism on the PCR products of genomic DNA extracted from peripheral blood mononuclear cells.\(^\text{19}\) Extracted DNA fragments were amplified using oligonucleotide primers for the Cys282Tyr and His63Asp loci synthesised according to previously reported sequences.\(^\text{19}\) Amplification, digestion, and visualisation of the PCR products were performed as previously reported.\(^\text{19}\)

Statistical analysis

Statistical analysis of the data was performed using SigmaStat (SPSS Science Software UK Ltd, Birmingham, UK). Differences between nominal variables were analysed by \(\chi^2\) tests. Differences among continuous variables were evaluated using the Kruskal-Wallis test. Logistic regression analysis with a stepwise approach was applied to determine those variables independently associated with the presence of liver iron and the grade and stage of liver disease. Consent to carry out this study was provided by the West Glasgow Hospitals University NHS Trust ethics committee.

RESULTS

HFE gene data

Table 1 shows the results of analysis for the Cys282Tyr and His63Asp mutations in 164 patients with chronic hepatitis C. Seventy four patients (45%) carried at least one of the mutations. The allele frequencies for the Cys282Tyr and His63Asp mutations were 12.2% and 14.9%.

Seven (4.2%) patients were homozygous for one of the mutations (three Cys282Tyr, four His63Asp) and were excluded from further analysis (table 2). All three patients homozygous for the Cys282Tyr mutation had staineable iron on liver biopsy. LICs and transferrin saturations were elevated in two (66.7%) of these patients but there was little evidence of associated hepatic inflammation or fibrosis on liver biopsy. Three patients homozygous for the His63Asp mutation had elevated serum iron studies (two transferrin saturation, one ferritin) but none had stainable iron on liver biopsy. LIC was not increased in either of the His63Asp homozygotes in whom it was estimated. One (2%) patient homozygous for the His63Asp mutation was cirrhotic.

Clinical data

To ensure that the role of sex and menstruation on iron metabolism did not bias the study, data for male and female patients in each of the four groups were assessed separately. The proportion and ages of male and female patients in each of the four groups were comparable. Analysis excluding female patients, and hence the effects of menstruation, did not affect the results for any clinical, serum, or liver biopsy parameter. Analysis excluding the 10 non-Caucasian patients, two of whom were heterozygous for the His63Asp mutation, did not influence the results and all analyses discussed included these patients. In comparison with interferon naive patients, those previously treated with interferon therapy had comparable serum and liver iron studies and the severity of HCV related liver injury and all analyses included these patients.

<table>
<thead>
<tr>
<th>Mutation status</th>
<th>No (%)</th>
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<tbody>
<tr>
<td>Cys282Tyr/Cys282Tyr</td>
<td>3 (1.8)</td>
</tr>
<tr>
<td>His63Asp/His63Asp</td>
<td>4 (2.4)</td>
</tr>
<tr>
<td>Cys282Tyr/His63Asp</td>
<td>8 (4.9)</td>
</tr>
<tr>
<td>Cys282Tyr/wild type</td>
<td>26 (15.8)</td>
</tr>
<tr>
<td>His63Asp/wild type</td>
<td>33 (20.1)</td>
</tr>
<tr>
<td>Wild type/wild type</td>
<td>90 (54.9)</td>
</tr>
<tr>
<td>Total</td>
<td>164 (100)</td>
</tr>
</tbody>
</table>

Table 1 Distribution of HFE gene mutations in patients with chronic hepatitis C infection
In table 3, the remaining 157 patients, including 67 with heterozygous HFE mutations and 90 without, were compared with regard to demographic, clinical, and virological data. No differences with respect to any of these features were found. One hundred and fifty-four patients were RT-PCR positive for HCV RNA of whom 64 were infected with genotype 1 and 72 with genotype 3.

**Serum iron markers**

The association between serum iron markers and HFE gene status indices is shown in table 4. No significant differences were observed. Sixteen (10%) patients had elevated ferritin levels, six (9%) carrying a HFE mutation (one His63Asp/Cys282Tyr, one Cys282Tyr/WT, four His63Asp/WT) and 10 (11%) without (p = 0.84). Transferrin saturation was elevated in 36 (23%) patients, 18 (27%) with (three Cys282Tyr/His63Asp, three Cys282Tyr/WT, 12 His63Asp/WT), and 18 (20%) without a HFE mutation (p = 0.67). Either serum ferritin or transferrin saturation was elevated in 44 (28%) patients, 18 (27%) of the 67 patients with and 23 (26%) of the 90 patients without HFE gene mutations (p = 0.54). Patients with elevated serum iron markers were more commonly male (37/44 (84%) v 62/113 (55%); p = 0.001), drank significantly more alcohol (median 12.5 v 4 U/week; p < 0.001), more frequently had stainable iron on liver biopsy (13 (29%) v 11 (10%; p = 0.004), had higher LICs (median 0.540 v 0.375; p = 0.009), and had more active chronic hepatitis (median necroinflammatory scores 5 v 4; p = 0.017) with more fibrosis (median fibrosis scores 2 v 1; p = 0.025) than patients with normal iron studies.

**Liver biopsy indices**

The association between necroinflammatory and fibrosis scores, stainable iron, LICs, and HFE gene status is also shown in table 4. No significant differences were observed.

Eleven (7%) patients had scores of 4 or more for stage of fibrosis, two (3%) of the 67 patients carrying a HFE mutation compared with nine (10%) of the 90 without a HFE mutation (p = 0.16). Six (4%) patients had cirrhosis, one (1%) with and five (6%) without HFE mutations (p = 0.37). By stepwise regression using a backwards approach, fibrosis scores could be predicted by a combination of age, alcohol consumption, and liver biopsy scores for interface hepatitis and confluent necrosis (all p < 0.001).

Stainable iron was detected on 24 (15%) biopsies, with hepatocyte staining alone in 11, macrophage staining in five, and staining of both in eight. No patient had an iron staining grade of 3 or 4 for hepatocyte iron and only one patient had macrophage iron staining of grade 3. Hepatocyte or macrophage iron staining was present on the liver biopsy of 13 (19%) of the 67 patients with a HFE mutation (three Cys282Tyr/His63Asp, six Cys282Tyr/WT, four His63Asp/WT) and 11 (12%) of the 90 patients without (p = 0.31). Patients with stainable iron were older (p = 0.007), with higher serum ferritins (p < 0.001), and had higher LICs (p < 0.001). There was no significant difference in the grade...
and stage of liver disease between patients with and without iron staining on liver biopsy.

A total of 115 of the 157 non-homozygous patients had LICs measured: five (3%) had LICs >1400 µg/g dry wt (median 1650 (range 1480–3400)) and only one (His63Asp/WT) carried a HFE mutation. Three (60%) patients with an elevated LIC had stainable iron on liver biopsy (one hepatocyte staining alone, two with hepatocyte and macrophage staining) compared with 21 (14%) of the 152 patients with a normal LIC (p=0.03). Patients with elevated LICs were older (p=0.05), acquired HCV infection earlier (p=0.02), had higher serum ferritins (p=0.003), and had more severe necroinflammatory activity on liver biopsy (p=0.02). There was a trend towards higher fibrosis scores in those patients with elevated LICs although this did not reach statistical significance (median 2 v 1; p=0.06). By stepwise regression analysis with a backwards approach, LICs could be predicted by a combination of serum ferritin, transferrin saturation, and hepatocyte staining with iron on liver biopsy (p=0.02). There was a trend towards patients with elevated LICs being more commonly male, drank more alcohol, and had more cirrhosis compared with 127 normal controls of whom 11 (8.7%) were cirrhotic (p=0.01). Heterozygotes had more advanced liver disease (p=0.01) and more commonly had iron staining on Perl's stained liver sections (p=0.02). Further studies by other groups have supported 12–13 and refuted 14–15 these findings. Some studies included small numbers of homozygous patients. 12–13–15 Factors, which are known to be associated with the progression of HCV related liver disease, were controlled for inconsistently in these studies. The lack of association between carriage of HFE mutations and the stage of fibrosis in this study may have resulted from excluding all homozygous patients from the analysis and controlling for many possible confounding variables, including sex, age at HCV acquisition, duration of HCV infection, route of HCV infection, HCV genotype, current alcohol consumption, and past history of alcohol excess. In this study, factors associated with stage of fibrosis were patient age and alcohol consumption at time of biopsy, which is compatible with current understanding. 16 In addition, the grade of hepatitis, in particular scores for interface hepatitis and the presence of confluent necrosis, were predictors of fibrosis. This is in keeping with studies that have shown these features on liver biopsy to be associated with progressive disease and a worse prognosis in chronic hepatitis. 17–19

Patients carrying a HFE mutation had no evidence of increased serum iron studies or intrahepatic iron deposition, assessed both directly and indirectly. The prevalence in this study of increased serum iron stores in patients with chronic HCV infection is comparable with that reported elsewhere, with 28% of patients having an elevated ferritin or transferrin saturation. 20 Patients with elevated serum iron markers were more commonly male, drank more alcohol, and had more active chronic hepatitis with more liver fibrosis. This suggests that elevated serum iron markers in patients with chronic HCV infection are the result of active hepatitis, either through leakage from damaged hepatocytes or as part of the systemic inflammatory response. 21

**Table 4** Relationship between HFE genotype, serum iron indices, liver biopsy findings, stainable iron, and hepatic iron indices

<table>
<thead>
<tr>
<th></th>
<th>Hist63Asp/Cys282Tyr (n=8)</th>
<th>Cys282Tyr/WT (n=26)</th>
<th>Hist63Asp/WT (n=33)</th>
<th>WT/WT (n=90)</th>
<th>p Value</th>
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<tbody>
<tr>
<td>Serum ferritin (ng/ml) (mean (SD))</td>
<td>138 (145)</td>
<td>106 (115)</td>
<td>171 (270)</td>
<td>162 (369)</td>
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<tr>
<td>Serum iron (µmol/l) (mean (SD))</td>
<td>25.1 (10.1)</td>
<td>17.8 (7.1)</td>
<td>26.1 (12.4)</td>
<td>22.7 (11.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>Serum transferrin (g/l) (mean (SD))</td>
<td>2.4 (0.3)</td>
<td>2.8 (0.6)</td>
<td>2.8 (0.3)</td>
<td>3.1 (1.6)</td>
<td>0.01</td>
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<tr>
<td>Transferrin saturation [%] (mean (SD))</td>
<td>45.9 (18.1)</td>
<td>32.2 (15.4)</td>
<td>41.0 (17.6)</td>
<td>34.8 (20.9)</td>
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<tr>
<td>Grade of inflammation (0–18)</td>
<td>5 (1–6)</td>
<td>4 (1–9)</td>
<td>4 (1–9)</td>
<td>5 (0–13)</td>
<td>0.22</td>
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<tr>
<td>Interface hepatitis (0–4)</td>
<td>1 (0–2)</td>
<td>1 (0–3)</td>
<td>1 (0–2)</td>
<td>1 (0–4)</td>
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<tr>
<td>Confluent necrosis (0–6)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–0)</td>
<td>0 (0–6)</td>
<td>0.20</td>
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<td>Parenchymal inflammation (0–4)</td>
<td>2 (1–3)</td>
<td>2 (0–3)</td>
<td>2 (0–4)</td>
<td>2 (0–4)</td>
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<tr>
<td>Portal inflammation (0–4)</td>
<td>1 (0–2)</td>
<td>1 (1–4)</td>
<td>1 (0–3)</td>
<td>1 (0–3)</td>
<td>0.59</td>
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<tr>
<td>Stage of fibrosis</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>10</td>
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<td>1</td>
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<td>Stainable iron—hepatocyte</td>
<td>0</td>
<td>5</td>
<td>22</td>
<td>29</td>
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<td>Stainable iron—macrophage</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
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<tr>
<td>No with hepatic iron estimation</td>
<td>5</td>
<td>22</td>
<td>24</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Liver iron concentration (µg/g) (mean (SD))</td>
<td>462 (286)</td>
<td>535 (321)</td>
<td>508 (320)</td>
<td>544 (568)</td>
<td>0.82</td>
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<tr>
<td>Hepatic iron index (mean (SD))</td>
<td>0.23 (0.10)</td>
<td>0.26 (0.15)</td>
<td>0.24 (0.14)</td>
<td>0.25 (0.22)</td>
<td>0.66</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The prevalence of both HFE gene mutations in our population is higher than that reported in studies of HCV infected populations from Europe, England, and Brazil. 22 This may reflect the high prevalence of HFE mutations in the local population, which is largely of Nordic and Celtic extraction, rather than a susceptibility to HCV infection in carriers of HFE mutations. 4

There was no evidence in this study that heterozygosity for either HFE mutation was associated with more severe grade or stage of HCV related liver disease. This contrasts with the study by Smith et al who first proposed an association between carriage of HFE mutations and increased hepatic fibrosis scores. 11 In that study, 10 Cys282Tyr heterozygotes of whom four (40%) had cirrhosis were compared with 127 normal controls of whom 11 (8.7%) were cirrhotic (p=0.01). Heterozygotes had more advanced liver disease (p=0.01) and more commonly had iron staining on Perl's stained liver sections (p=0.02). Further studies by other groups have supported 12–13 and refuted 14–15 these findings. Some studies included small numbers of homozygous patients. 12–13–15 Factors, which are known to be associated with the progression of HCV related liver disease, were controlled for inconsistently in these studies. The lack of association between carriage of HFE mutations and the stage of fibrosis in this study may have resulted from excluding all homozygous patients from the analysis and controlling for many possible confounding variables, including sex, age at HCV acquisition, duration of HCV infection, route of HCV infection, HCV genotype, current alcohol consumption, and past history of alcohol excess. In this study, factors associated with stage of fibrosis were patient age and alcohol consumption at time of biopsy, which is compatible with current understanding. 16 In addition, the grade of hepatitis, in particular scores for interface hepatitis and the presence of confluent necrosis, were predictors of fibrosis. This is in keeping with studies that have shown these features on liver biopsy to be associated with progressive disease and a worse prognosis in chronic hepatitis. 17–19

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Despite frequently elevated serum iron studies, few patients had direct or indirect evidence of increased liver iron, with only 3% of patients having an elevated LIC and 15% of biopsies having stainable iron almost exclusively of grades 1 or 2. The prevalence of elevated LIC in this study was lower than that reported in earlier studies of patients with chronic hepatitis C, where a prevalence of 10–36% has been recorded. There was no evidence that carriage of the HFE mutations was associated with liver iron accumulation. This finding is consistent with earlier studies exploring the role of HFE mutations in HCV that assessed liver iron histologically and biochemically was unrelated to the grade and stage of HCV related liver injury. The mechanism by which liver iron accumulates in a small number of patients is unclear. These patients have significantly more liver inflammation and a trend towards increased fibrosis compared with those with normal LICs but there is considerable overlap between the two groups. Whether this iron accumulation is the cause or result of liver injury is unclear.

In summary, we found that although many patients with chronic HCV infection have elevated serum iron studies, few have significant iron deposition within the liver. Carriage of the recognised mutations in the HFE gene, although frequently observed, do not account for the elevated serum iron studies and liver iron deposition that is present nor are they associated with any clinical, biochemical, virological, or pathological feature. The severity of HCV related liver injury could be predicted by patient age, alcohol consumption, and histological grade of interface hepatitis and confluent necrosis, as previously described, and overall the concentration of liver iron did not have a significant role in the progression of HCV related liver injury. These findings do not support a role for iron depletion by venesection in patients with chronic HCV infection, including those with elevated serum iron studies.

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