



# Post-mortem liver and bone marrow iron quantification in haemodialysis patients: A prospective cohort study

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## Summary

**Background** Magnetic resonance liver scans indicate that iron overload is common in haemodialysis (HD) patients. However, histological evidence is scarce.

**Methods** Liver biopsy and bone marrow aspirate were obtained in the first 24h post mortem from 21 adult HD patients. Biochemical liver iron content (LIC) was quantified by electrothermal atomization atomic absorption spectrophotometry. Tissue iron deposition was graded in the liver and bone marrow using Scheuer and Gale's criteria, respectively.

**Findings** Median LIC was 42.5 (22.9-69.7)  $\mu\text{mol/g}$  and the majority ( $n=11$ ; 57%) had mild to moderate liver iron overload (LIC  $>36 \mu\text{mol/g}$ ). Scheuer grade was 2 (1-3) and 13 (62%) of liver biopsies had increased ( $> 1$ ) iron deposition. In the bone marrow, median Gale's grade was 3 (3-4) and 9 (45%) patients had increased ( $> 3$ ) iron content.

Contrary to old autopsy studies, done in the pre-erythropoiesis-stimulating agents (ESAs) era, both liver and bone marrow were iron replete and showed a positive correlation ( $r=0.71$ ,  $p<0.001$ ).

Ferritin proved to have a good diagnostic accuracy for liver iron overload (0.87 95% CI 0.71-1.00) with an optimal cut-off value of 422 ng/ml. Haemoglobin was negatively associated with both LIC ( $r= -0.46$ ,  $p=0.04$ ) and iron content in the bone marrow ( $p=0.04$ ). Patients with increased LIC had higher resistance to ESAs ( $p=0.02$ ), yet no association with previous IV iron therapy.

**Interpretation** In the majority of HD patients there was iron accumulation in both the liver and bone marrow that associated with anaemia severity and resistance to ESAs, suggesting a blocking mechanism of iron's utilization.

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**Keywords:** Anaemia; Chronic kidney disease; Haemodialysis; Liver iron content; Electrothermal atomization atomic absorption spectrophotometry; Bone marrow

**Abbreviations:** CKD, Chronic kidney disease; HD, Hemodialysis; LIC, Liver Iron Content; ETA AAS, Electrothermal Atomization Atomic Absorption Spectrophotometry

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## Introduction

Severe iron overload among chronic kidney disease (CKD) stage 5 patients was common during the pre-erythropoiesis-stimulating agents' (ESA) era, when blood transfusions were often used to treat anaemia and when intravenous (IV) iron therapy was given without

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### Research in context

#### *Evidence before this study*

Iron overload associated with haemodialysis (HD) was documented in old autopsy studies, when blood transfusions were often given to patients, prior to the introduction of erythropoiesis-stimulating agents (ESAs). Repeated administration of high doses of intravenous iron, despite elevated serum ferritin levels, is becoming a common practice in HD and its long-term consequences are unknown. MRI studies have estimated iron overload to be common in present-day hemodialysis patients.

#### *Added value of this study*

This is the first study in the post-ESA era to quantify iron levels in liver and bone marrow of deceased haemodialysis patients, in order to know the actual state of iron deposition in tissues by gold standard methods: biochemical and histological grading. The results show that none of the 21 patients had iron deficiency in the bone marrow, and the majority had excess iron in both the liver and bone marrow. This is contrary to what was described in the pre-ESA era, when despite hepatosplenic siderosis there was a scarcity or absence of iron in the bone marrow. Histological findings show that iron accumulated in the reticuloendothelial cells, without liver fibrosis.

#### *Implications of all the available evidence*

The finding that patients with higher liver iron deposits were the ones with higher resistance to ESAs and more severe anaemia, suggest a blockage of iron utilization. Serum ferritin proved to be a valuable tool accurately reflecting the accumulation of iron in the liver and validating international guidelines suggesting prudence when administering iron in patients with serum ferritin levels greater than 500ng/mL.

concomitant ESA administration.<sup>1–6</sup> In pre-ESA era, autopsy studies have found that while iron stores in the liver and spleen were high, there was scarcity or absence of stainable iron in the bone marrow of patients with hepatosplenic siderosis and high serum levels of ferritin.<sup>5,6</sup> Since the introduction of ESA's, concern about iron overload waned and IV iron use has been liberalized and even encouraged to optimize erythropoiesis and recently thought to improve cardiovascular outcomes.<sup>7</sup>

An increasing number of magnetic resonance imaging (MRI)<sup>8–12</sup> studies now show that a significant proportion of haemodialysis (HD) patients receiving iron and ESA according to current guidelines have hepatic iron overload. However, histological evidence of iron overload is scarce, as most studies in HD patients were done in the pre-ESA era. A thorough understanding of

iron metabolism and storage in patients suffering from chronic kidney disease is crucial to safely and judiciously guide anaemia therapy. The primary objective of this study was to determine liver and bone marrow iron content in HD patients by biochemical quantitative and histological semi-quantitative methods. Secondary objective was to explore the association between iron stores and clinical, laboratorial markers of iron status and anaemia therapy.

### Methods

#### Design, setting, and participants

This was a single-centre prospective cohort study of adult patients on chronic haemodialysis treatment that died at the Hospital Fernando da Fonseca between November 2013 and May 2016.

#### Ethics

Since bone marrow aspiration and liver biopsy are medical invasive procedures, we decided to conduct a post-mortem study that was approved by the Institution's Ethical Board. All research procedures were conducted according to the principles of the Declaration of Helsinki of 1975 as revised in 2013. Informed consent was obtained from next of kin for all enrolled patients, following ICJME recommendations.<sup>13</sup>

A liver biopsy and bone marrow aspirate were performed in the first 24h after death among those who met the following eligibility criteria.

**Inclusion criteria:** Adults (aged >18 years), CKD stage 5 under regular haemodialysis for at least 3 months, with anaemia or under current therapy with ESA or IV iron.

**Exclusion criteria:** blood transfusion in the previous 2 weeks, acute liver failure, cirrhosis, abnormal liver blood tests, HIV infection, primary haemochromatosis or active malignancy.

Percutaneous liver biopsy was performed with ultrasound guidance in the right lobe with a 16-gauge semi-automatic needle with retrieval of two specimens. One specimen was put in a dry decontaminated tube without iron and analysed to quantify hepatic iron by Electrothermal Atomization Atomic (ETA) Absorption Spectrophotometry (AAS) method at the Laboratory of Toxicology in Porto's Faculty of Pharmacy. The liver iron content (LIC) was measured and reported as  $\mu$ moles per gram of dry weight of liver.

Another specimen was placed in a 10% buffered formaldehyde saline solution, processed routinely, embedded in a paraffin block. After paraffin embedding, sections were obtained for hematoxylin-eosin and Perls' Prussian blue staining.

Bone marrow aspirate was obtained from the sternum and prepared with a minimum of 5 slides per

patient, stained with a commercial kit for Prussian blue's HematoGnost Fe<sup>®</sup>, according to manufacturer's instructions.<sup>14</sup>

All investigators responsible for iron quantification were blinded for patients' identity and clinical data.

### Operational definitions

LIC was classified as normal ( $\leq 36 \mu\text{mol/g}$ ), mild ( $37\text{--}100 \mu\text{mol/g}$ ), moderate ( $101\text{--}200 \mu\text{mol/g}$ ) or severe ( $> 201 \mu\text{mol/g}$ ) as in previous studies.<sup>15–18</sup>

Histological semi-quantitative analysis of hepatic iron was graded according to Scheuer's classification system, ranging from 0 to 4, higher grades representing increasing levels of iron content. Scheuer grades above 1 are considered to represent iron overload.<sup>19,20</sup>

Histological semi-quantitative analysis of bone marrow iron content (BMIC) was graded according to Gale's criteria<sup>21,22</sup> ranging from 0 to 6 (none to very heavy stainable iron). Bone marrow findings were classified as iron deficient (score of 0 or 1), normal (graded from 2–3) or iron overload (graded from 4–6).

### Data source and variables

Data were retrieved from medical records both in-hospital and from the outpatient haemodialysis facilities. The following baseline characteristics were recorded: age, sex, ethnicity, etiology of CKD, dialysis vintage (months), Charlson Comorbidity Index (CCI), body weight (kg), presence of diabetes, type of vascular access, cause of death. Laboratory data obtained were the last available before death and included: haemoglobin (Hb, g/dl), ferritin (ng/ml), transferrin saturation (TSAT, %), intact parathyroid hormone (iPTH, pg/ml), albumin (g/dl), C-reactive protein (mg/dl) and white blood cell count (WBC,  $\times 10^9/\text{L}$ ). Data pertaining to anaemia treatment included cumulative 6 and 12 months' IV iron administered before death (mg), ESA therapy and dose, erythropoietin resistance index (ERI) calculated by the weekly EPO dose (U/kg/week) divided by the haemoglobin level (g/dL)<sup>23</sup> and red blood cell transfusions in the previous 12 months. We used a darbepoetin to epoetin conversion of 1:200 IU.<sup>24</sup>

### Statistics

Categorical variables were presented as proportions and continuous variables as median and inter-quartile ranges (IQR: 25th percentile–75th percentile). Missing values were completely at random and therefore no imputation was performed. Comparison between groups was made with Mann-Whitney U and Chi-square test for continuous and categorical data respectively. Fisher's exact test was used when one or more expected values were less than 5. Univariable comparisons between continuous and categorical variables were performed with Kruskal-Wallis test. The strength of

linear association between continuous or ordinal variables was performed with Spearman's rank correlation coefficient. AUC of receiver operating characteristic (ROC) curve analysis was used to measure the diagnostic accuracy and optimal cut-off point of ferritin to detect LIC overload. Since we did not have a preference between sensitivity and specificity we chose the value from the ROC curve that maximized their summation, considering that at this point the youden index is also maximum.<sup>25–27</sup> Due to the small sample size and expected low precision of the estimate of results, multivariate analysis to explore variables associated with iron overload was not performed. The threshold for statistical significance was defined as  $p=0.05$  (2-tailed). Statistical analysis was performed using IBM SPSS Statistics, version 27 (IBM Corp, North Castle, NY, US).

### Role of Funding Source

No funding was received for the study.

### Results

A total of 24 patients were referred to the investigation team and assessed for inclusion in the study. Two were excluded due to refusal to participate and one patient was excluded due to an error sampling of the liver biopsy.

Of 21 patients included, ( $n=10$ , 47.6%) were male, median (IQR) age was 76.0 (67.5–85.5) years old, most were Caucasian ( $n=18$ , 85.7%). Median (IQR) dialysis vintage was 47.0 (12.5–104.0) months and Charlson Comorbidity index was 10.0 (7.5–11.0). A third of patients ( $n=7$ , 33%) had diabetes, and approximately half of the patients ( $n=11$ , 52.4%) used an arteriovenous fistula as vascular access. The major causes of death were infection ( $n=9$ , 42.9%) and cardiovascular disease ( $n=6$ , 28.6%).

Baseline clinical characteristics of the 21 patients included are summarized in [Table 1](#).

The majority of patients ( $n=19$ , 90%) had anaemia according to WHO definition and nearly half ( $n=11$ , 52%) had haemoglobin (Hb) below 10g/dL.

Median (IQR) Hb was 9.8g/dL (8.5–11.4), and ferritin was 494ng/ml (136–851). Transferrin saturation (TSAT) was available for only 16 patients and the median (IQR) value was 19.9% (13.3–26.0) ([Table 2](#)).

### Previous anaemia therapy

All patients were receiving ESA therapy, the majority ( $n=15$ , 71%) treated with beta-epoetin and the remainder with darbepoetin alfa, with median dose of 5000 units per week ([Table 2](#)). Median ERI was 9.6 (4.2–16.6). The majority of the patients ( $n=19$ , 90.5%) were receiving maintenance IV iron treatment with 6 months' cumulative dose of 800mg (300–1250). In the last 12 months

	Population N (%); Median [IQR]	LIC (n=21)		BMIC (n=20)	
		Normal (n=9)	Increased (n=12)	Normal (n=11)	Increased (n=9)
Age (years)	76.0 [67.5-85.5]	76.0 [69.5-82.0]	80.0 [66.3-85.8]	77.0 [74.0-87.0]	75.5 [64.5-82.0]
Male	10 (47.6)	4 (44.4)	6 (50.0)	7 (63.6)	2 (22.2)
Race					
Black	3 (14.3)	2 (22.2)	1 (8.3)	1 (9.1)	2 (22.2)
White	18 (85.7)	7 (77.8)	11 (91.7)	10 (90.9)	7 (77.8)
Dialysis vintage (months)	47.0 [12.5-104.0]	75.0 [12.5-134.0]	47.0 [15.3-73.5]	40.0 [8.0-75.0]	57.0 [31.0-144.0]
Charlson Comorbidity Index (CCI)	10.0 [7.5-11.0]	9.0 [7.0-10.5]	10.0 [8.0-11.0]	10.0 [9.0-11.0]	8.0 [5.5-11.5]
Diabetes	7 (33.3)	1 (11.1)	6 (50.0)	3 (27.3)	3 (33.3)
Body weight (Kg)	55.0 (49.5-61.0)	53.0 (49.5-63.5)	55.2 (47.7-58.6)	55.0 (52.0-59.0)	50.0 (48.0-61.5)
Vascular access					
Fistula	11 (52.4)	6 (66.7)	5 (41.7)	5 (45.5)	6 (66.7)
Graft	2 (9.5)	0 (0)	2 (16.6)	0 (0)	1 (11.1)
Catheter	8 (38.1)	3 (33.3)	5 (41.7)	6 (54.5)	2 (22.2)
CKD's etiology					
Hypertension	3 (14.3)	1 (11.1)	2 (16.7)	2 (18.2)	1 (11.1)
Diabetes	4 (19.0)	0 (0)	4 (33.3)	2 (18.2)	2 (22.2)
Other	6 (28.6)	5 (55.6)	1 (8.3)	5 (45.5)	1 (11.1)
Uncertain	8 (38.1)	3 (33.3)	5 (41.7)	2 (18.2)	5 (55.6)
Cause of death					
Infection	9 (42.9)	4 (44.4)	5 (41.7)	6 (54.6)	3 (33.3)
Cardiovascular	6 (28.6)	4 (44.4)	2 (16.6)	3 (27.2)	3 (33.3)
Palliative care	3 (14.3)	0 (0)	3 (25.0)	0 (0)	3 (33.3)
Other	3 (14.3)	1 (11.1)	2 (16.7)	2 (18.2)	0 (0)

**Table 1: Patient clinical characteristics.**  
Comparison between groups of normal vs increased LIC and BMIC.  
LIC: Liver iron content; Normal LIC  $\leq 36 \mu\text{mol/g}$ ; Increased LIC  $> 36 \mu\text{mol/g}$ ; BMIC: bone marrow iron content; Normal BMIC: Gale's grade 0-3; Increased BMIC: Gale's grade 4-6.  
\*p-value  $> 0.05$  for all comparisons between groups (Mann Whitney, Chi-square or Fisher's exact test as appropriate).

before death the cumulative dose of iron was 1500mg (650-2175). The iron formulation used was iron sucrose in every patient.

The median (IQR) number of days between iron administration and death was 21.5 (9.0-83.0). Three patients (14.3%) received packed RBC transfusions in their last 12 months.

### Quantification of iron stores

**Liver.** The median (IQR) liver iron content (LIC) was 42.5  $\mu\text{mol/g}$  (22.9-69.7). Nine patients (43%) had normal LIC, while the remainder had mild (9 patients, 43%) to moderate (3 patients, 14%) overload.

Iron deposition was also evaluated in histological sections of the liver and graded according to increasing iron deposition. While iron stains of the liver are expected to be negative in most instances (i.e. Scheuer's grade  $\leq 1$ ),<sup>19</sup> in our study, 13 (62%) liver biopsies had increased iron deposition, and the median (IQR) Scheuer grade was 2 (1-3) (Table 3). There was a positive association between the two methods of iron measurement

in the liver ( $X^2(4)=11.47$ ;  $p=0.022$ ) (Kruskal-Wallis) (Figure 1)

Six patients (29%) had severe (grade 3-4) iron deposition as documented in their liver biopsies, characteristic of secondary (acquired) iron overload, with reticuloendothelial system Kupffer cell's filled with deposits of fine and coarse granules of iron, however without liver fibrosis (Figure 2).

**Bone marrow.** In one patient, bone marrow sample was not considered adequate for diagnosis, leaving 20 patients for analysis.

The median (IQR) Gale's iron grade in the bone marrow was 3 (3-4).

None of the patients had iron-depleted bone marrow (grade 0-1).

Three patients (15%) were classified as grade 2, eight patients (40%) grade 3, seven patients (35%) grade 4 and two patients (10%) grade 5.

Grade 2 and 3 represent the normal iron status, with a moderate presence of small iron particles in reticulum cells throughout the marrow fragment.

Laboratorial Parameters	Population (n=21) Median [IQR]	LIC		BMIC	
		Normal (n=9)	Increased (n=12)	Normal (n=11)	Increased (n=9)
Hb (g/dl)	9.8 [8.5-11.4]	12.1 [10.5-13.5]*	8.8 [8.3-9.8]	11.7 [9.1-12.8]*	8.8 [7.9-10.2]
Ferritin (ng/ml)	494 [136-851]	110 [88-262]*	717 [253-1240]	211 [110-717]	882 [190-1531]
TSAT (%) n=16	19.9 [13.3-26.0]	13.6 [10.0-26.8]*	23.2 [19.8-38.1]	21.0 [13.6-34.2]	22.8 [10.0-38.1]
iPTH (pg/ml) n=20	224 [115-480]	352 [248-531]	208 [123-303]	300 [197-454]	164 [108-775]
Albumin (g/dl)	3.3 [3.1-3.7]	3.5 [3.3-3.8]	3.3 [2.7-3.6]	3.6 [3.2-3.7]	3.2 [2.8-3.5]
C-reactive protein (mg/dl), n=20	1.7 [0.7-9.4]	1.2 [0.7-2.8]	1.2 [0.1-13.7]	1.3 [0.7-5.1]	1.2 [0.3-14.7]
WBC (x10 <sup>9</sup> /L)	9.5 [7.4-15.4]	10.6 [7.7-15.1]	10.7 [7.7-16.5]	9.6 [8.3-15.7]	13.8 [8.4-16.4]
Anaemia Therapy					
IV Iron 6 months (mg)	800 [300-1250]	800 [450-1250]	700 [125-1475]	720 [200-1400]	1100 [300-1250]
IV Iron 12 months (mg)	1500 [650-2175]	1300 [450-1920]	1600 [875-2737]	1500 [500-2250]	1300 [750-2500]
EPO (IU/week)	5000 [3000-9000]	2000 [1050-6500]	6000 [4000-13500]	5000 [2000-9000]	4000 [2750-6000]
ERI	9.6 [4.2-16.6]	3.3 [1.7-11.7]	14.9 [6.6-21.9]*	9.6 [3.3-17.8]	7.9 [4.1-15.2]

**Table 2: Laboratorial parameters and anaemia therapy.**

Comparison between groups of normal vs increased LIC and BMIC.

LIC: Liver iron content measured by Electrothermal Atomization Atomic Absorption Spectrophotometry ( $\mu\text{mol/g}$ ) in liver biopsy; Normal LIC  $\leq 36 \mu\text{mol/g}$ ; Increased LIC  $> 36 \mu\text{mol/g}$ ;

BMIC: bone marrow iron content, graded according to Gale's criteria in bone marrow aspirate; Normal BMIC: Gale's grade 0-3; Increased BMIC: Gale's grade 4-6; Hb: haemoglobin; TSAT: transferrin saturation; iPTH: intact parathyroid hormone; WBC: white blood cell count; IV iron 6 and 12 months: cumulative intravenous iron dose administered during the last 6 and 12 months respectively; EPO: epoetin; conversion of darbepoetin to epoetin used was 1:200 U; ERI: erythropoietin resistance index defined as weekly epoetin (EPO) dose per kg body weight divided by the haemoglobin level.

\* p-value  $< 0.05$  for comparison between groups (Mann Whitney test).

Scheuer's Classification	Population n (%)	LIC		BMIC	
		Normal (n=9)	Increased (n=12)	Normal (n=11)	Increased (n=9)
Grade 0	1 (4.8)	1 (11.1)	0 (0)	1 (9.1)	0 (0)
Grade 1	7 (33.3)	5 (55.6)	2 (16.7)	6 (54.5)	0 (0)
Grade 2	7 (33.3)	3 (33.3)	4 (33.3)	3 (27.3)	4 (44.4)
Grade 3	4 (19.0)	0 (0)	4 (33.3)	0 (0)	4 (44.4)
Grade 4	2 (9.5)	0 (0)	2 (16.7)	1 (9.1)	1 (11.1)
Iron overload	13 (62)	3 (33.3)	10 (83.3)*	4 (36.4)	9 (100)*

**Table 3: Histological hepatic iron graded according to Scheuer's classification system.**

Scheuer's normal hepatic iron: grade [0-1]; Scheuer's hepatic iron overload: grade [2-4]; LIC: Liver iron content; Normal LIC  $\leq 36 \mu\text{mol/g}$ ; Increased LIC  $> 36 \mu\text{mol/g}$ ; BMIC: bone marrow iron content; Normal BMIC: Gale's grade [0-3]; Increased BMIC: Gale's grade [4-6]; Iron overload by Scheuer's classification of liver's histology was associated with increased biochemical LIC and increased BMIC according to Gale's grading.

\* p-value  $< 0.05$  (Fisher's exact test).

Almost half of the patients (45%) had an increased (>grade 3) iron content in the bone marrow, where clumps of iron are seen across the fragment (Figure 3).

### Correlation between liver and bone marrow iron stores and laboratorial parameters

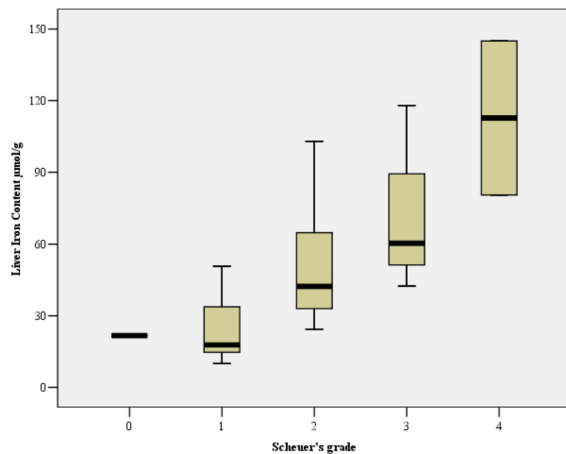
Semi-quantitative iron scores in liver and bone marrow had a significant positive correlation ( $r=0.71$ ,  $p<0.001$ ) (Spearman's rho), as increased liver iron stores associated with higher bone marrow iron deposits (Table 3).

There was a strong positive correlation between LIC and ferritin ( $r=0.86$ ,  $p < 0.001$ ) (Spearman's rho)

(Figure 4) and also with TSAT ( $r=0.56$ ,  $p=0.02$ ) (Spearman's rho).

Ferritin had a good diagnostic accuracy for iron overload (LIC  $> 36 \mu\text{mol/g}$ ) with an AUC-ROC of 0.87 (95%CI 0.71-1.00). The optimal cut-off value was 422 with a sensitivity of 83.3% and a specificity of 77.8%.

Haemoglobin was negatively associated with LIC ( $r= -0.46$ ,  $p=0.04$ ) (Spearman's rho), and with iron content in the bone marrow ( $r= 0.626$ ,  $p=0.003$ ) (Spearman's rho). LIC did not associate with C-reactive protein, dialysis vintage or other clinical parameters (Tables 1-3).



**Figure 1.** Biochemical measurement of liver iron (LIC- liver iron content, measured by Electrothermal Atomization Atomic (ETA) Absorption Spectrophotometry (AAS) method was positively associated with histological iron deposition graded according to Scheuer's classification [ $X(4)=11.47$ ;  $p=0.022$ ].

#### Correlation between therapy and iron stores

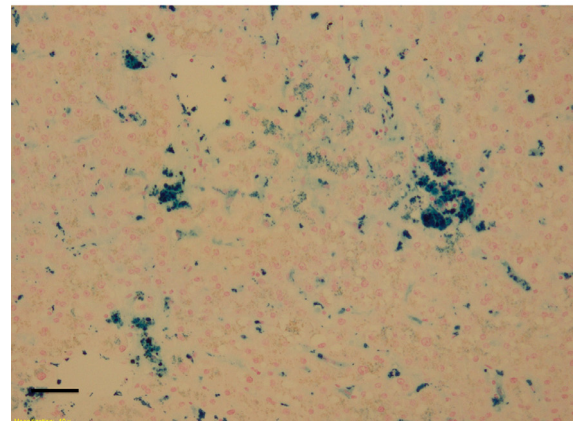
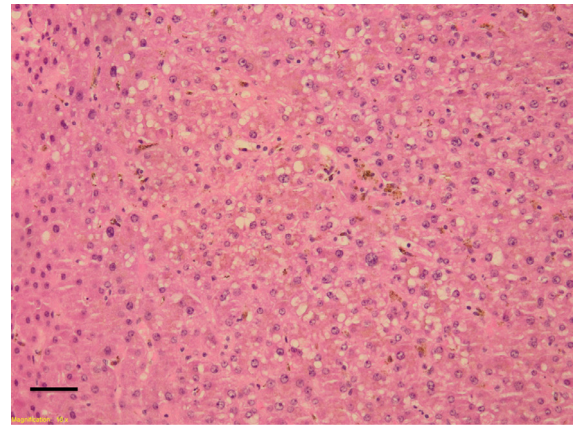
Resistance to ESA therapy (ERI) was higher in patients with increased LIC (Table 2) ( $p=0.02$ ) (Mann-Whitney). There was no association between iron stores in the liver or bone marrow and previous cumulative dose of IV iron in the last 6 or 12 months. Moreover, no association was found with blood transfusions or ESA dose (Supplementary data, Figure S1–8).

#### Discussion

This study conducted in a population of haemodialysis patients found that median LIC is higher than that considered to be normal and that the majority of patients are categorized as having increased liver iron stores both by ETA AAS quantification (57%) and semi-quantitative histological evaluation of iron deposition (62%).

Moreover, 45% of patients also have increased iron content in the bone marrow. These results are in contrast with findings published during the pre-ESA era, where Ali found that iron stores in the bone marrow were scarce in the presence of liver and spleen iron overload.<sup>5,6</sup> They support the more recent bone marrow findings in bone histomorphometry<sup>28</sup> that show iron repletion in the majority of haemodialysis patients under current anaemia treatment, that is with ESA's. In other secondary haemosiderosis settings, iron accumulation is usually documented in the liver, spleen and bone marrow.<sup>29</sup> It is possible that ESA's have changed Ali's paradox.<sup>5</sup> As in other studies,<sup>9,28</sup> we also found that serum ferritin levels have a positive correlation with iron deposits in both liver and bone marrow, suggesting that ferritin does adequately reflect iron stores.

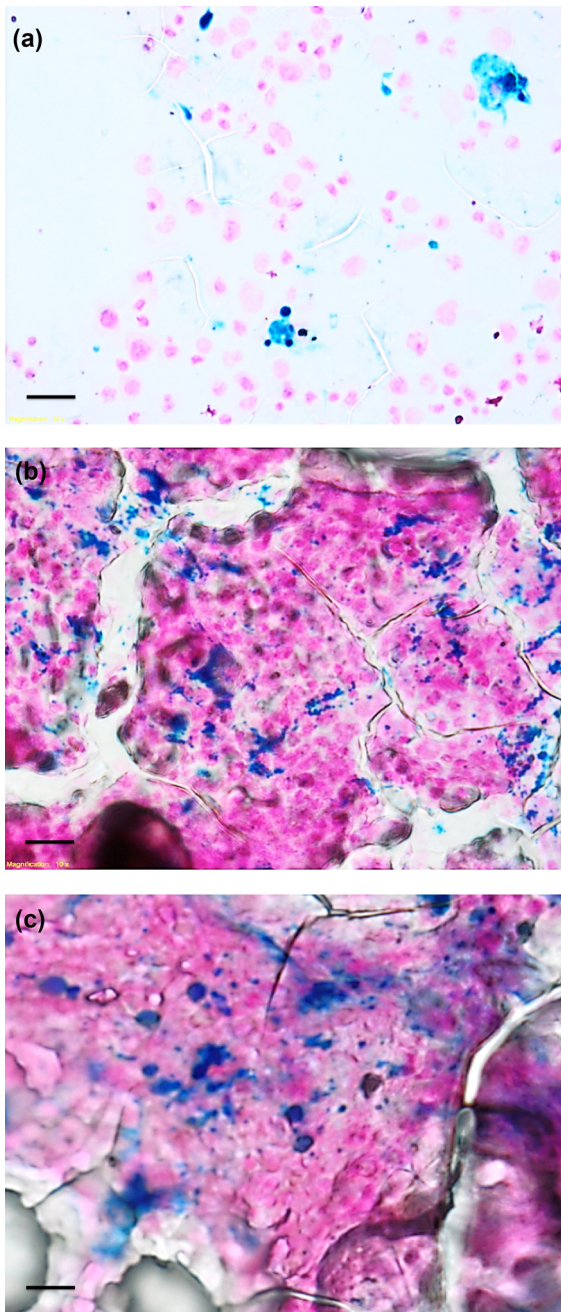
In this work, ferritin predicted an accurate diagnosis of liver iron overload (0.87 95% CI 0.71–1.00) with an



**Figure 2.** Hepatic parenchyma of a patient with severe iron deposition characteristic of secondary (acquired) iron overload. a) HE staining 10x (scale bar 100 µm): Hepatic parenchyma with preserved architecture, showing haemosiderin pigment in Kupffer cells, without significant deposits in hepatocytes. b) Perls' Prussian blue stain 10x (scale bar 100 µm): fine and coarse granules of haemosiderin pigment (blue stain) in the cytoplasm of Kupffer cells, visible in 10x magnification, graded 4 according to Scheuer's criteria.

optimal cut-off value of 422ng/ml. This value is not far from the 500ng/ml suggested by international guidelines,<sup>30,31</sup> which recommend caution considering further IV iron administration. PIVOTAL study<sup>7</sup> used IV iron allowing the upper limit of ferritin to be 700ng/ml, without short-term deleterious clinical consequences. Nowadays several guidelines<sup>32,33</sup> are advocating higher ferritin targets than the KDIGO limit of 500ng/ml in HD patients.<sup>30</sup>

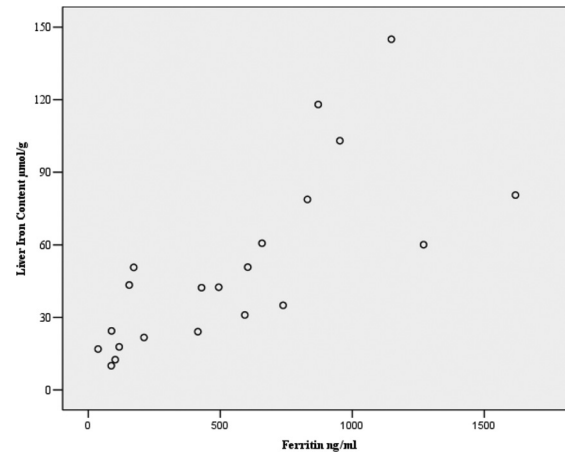
From our observations, we can suggest that giving iron to patients with ferritin above 500ng/ml means giving iron to patients with iron-replete deposits. Besides, since none of these patients had iron deficiency in the bone marrow, the classification “functional iron deficiency” applied to those with TSAT <25% could be better referred to as “functional iron unavailability”. In the sample studied, ESA hyporesponsiveness was



**Figure 3.** Bone marrow fragments from patients of this study, stained with Perl's Prussian blue, graded according to Gale's criteria. a) 20x (scale bar 50  $\mu\text{m}$ ): numerous small iron particles throughout the fragment in reticulum cells - Grade 3. b) 40x (scale bar 25  $\mu\text{m}$ ): large iron particles with tendency to aggregate into clumps - Grade 4 c) 40x (scale bar 25  $\mu\text{m}$ ): dense large clumps of iron throughout the fragment - Grade 5

associated with iron excess, not deficiency, as commonly thought to be the case in HD patients.<sup>34</sup>

Although the diagnostic accuracy of serum ferritin may be influenced by factors not associated with iron



**Figure 4.** Ferritin had a strong positive correlation with liver iron content (LIC) measured by Electrothermal Atomization Atomic (ETA) Absorption Spectrophotometry (AAS) method I ( $\mu\text{mol/g}$ ); Spearman's correlation coefficient test ( $r = 0.86$ ;  $p < 0.001$ ).

status such as inflammation and malnutrition,<sup>35</sup> in our study we did not find an association between iron stores with inflammation/malnutrition parameters such as C-reactive protein, WBC, albumin or the Charlson comorbidity index. Unfortunately, hepcidin was not included in this study, as it is not used as a routine tool in clinical practice. Hepcidin is known to be stimulated by iron overload and to a lesser extent by inflammation.<sup>36,37</sup> Increased hepcidin limits iron availability by suppressing iron export from macrophages, that become iron-laden.<sup>38</sup> This could contribute towards explaining the sequestration of iron in reticuloendothelial (RES) cells seen in our study population, compatible with secondary haemosiderosis.

The presence of excess iron in the liver or bone marrow was not associated with liver fibrosis and therefore does not represent a worrisome finding.<sup>39</sup> However, the fact that RES cells are chronically overloaded with iron may be a matter of concern, as the usual functions of these cells in the immune surveillance system may be compromised.<sup>40-42</sup> Besides, iron-laden macrophages are known to be a proatherogenic phenotype,<sup>43</sup> and their presence in haemodialysis patients may add to the already elevated risk of atherogenesis.<sup>44-46</sup>

In the patients studied, there was a paradoxical negative correlation between haemoglobin levels and iron stores. Also, those with higher liver iron deposits were the ones with a higher resistance index to erythropoiesis-stimulating agents.

These findings suggest that iron is not available for erythropoiesis, but is instead blocked in replete RES cells. The trend towards a negative association between haemoglobin and ferritin is also seen in large observational studies in incident haemodialysis patients, such as DOPPS and USRDS,<sup>47,48</sup> indicating the need to

seek the mechanisms behind CKD's iron disturbed metabolism.

Finally, there was no significant association between the dose of IV iron administered in the previous 6 and 12 months and LIC, iron semi-quantitative scores, ferritin or TSAT.

On the basis of our study, we hypothesize that iron deposits and their laboratory surrogates do not reflect the cumulative iron dose due to the fact that they are the result of a balance between gains and losses that could not be assessed in this study.

IV iron's contribution to iron overload in CKD is well established, as previous autopsy studies<sup>3</sup> and MRI cohort studies<sup>8,11</sup> show that liver iron content increases and decreases in parallel with IV iron administration or suspension.

This study has several limitations. First, the sample is small. Second, a majority of the patients were old with high comorbidity indexes. MRI scan studies have estimated liver iron overload to be moderate to severe in most haemodialysis patients,<sup>8,11</sup> which is higher than the findings of this study, in which most patients had mild to moderate liver iron overload. DOPPS Practice Monitor<sup>49</sup> shows that in the United States in September 2020, *circa* 75% of patients had ferritin levels above 500ng/mL, while in our study only 48% of the patients had ferritin above that level. The IV iron doses used in our study population were relatively low compared to the usual practice in other settings and thus, the sample studied may not be representative of other haemodialysis patients treated with more aggressive iron regimens.<sup>7,8,12,50</sup> The absence of a control group is a limitation of this study. However, normal values for liver iron concentration measured by biochemical methods are well established and are gold standard.<sup>15,18</sup> They were used in studies comparing biochemically measured liver iron and estimated iron content by T2 MRI.<sup>8,51,52</sup> Also semi-quantitative scales to grade iron deposition in liver and bone marrow are based upon seminal studies that have established the "normal" and "increased" iron content.<sup>19,21</sup> Hereditary haemochromatosis was not excluded by genetic test, as it is prevalent in Europeans and could explain iron overload on an individual basis. However, liver histology findings were not compatible with primary haemochromatosis in any patient. Iron deposits are not restricted to liver or bone marrow in secondary haemosiderosis<sup>29</sup>, however in this study we did not search for other organ deposition, as it would not have been feasible using a percutaneous technique and would have raised additional ethical and logistical concerns. The reassuring finding of no liver fibrosis does not exclude the possibility of the contribution of iron to other organ damage in these patients, as described in other iron overload settings.<sup>29,53</sup> Despite these limitations, we believe that our findings add to the literature about iron status in haemodialysis patients. To the best of our knowledge, this is the first study in

the post-ESA era to describe the correlation between ferritin and the gold standard quantitative method (ETA AAS) for determining iron content in the liver as well as its correlation with a semi-quantitative method of iron content in the bone marrow, in the same population. In addition, the histological data facilitated a simultaneous evaluation of liver iron deposition and its consequences, where no liver fibrosis was seen. Overall, this study shows that in the majority of haemodialysis patients receiving current anaemia treatment there was concomitant iron accumulation in the liver and bone marrow, suggesting that iron is not available for erythropoiesis, but instead it is blocked in RES cells. Ferritin showed a good diagnostic accuracy for iron overload, with an optimal cut-off value of 422ng/ml.

This finding reinforces the International guidelines (KDIGO) recommendations not to administer routinely iron to patients with ferritin levels consistently above 500ng/mL.

Future studies should address the mechanisms behind iron blockage in stores, its clinical consequences as well as strategies to improve the utilization of iron.

#### Declaration of interests

None of the authors have competing interests to declare.

#### Contributors

PC and PF had the idea of this study and have contributed with conception and design, data collection, statistical analysis and interpretation. PC, PF and AL have accessed and verified the underlying data. AL built the tables. PC and PF have critically revised the manuscript.

AS and LS have prepared and analysed bone marrow, RM was responsible for hepatic histology and iron's grading according to Scheuer's classification. LB and ES for liver iron quantification by electrothermal atomization atomic absorption spectrophotometry. All authors read and approved the final version of the manuscript. PC was responsible for the decision to submit the manuscript.

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### Data sharing statement

Data sharing requests will be considered by the co-authors upon written request to the corresponding author. Anonymized participant data or other pre-specified data will be available subject to a written proposal and a signed data sharing agreement.

### Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103921.

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