Low hepatic iron concentration: evaluation of two complementary methods, colorimetric assay and iron histological scoring

Françoise Imbert-Bismut, Frederic Charlotte, Bruno Turlin, Lina Khalil, Annie Piton, Pierre Brissot, Yves Le Charpentier, Jacques Delattre, Pierre Opolon, Yves Deugnier, Thierry Poinard

Abstract

Aims—To validate a method of assessment of low hepatic iron concentration based on a biochemical colorimetric assay plus histological scoring.

Methods—The within-day and day to day precision of the iron colorimetric assay was determined on frozen rat liver. The coefficient of variation (CV) of iron measurement in two separate samples from the same liver was determined for 21 deparaffinised human biopsies. The intra- and interlaboratory variability of the colorimetric assay and histological scoring were assessed on 38 deparaffinised liver biopsies.

Results—For the within-day test, the CV was 11% (5.1 (0.6) $\mu\text{mol/g}$ dry weight (dw), mean (SD) iron concentration). For the day to day test, the CV was 19.5% (8.2 (1.6) $\mu\text{mol/g}$ dw). The CV was 14.7% for iron concentration determined in two separate samples from the same liver. By correlation and κ concordance tests, the intra- and interlaboratory variability of the hepatic iron colorimetric assay and iron histological scoring was slight. Absence of stainable iron corresponded to a liver iron concentration $\leq 20 \mu\text{mol/g}$ dw.

Conclusions—A combination of two complementary methods, colorimetric measurement and histological scoring, is an accurate and reliable way of determining low iron concentrations in deparaffinised human liver biopsies. In secondary haemosiderosis, such methods would be essential for investigating the role of low iron overload in fibrogenesis and during the response to antiviral treatment in chronic viral hepatitis.

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explained by the difference in the sensitivity of assessment methods for the low liver iron content usually found in patients with hepatitis C.⁴⁻¹¹ A high liver iron content can be evaluated by two direct methods: colorimetric measurement of iron concentration and histological scoring of stainable iron.¹²⁻¹³ The quantitative chemical method assesses all liver iron forms, whereas the semiquantitative histological analysis evaluates only the haemosiderin iron form and not the unstainable ferritin iron form.¹⁴⁻¹⁵ The aim of this study was therefore to validate these two methods for low iron levels by (1) determining the accuracy of the colorimetric measurement of hepatic iron concentration, (2) estimating the intralaboratory and interlaboratory variability in colorimetric assessment and histological scoring, and (3) evaluating histological iron scoring using the colorimetric iron assay

Methods

LIVER SAMPLE SELECTION

A frozen Wistar rat liver was used for the study of reliability criteria for determining iron concentration. The variability of the iron concentration measurement and histological scoring was evaluated in 4% formalin fixed, paraffin embedded human hepatic biopsies that were large enough for several chemical iron determinations. These liver specimens were obtained by percutaneous needle or wedge biopsies in 13 and 25 cases, respectively. Clinical data and the results of hepatic iron concentration measurements and histological scores are listed in table 1. There were 38 patients (median age 49 years): 18 men (median age 56 years, range 28 to 74) and 20 women (median age 48 years, range 19 to 72).

IRON HISTOLOGICAL SCORING

Liver sections (3 μm) were stained using Perls' reaction. We followed the quantitative histological grading previously used and modified by Deugnier *et al.*¹⁶ The total iron score (0-60) was equal to the sum of hepatocytic, sinusoidal, and portal scores multiplied by a heterogeneity coefficient defined according to iron distribution as follows: 1/3, very heterogeneous; 2/3, moderately heterogeneous; 3/3, homogeneous. To evaluate interobserver variation, the same set of slides was independently reviewed by two pathologists (FC and BT) without previous knowledge of the corresponding biochemical results. For intraobserver variation, each

Laboratoire de
Biochimie, Hôpital
Pitié-Salpêtrière 75013
Paris, France
F Imbert-Bismut
L Khalil
A Piton
J Delattre

Laboratoire
d'Anatomie
Pathologique, Hôpital
Pitié-Salpêtrière
F Charlotte
Y Le Charpentier

Laboratoire
d'Hépatogastroentérologie,
Hôpital Pitié-Salpêtrière
P Opolon
T Poinard

Laboratoire
d'Anatomie
Pathologique B, U-49
de l'INSERM, CHRU
Pontchaillou 35033
Rennes, France
B Turlin

Clinique des Maladies
du Foie and Unités de
Recherches
Hépatologiques, U-49
de l'INSERM
P Brissot
Y Deugnier

Correspondence to:
Dr Imbert-Bismut.

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Recent studies have emphasised the pathophysiological role of iron in various liver diseases with low iron excess, such as viral chronic hepatitis C.¹ Experimental investigations have suggested that iron may play a role in liver fibrogenesis as a cofactor in lipid peroxidation.²⁻³ Some clinical studies have shown that the hepatic iron content may influence the response to interferon alfa treatment in chronic hepatitis C,⁴⁻⁷ while others have not.⁸⁻¹⁰ This discrepancy could partly be

pathologist re-examined the slides after a three month interval.

DETERMINATION OF HEPATIC IRON CONCENTRATION

Paraffin embedded specimens were deparaffinised initially by melting the paraffin at 60°C until the samples were released from the block, followed by three successive immersions of 30 minutes each in xylene and then in ethanol. Next, specimens were oven dried for 24 hours and weighed on a microbalance with 0.01 mg precision. The mean (SD) dry weight (dw) of liver specimens was 1.18 (0.36) mg (range 0.60 to 1.76 mg). Hepatic iron concentration was measured using the colorimetric method described by Barry and Sherlock.¹⁷ Chemical assay and histological scoring had to be performed on a representative liver sample, the dry weight and the length of which had to be ≥ 0.50 mg and ≥ 5 mm, respectively. In each assay, it was essential that the absorbance of the blank assay was < 0.0060 to verify that reagents and washed glassware were uncontaminated by iron. The absorbance reading was performed by a Beckman DU 640 spectrophotometer. A linear calibration was obtained for an iron concentration range of 0 to 14.9 $\mu\text{mol/litre}$. Under these conditions, the analytical sensitivity of the method was about 0.3 $\mu\text{mol/litre}$. Iron concentration was determined in two different samples from the same liver biopsy and each sample was assayed in duplicate. Results are expressed as $\mu\text{mol/g dw}$

of liver tissue. The chemical determination of liver iron was performed without reference to histological scoring.

RELIABILITY CRITERIA FOR THE COLORIMETRIC MEASUREMENT OF HEPATIC IRON CONCENTRATION

Day to day reproducibility of the assay was determined over five days by measuring the iron content of 22 samples from the same rat liver. Within-day precision was determined by repeated iron concentration measurement of 10 samples from the same rat liver.

VARIABILITY IN COLORIMETRIC MEASUREMENT OF HEPATIC IRON CONCENTRATION AND IN IRON HISTOLOGICAL SCORING

The distribution of stainable hepatic iron was heterogeneous in five liver biopsies and homogeneous or not present in the other 33. Two groups of biopsies were defined as follows: (A) with homogeneous iron distribution or without iron; (B) biopsies from group A plus five biopsies with heterogeneous iron distribution. Variability of iron concentration determination was evaluated in the same laboratory as follows: intraoperator variations (LK) for biopsies of groups A (n=28) and B (n=33), and inter-operator variations (LK, FIB) for groups A (n=33) and B (n=38). Interlaboratory variations (LK, JYR and FIB, JYR) were assessed for group A (n=19).

Intralaboratory, intraobserver (FC), and interobserver (FC, BT) variability of stainable iron scoring was evaluated for groups A (n=33) and B (n=38).

STATISTICAL METHODS

Intraoperator and interoperator variations for iron concentration measurement were estimated by Bland–Altman plots,¹⁸ by the difference between result means, and by the κ concordance test.¹⁹ Intraobserver and inter-observer variations in iron histological scoring were estimated by the κ concordance test.¹⁹

Results

VARIABILITY IN IRON HISTOLOGICAL SCORING

Stainable iron was observed in 16 of 38 biopsies, with an iron histological scoring range between 1 and 25 (table 2). In four cases iron histological scoring was ≥ 10 . The strength of intraobserver (FC) agreement was substantial for all histological items. If the five liver biopsies with heterogeneous iron distribution were excluded, the degree of intraobserver (FC) concordance was almost perfect except for the sinusoidal iron score. The degree of interobserver (FC, BT) concordance was lower, the strength of agreement being moderate for the sinusoidal iron score and substantial for the hepatocytic and portal iron scores. If the livers with heterogeneous iron distribution were excluded, no significant modification to the degree of interobserver concordance was observed except in the case of the portal iron score, where the strength of agreement was almost perfect.

Table 1 Clinical, biochemical, and histological data on 38 patients

Patient	Clinical condition	Sex	Age (years)	Hepatic iron ($\mu\text{mol/g dw}$)	Histological grade
1	Viral hepatitis C	F	40	3	0
2	Viral hepatitis C	M	47	3	0
3	Viral hepatitis C	F	62	4	0
4	Viral hepatitis C	M	56	4	0
5*	Viral hepatitis C	M	29	4	1 (1/3)
6	Viral hepatitis C	F	47	5	0
7	Viral hepatitis C	F	48	5	0
8	Viral hepatitis C	M	32	6	0
9	Viral hepatitis C	F	62	7	0
10	Viral hepatitis C	M	28	8	3
11	Viral hepatitis C	F	58	9	0
12	Viral hepatitis C	M	74	11	1
13*	Viral hepatitis C	M	69	16	2 (2/3)
14	Viral hepatitis C	F	72	22	3
15	Viral hepatitis C	F	49	48	25
16	Viral hepatitis B+C	F	60	14	0
17	Viral hepatitis B	M	73	8	0
18*	Steatosis	M	57	16	2 (1/3)
19	Steatosis	M	71	70	9
20	Alcoholic hepatitis	F	45	7	0
21	Secondary haemosiderosis	F	29	204	15
22	Secondary haemosiderosis	M	36	365	24
23	Liver trauma	F	65	7	0
24	Liver trauma	F	19	8	0
25	Liver trauma	F	32	8	0
26	Liver trauma	M	29	9	0
27	Liver trauma	F	30	9	0
28	Liver trauma	M	47	11	0
29	Liver trauma	M	35	16	0
30	Vesicular lithiasis	M	69	26	4
31	Haemolytic anaemia	F	21	61	6
32	Liver congestion	F	27	3	0
33	Liver congestion	F	52	4	0
34	Amyloidosis	F	49	16	0
35*	Hepatocellular carcinoma	M	71	12	1 (1/3)
36	Nodal lymphoma	M	68	44	6
37	Metastases (neuroendocrine carcinoma)	F	65	20	3
38*	Metastases (adenocarcinoma)	M	56	33	12 (2/3)

*Patients 5, 13, 18, 35, and 38: iron heterogeneously distributed (heterogeneity coefficient). dw, dry weight.

Table 2 Intraobserver and interobserver variations in iron histological scoring

Variable	κ Test
Hepatocyte iron score	
Intraobserver variation (FC)	
A (n=33)	0.87*
B (n=38)	0.74*
Interobserver variation (FC, BT)	
A (n=33)	0.62*
B (n=38)	0.62*
Sinusoidal iron score	
Intraobserver variation (FC)	
A (n=33)	0.73*
B (n=38)	0.78*
Interobserver variation (FC, BT)	
A (n=33)	0.52*
B (n=38)	0.59*
Portal iron score	
Intraobserver variation (FC)	
A (n=33)	0.82*
B (n=38)	0.77*
Interobserver variation (FC, BT)	
A (n=33)	0.84*
B (n=38)	0.62*
Iron histological score	
Intraobserver variation (FC)	
A (n=33)	0.82*
B (n=38)	0.72*
Interobserver variation (FC, BT)	
A (n=33)	0.65†
B (n=38)	0.69†

A, biopsies not including the five biopsies with heterogeneous iron distribution; B, biopsies with and without stainable iron; FC, BT, initials of investigators.

* κ for three classes of different iron histological score (0; 0–5; >5).

† κ for two classes of different iron histological score (0; >0).

PRECISION OF COLORIMETRIC MEASUREMENT OF HEPATIC IRON CONCENTRATION

The within-day and day to day precision tests were satisfactory, since the coefficients of variation (CV) were 11% (mean iron concentration 5.1 (0.56) $\mu\text{mol/g dw}$) and 19.5% (mean 8.2 (1.6) $\mu\text{mol/g dw}$), respectively. For 21 human biopsies in group A which were tested in duplicate (iron concentration $\leq 20 \mu\text{mol/g dw}$), the standard error for a single iron concentration determination corresponded to a CV of 14.7%.

VARIABILITY IN CHEMICAL DETERMINATION OF HEPATIC IRON CONCENTRATION

These results are shown in table 3 and fig 1. Median hepatic iron concentration was 9 $\mu\text{mol/g dw}$ (range 3 to 365) for the 38 biopsies. Hepatic iron content was $\leq 20 \mu\text{mol/g dw}$ in 28 biopsies, between 20 and 60 $\mu\text{mol/g dw}$ in seven, and $\geq 60 \mu\text{mol/g dw}$ in three.

In the same laboratory, the intraoperator and interoperator variability of the iron colorimetric assay was slight. Between the two laboratories, using the same technique, the differences in the iron concentration results were minor. The κ test showed an almost perfect strength of agreement for iron concentration classes 0 to 20, 20 to 50, and $> 50 \mu\text{mol/g dw}$ when determined by two operators in the same laboratory or in two different laboratories. The concordance of results was substantial for iron concentration classes 0 to 10, 10 to 20, 20 to 50, and $> 50 \mu\text{mol/g dw}$ when iron concentrations were determined by the same operator in the same laboratory. The κ tests for groups A and B were similar.

COMPARISON BETWEEN COLORIMETRIC DETERMINATION OF IRON CONCENTRATION AND HISTOLOGICAL SCORING

The clinical data and the results of iron concentration and histological scoring are summarised in table 1. Comparisons between iron concentration and histological scoring results are shown in fig 2. For no stainable iron, the κ test showed a substantial strength of agreement when the iron concentration was $\leq 15 \mu\text{mol/g dw}$ ($\kappa = 0.74$). It was almost perfect when the iron concentration was $\leq 20 \mu\text{mol/g dw}$ ($\kappa = 0.86$).

Table 3 Variability of determination of hepatic iron concentration

Variable	A (SD)	κ Test
Intralaboratory variation		
Intratechnician (LK)		
A (n=28)	6.11 (24.10)	0.78*
B (n=33)	5.57 (22.31)	0.77*
Intertechnician (LK, FIB)		
A (n=33)	-3.09 (7.34)	0.93†
B (n=38)	-3.71 (7.57)	0.81†
Interlaboratory variation		
Intertechnician (LK, JYR)		
A (n=19)	-1.00 (16.43)	0.82†
Intertechnician (FIB, JYR)		
A (n=19)	2.84 (22.88)	0.91†

A, biopsies not including the five with heterogeneous iron distribution; B, biopsies with and without stainable iron.

Δ, difference between means (expressed as $\mu\text{mol/g dry weight}$).
* κ for four classes of iron concentration (0–10; 10–20; 20–50; $> 50 \mu\text{mol/g dry weight}$).

† κ for three classes of iron concentration (0–20; 20–50; $> 50 \mu\text{mol/g dry weight}$).

Figure 1 Variability of hepatic iron concentration (HIC) expressed as $\mu\text{mol/g dw}$. Top: Intralaboratory, intraoperator: difference between LK1 and LK2 HIC measurement (log HIC) against the mean of the pair. Middle: Intralaboratory, interoperator: difference between LK1 and FIB HIC measurement (log HIC) against the mean of the pair. Bottom: Interlaboratory, interoperator: difference between LK1 and JYR HIC measurement (log HIC) against the mean of the pair.

haemochromatosis^{1 28} and in clinical research on cofactors involved in liver fibrogenesis.

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