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Clinical and histological characterization of liver disease in patients with transfusion-dependent β-thalassemia. A multicenter study of 117 cases

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A B S T R A C T

Background and Objectives. Updated information on liver disease in transfusion-dependent β-thalassemia is lacking. We conducted a multicenter study within the Cooleycare Group to describe the clinical and histopathological features of liver disease in currently treated thalassemics.

Design and Methods. Two-hundred and three thalassemics with laboratory signs of liver disease were eligible. Liver biopsy was performed in the 129 (63.5%) who consented (age 26±7 years). Biological samples were sent to the central laboratory.

Results. Anti-hepatitis C virus (HCV) antibodies were found in 118 patients (91%), 85 (72%) of whom were viremic. Ninety-one patients (70%) had abnormal aminotransferase concentrations. In the 117 liver biopsies that met the criteria for evaluation (88%), the median Ishak's necroinflammatory and fibrosis scores were 4 (range, 0-9) and 2 (range, 0-6), respectively. Significant fibrosis (score ≥3) was found in 53 (45%); 9 (8%) had cirrhosis. At multivariate analysis, necroinflammation was related to HCV viremia, and fibrosis to increased serum aminotransferases, higher iron stores (including serum ferritin, Deugnier's total iron score, and liver iron content) and male gender ($p<0.05$). In HCV-RNA negative subjects, the median total iron score was 27 (range, 0-52). Iron accumulated in both mesenchymal cells and hepatocytes, and the presence of a lobular gradient was interpreted to indicate intestinal hyperabsorption.

Interpretation and Conclusions. Transfusion-dependent thalassemics have mild liver necroinflammation, mainly attributable to HCV infection. Significant fibrosis is frequent, and its progression is mostly influenced by iron overload which, with current therapy regimens, may be attributable to both erythrocyte catabolism and iron hyperabsorption.

Key words: liver disease, thalassemia, blood transfusion, hepatitis C, iron overload.

Chronic liver disease affects a high proportion of patients with β-thalassemia major.[1-4] During the past twenty years, the survival of thalassemic patients has improved due to better blood transfusion protocols and the increasing use of chronic iron chelation.[1,5,6] As life expectancy prolongs beyond the third decade, the consequences of chronic liver disease will likely increase.

The main causes of liver injury in thalassemics are hepatitis C virus (HCV) infection and hepatic iron overload, both secondary to the regular transfusion regimen.[3] The possible role of other factors — including unidentified infectious agents, iron-induced glucose intolerance, and chronic use of some medications — is currently under debate.[2,3,7-10] In spite of its clinical relevance, thalassemia-associated liver damage has been insufficiently characterized. Most histological studies were published before the identification of HCV and the adoption of regular deferoxamine treatment.[11-14] Hepatic lesions were described using obsolete classification systems which provided little information on the degree of liver fibrosis, the variable most closely related to disease progression. The information collected in recent years focused on particular categories of patients, including those receiving antiviral drugs[15] and oral chelators,[7,10,16] or was limited to patients cured of thalassemia by bone marrow transplantation.[17,18] Thus, an extensive etiological and histological update on liver disease in β-thalassemia major is warranted. This multicenter investigation was conducted within Cooleycare, a co-operative program developed in 1984 to improve the quality of treatment in β-thalassemia.[2,3,19,20-24] It was aimed at describing the pathological fea-

tures of liver disease in thalassemics, and at exploring their relations with iron overload and chronic HCV infection.

Design and Methods

Study design and population base

The study population was selected from the cohort of 1100 β-thalassemic patients of the Cooleycare Study program,[2,3,19,20-24] which currently includes 33 clinical centers in Italy. Five centers participated in the present study. Centers were selected on the basis of the following criteria: 1) number of patients attending the center (at least 20); 2) location in Italy (at least one center for northern, central, and southern Italy); 3) willingness to participate. The 177 thalassemic patients who were on active follow-up at these centers on December 1, 2000 were identified, and a liver biopsy was proposed to all the patients who were eligible.

The study was conducted according to the Declaration of Helsinki and received approval from the Ethics Committee of IRCCS Ospedale Maggiore, Milan. Written informed consent was obtained from all study patients.

Inclusion criteria

For enrollment, patients were required to meet all the following inclusion criteria: (i) clinical and genetic diagnosis of β-thalassemia major; (ii) clinical follow up of at least 36 months before study entry; (iii) presence of at least one of the three following laboratory indicators of liver disease: (a) anti-HCV reactivity; (b) alanine aminotransferase (ALT) levels intermittently or persistently above the upper reference limits (40 U/L in males, 30 U/L in females), as evaluated at each transfusion event during the pre-enrollment follow-up period of 3 years; (c) mean serum ferritin levels above 1000 ng/mL, as assessed in the 12 months before enrolment.

Exclusion Criteria

Patients were not eligible for the study if they had one or more of the following: (i) a history of congenital liver disease; (ii) a history of alcohol abuse (more than 80 g of ethanol per day), or intravenous use of illicit drugs; (iii) a platelet count of less than 75×10^9/L, platelet function disorders, or clotting abnormalities; (iv) severe heart disease or other medical conditions contraindicating liver biopsy; (v) antibodies to human immunodeficiency virus; (vi) previous performance of a liver biopsy between 36 and 60 months before study entry.

Sample collection and shipping

Liver biopsies were performed under local anesthesia and ultrasound control with a 16- or 18-gauge needle. Liver specimens were paraffin-embedded by a local pathologist, and at least 3 unstained histological preparations (thickness, 4 microns) were mounted on slides treated with either albumin, poly-L-lysine or equivalent reagents. If patients had undergone liver biopsies within 36 months before enrollment, the paraffin-embedded blocks were retrieved from the local archive, and slides were similarly prepared. Centers were required to ship slides, blocks and a serum sample to the central Cooleycare laboratory in Milan, along with a record reporting clinical and laboratory data collected in the pre-enrollment period.

Virological testing

Determinations of anti-HCV and antibodies to human immunodeficiency virus type 1-2 were performed by the central laboratory as a part of the Cooleycare program which requires regular serological screening.[2,20-24] In patients with confirmed anti-HCV reactivity, serum HCV RNA was determined by reverse transcriptase polymerase chain reaction (RT-PCR) (Amplicor HCV, Roche Molecular Systems, Basel, Switzerland).

Histological Methods

Liver biopsy specimens were coded, stained with standard techniques, and evaluated by a single pathologist (MM) who was unaware of the patients' data and clinical status. To be adequate for analysis, specimens had to contain 5 or more portal tracts. The degree of hepatic inflammation and fibrosis was reported according to the method developed by Ishak *et al.*,[25] which provides a semi-quantitative evaluation of the necroinflammatory activity (*grading*, from 0 to 18) and fibrosis (*staging*, from 0 to 6). As described elsewhere,[26] fibrosis progression per year was defined as the ratio between the fibrosis stage in Ishak units and the estimated duration of infection (i.e., the age of the patient). The median duration from infection to cirrhosis was calculated dividing 5 (i.e., Ishak units corresponding to incomplete cirrhosis) by the median rate of fibrosis progression per year.

Iron accumulation was assessed according to the total iron score, developed by Deugnier *et al.*[27-29] The study protocol also included the measurement of liver iron content by atomic absorption spectrophotometry.[30] Due to local regulations, one center was not allowed to provide the paraffin-embedded blocks to the central laboratory. Therefore, liver iron content results were analyzed in a subgroup of patients.

Data analysis and presentation

We assumed that liver disease commenced within the first year of life in of our patients. This was deduced from the transfusion requirement of thalassemic patients (i.e., at least 1 red cell unit every 15-20 days from early childhood),[1181] the risk of acquiring post-transfusion hepatitis in Italy before 1991 (i.e., 1 in 50 blood units),[3,31] and the amount of iron needed to completely saturate serum transferrin (i.e., that contained in 10-15 red cell units).[32]

Statistical analyses were performed using the Statistical Analysis System package 6.12 (SAS, Cary, NC, USA). The ages at the attainment of a grading score of 5 or more and of a staging score of 3 or more were modelled as a function of a set of possible risk factors and fitted with Cox's semiparametric model.[33] After data plot analysis, the following dichotomous covariates were taken into account: gender (*female*, *male*), HCV-RNA (*negative*, *positive*), ALT-pattern (*normal*, *abnormal*), serum ferritin (*<2500 ng/mL*, *≥2500 ng/mL*), total iron score (*≤25*, *>25*), and liver iron content (*≤6 mg/g dry weight*, *>6 mg/g, dry weigh*t).

The absence of severe violations of the proportional hazard assumption was ascertained by inspection of the plot of log (cumulative hazard) vs. log (time of exposure to HCV). *p* values of less than 0.05 were considered statistically significant.

For the calculation of the 95% confidence intervals for median values, we used the binomial-based method. When appropriate, Student's t test, χ^2 test, and Mann-Whitney test were used.

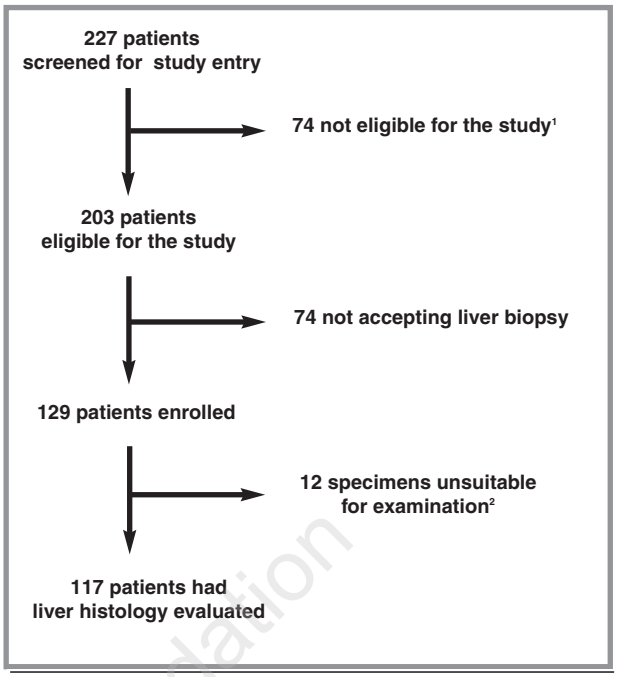

Figure 1. Selection procedure of the study subjects. [1]Due to: anti-HCV negative, persistently normal ALT levels, and serum ferritin levels below 1000 ng/mL (n=22); intravenous drug abuse or alcoholism (n=2); platelet count of less than 75×10^9/L, platelet function disorders, or clotting abnormalities (n=2); severe heart disease or other medical contraindication to liver biopsy (n=11); anti-HIV reactivity (n=2); execution of a liver biopsy between 60 and 36 months before study entry (n=21); other reasons, including procrastination of liver biopsy, inclusion in the waiting list for BM transplantation (n=14). [2]Due to less than five portal tracts on microscopic examination.

Results

Baseline characteristics of the study patients

The procedure for the selection of the study subjects is illustrated in Figure 1. As reported in Table 1, enrolled patients did not differ significantly from eligible patients with regards to main demographic, virological and clinical characteristics.

Evaluation of necroinflammation and fibrosis

Median necroinflammatory and fibrosis scores were 4 (range, 0 to 9) and 2 (range, 0 to 6), respectively. HCV RNA positive patients had higher necroinflammatory scores than HCV RNA negative patients [median 5 (range, 0 to 9) vs. 2 (0 to 9); $p<0.05$ by Mann and Whitney test], while the fibrosis score distributions did not significantly differ in the two groups [median 2 (range, 0 to 6) in both groups, $p=0.086$]. Significant fibrosis (i.e., an Ishak fibrosis score equal to or greater than 3, which indicates bridging fibrosis) was present in 53 of 117 patients (45%; 95% CI, 36-54%). The prevalence of significant fibrosis was comparable

Table 1. Baseline characteristics of the eligible and enrolled patients.

	Patients Eligible	Patients Enrolled
N. of cases	203	129
N. of males	91 (45%)	65 (50%)
Age (years)	25±7	26±7
N. with abnormal serum ALT*	147 (72%)	91 (71%)
N. anti-HCV positive	181 (89%)	118 (91%)
N. HCV RNA positive	113 (56%)	85 (66%)
Mean serum ferritin (ng/mL)†	1890±1633	1702±1576
N. with diabetes or glucose intolerance	31 (15%)	12 (9%)
N. who had taken deferiprone	16 (8%)	8 (6%)
N. with previous antiviral therapy	14 (7%)	11 (8%)

**Based on multiple determinations collected at each transfusion during the 36 months before enrollment; †based on multiple determinations collected at each transfusion during the 12 months before enrollment.*

Table 2. Rates of fibrosis progression per year and the corresponding expected time from infection to cirrhosis among 117 thalassemic patients.

	N. of patients	*Rate of fibrosis progression per year*	*Expected duration for progression to cirrhosis (years)*
Overall patients	117	0.087 (0.077-0.107)	57 (47-65)
HCV RNA positive	80	0.101 (0.083-0.120)	49 (42-60)
HCV RNA negative	37	0.075 (0.059-0.111)	67 (45-85)

All values are expressed as median (95% CI). Estimates on fibrosis progression assumed linearity, according to Poynard et al.[26]

between HCV RNA positive (37 of 80, 46%; 95% CI 35-58%) and HCV RNA negative patients (16 of 37, 43%; 95% CI 27-60%). Overall, incomplete or definite cirrhosis was present in 9 patients (8 of whom were HCV RNA positive), this being a frequency of 8% (95% CI, 4-14%). The rates of fibrosis progression per year and the corresponding estimated time from infection to cirrhosis are given in Table 2. Figures are shown as median values because the rate of fibrosis progression was not normally distributed (*data not shown*). The analysis of selected subgroups of patients indicated that the time for progression to cirrhosis was slowest in non-viremic females (70 years; 95% CI, 39-130) and most rapid in viremic males (43 years; 95% CI, 30-53), particularly in the presence of persistently abnormal ALT levels (36 years, 95% CI, 30-53).

The univariate and multivariate analyses of the relations between necroinflammatory and fibrosis scores with several qualitative and quantitative variables are given in Table 3.

At multivariate analysis, necroinflammation was related to HCV viremia, and fibrosis to abnormal ALT, high ferritin levels, and male gender. With regards to iron overload, both serum ferritin (*<2500 ng/mL*, *≥2500 ng/mL*) and total iron score (*≤25*, *>25*) were included in the multivariate model. As for necroinflammatory score, the inclusion of either or both these markers of iron overload did not significantly affect the estimates of RR concerning the other terms in the model. As for fibrosis score, the inclusion of both marker gave the result that neither marker was associated with fibrosis progression, whereas both total iron score and serum ferritin appeared to be associated to fibrosis progression when entered into the model separately; for total iron score, the RR was 1.97 (95% CI, 1.08-3.6). This was due to the high correlation (r=0.74) between total iron score and ferritin (log-scale). Similar results were obtained when the multivariate analysis was repeated in the subgroup of 81 patients for whom liver iron content data were available. In this model, when liver iron content replaced serum ferritin, the RR was 2.23 (95% CI, 1.14-4.36). Figure 2 shows the necroinflammatory and fibrosis score progression as a function of age.

Table 3. Effect of different risk factors on the incidence of a necroinflammatory score equal to 5 Ishak units or higher (top) and of a fibrosis score equal to 3 Ishak units or higher (bottom), expressed as relative risks.

Risk Factor	*RR*[1]	*(95% CI)*	*RR*[2]	*(95% CI)*
Necroinflammatory score				
HCV-RNA (*positive* vs. *negative*)	6.21°	2.22-17.37	5.76**	1.90-17.48
ALT-pattern (*abnormal*" vs. *normal*)	2.58*	1.08-6.14	1.63	0.67-3.99
Serum ferritin (*≥2500 ng/mL* vs. *<2500 ng/mL*)	0.60-1.69	0.21	1.16	0.39-3.51
Gender (*male* vs. *female*)	0.79	0.79-1.42	1.04	0.58-1.88
Fibrosis score				
HCV-RNA (*positive* vs. *negative*)	1.29	0.71-2.34	1.51	0.76-3.02
ALT-pattern (*abnormal* vs. *normal*)	3.53°	1.39-8.94	2.84*	1.07-7.49
Serum ferritin (*≥2500 ng/mL* vs. *<2500 ng/mL*)	2.19*	1.16-4.11	2.17*	1.05 - 4.48
Gender (*male* vs. *female*)	1.75	0.99-3.07	1.79*	1.02-3.17

*[1]Estimates derived from univariate Cox models; [2] estimates derived from a multivariate Cox model (these estimates are adjusted for the effect of all the other terms in the model). *p<0.05; °p<0.01.*

The presence of diabetes and previous therapy with deferiprone was not related to histological features. The overall frequency of steatosis was 16 of 117 (14%; 95% CI, 7.5-20%). Steatosis was unrelated to all the considered variables.

Evaluation of hepatic iron

The grading scores for iron overload in parenchyma, sinusoidal cells, and portal tracts according to the results of HCV determination are reported in Table 4. In 2/117 patients (1.7%; age 19 and 37 years; mean serum ferritin, 514 and 450 ng/mL respectively), there was no stainable iron in the liver (i.e., total iron score was 0). Iron was absent from hepatocytes and limited

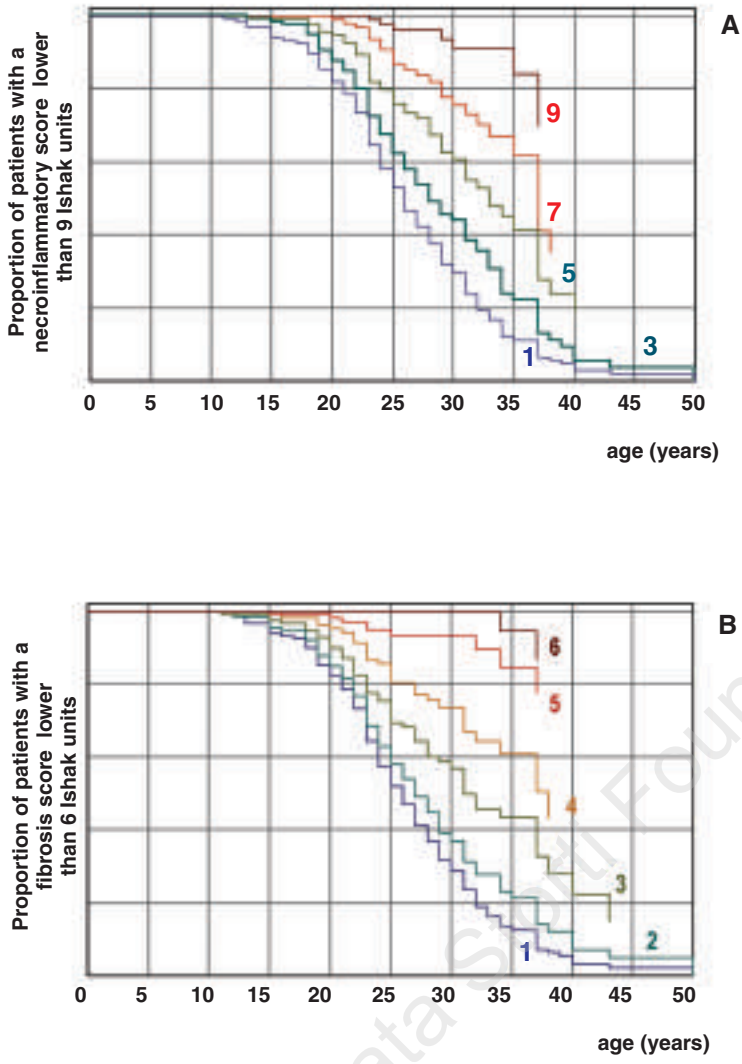

Figure 2. Panel A: Proportion of patients with a necroinflammatory score lower than 9 Ishak units as a function of age. Panel B: Proportion of patients with a fibrosis score lower than 6 Ishak units as a function of age.

to the Küpffer cells in another 3 patients (2.5%), all with mean ferritin levels ≤900 ng/mL and total iron score ≤5. Among the remaining 112 patients, parenchymal iron predominated in zone 1, with a decreasing gradient throughout the lobule from zone 1 to zone 3 (Figure 3). The lobular gradient was appreciable in all the 112 patients but 2, both with massive iron overload (i.e. total iron score of 34 and 35). The extent of iron deposition in patients with different degrees of hepatic iron overload is summarized in Table 5. In the subgroup of 81 patients for whom the measurement was possible, the median liver iron content was 6 mg/g dry weight (range, 0.6-29 mg/g dry weight).

Discussion

Of the patients initially evaluated, only a few (8%) were excluded because they were free from biochemical or virological signs of liver disease, confirming that hepatic disease is a dominant issue in the management of thalassemia.[2,3] The proportion of patients with evidence of chronic liver disease would have been even higher had more stringent ALT thresholds been used.[34]

The presence of HCV viremia was the only factor independently related to necroinflammation. This notwithstanding, the median necroinflammatory score was just three units higher in viremic patients than in non-viremic ones. This mildness of inflammatory lesions is in line with data collected in non-thalassemics who acquired HCV infection during infancy or adolescence.[26,35] With regards to HCV RNA negative patients, the paucity of the inflammatory infiltrate is an important argument against the clinical relevance of a putative blood-borne non-A, non-B, non-C chronic hepatitis agent. This is the first histological counterpart to previous serological studies among multitransfused patients, which were unable to document a pathogenetic role for several new-

Table 4. Grading for iron overload in parenchyma, sinusoidal cells, and portal tracts in 117 thalassemic patients, grouped according to the results of HCV determination.

	HCV RNA Neg. (n=37)	*HCV RNA Pos. (n=80)*	*Overall (n=117)*
Parenchymal Iron Score [0-12]			
Zone 1	6 (0-12)	6 (0-9)	6 (0-12)
Zone 2	6 (0-9)	3 (0-6)*	3 (0-9)
Zone 3	3 (0-9)	0 (0-6)*	0 (0-9)
Sinusoidal (Küpffer cells) Iron Score [0-12]			
	8 (0-12)	6 (0-10)*	6 (0-12)
Portal Iron Score [0-4]			
Connective tissue	1 (0-4)	1 (0-4)	1 (0-4)
Biliary ducts	0 (0-4)	0 (0-3)	0 (0-4)
Vascular walls	1 (0-4)	0 (0-3)*	1 (0-4)
Total Iron Score [0-60]			
	27 (0-52)	17.5 (0-39)*	20 (0-52)

*All distributions are expressed as median (range). Intervals in brackets indicate the grading scoring scale for iron deposition. *= p<0.05 as compared to HCV RNA negative patients (Mann-Whitney test).*

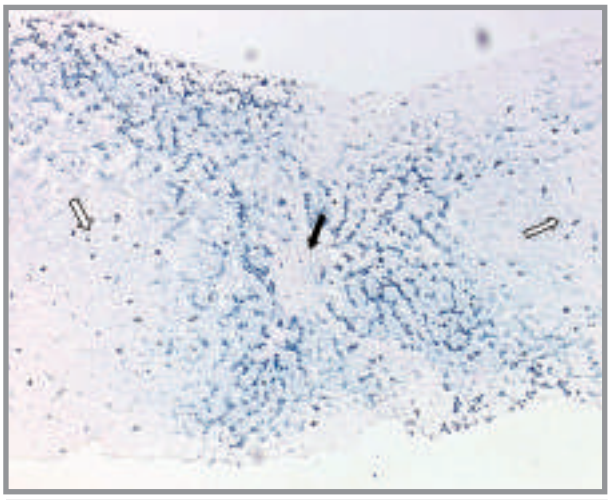

Figure 3. Iron storage is prevalent in periportal and periseptal hepatocytes (black arrow), while it is diffuse throughout the lobule in Kupffer's cells (white arrows).

ly identified transfusion-transmissible viruses, even in the presence of very high frequencies of infection.[3,4,23,24,36-39] The prevalence of clinically significant fibrosis was not negligible in our thalassemic cohort (overall, 45%). On the basis of a widely used mathematical model,[26] the median expected time to cirrhosis was 57 years. The progression to cirrhosis was more rapid in HCV RNA positive as compared to in HCV RNA negative patients (49 vs. 67 years), and at the time of liver biopsy 8 of the 9 thalassemics with cirrhosis had detectable HCV RNA in the serum. However, our data indicate that liver fibrosis is the result of multiple factors rather than being uniquely consequent to HCV infection. In fact, at multivariate analysis, fibrosis progression was unrelated to the presence of HCV RNA, being mainly associated with the degree of liver cell injury (ALT pattern) and, to a lesser extent, to male gender and ferritin levels. A recent investigation in patients cured of thalassemia by bone marrow transplantation identified HCV infection and iron overload as independent factors for disease progression.[18] Our findings indicate a less striking relationship between HCV viremia and fibrosis. The possible implications of the multifactorial nature of thalassemia-associated liver disease need to be discussed in greater detail. It should be considered that surviving after 40 years of age is now a realistic target for thalassemics born after 1970 (*C. Borgna-Pignatti, personal communication*). It is therefore foreseeable that a consistent proportion of these young patients will progress towards end-stage liver disease during their lifetimes. In addition, current estimates on fibrosis progression are based on a linear model, which might not be fully appropriate to predict liver disease outcome.[40] A patient's age at clinical evaluation is independent of the duration of infection in predicting

Table 5. Patterns of hepatic iron deposition and corresponding levels of serum ferritin in thalassemic patients with low, intermediate and high grade of iron overload.

	Low Total Iron Score (n=36)	*Intermediate Total Iron Score (n=44)*	*High Total Iron Score (n=37)*
Parenchymal Iron Score [0-12]			
Zone 1	6 (0-6)	6 (6-9)	9 (6-12)
Zone 2	0 (0-3)	3 (0-6)	6 (3-9)
Zone 3	0 (0-3)	0 (0-6)	6 (0-9)
Sinusoidal (Küpffer cells) Iron Score [0-12]			
	3 (0-9)	7 (1-10)	9 (2-12)
Portal Iron Score [0-4]			
Connective tissue	0 (0-3)	1 (0-4)	4 (0-4)
Biliary ducts	0 (0-0)	0 (0-3)	1 (0-4)
Vascular walls	0 (0-2)	1 (0-3)	1 (0-4)
Total Iron Score [0-60]			
	10 (0-15)	20 (16-25)	32 (26-52)
Mean Serum Ferritin (ng/mL)			
	741 (65-2200)	1173 (198-3917)	2532 (788-9800)

Low total iron score: 0-15; intermediate total iron score: 16-25; high total iron score: 26-60. All distributions are expressed as median (range). Intervals in brackets indicate the grading scoring scale for iron deposition.

HCV–related cirrhosis.[41]

Furthermore, the long-term follow-up of the Cooley-care cohort showed that aminotransferase flares are frequent among thalassemics, even in the absence of HCV infection.[2] The present data documented a worsening of the necroinflammatory activity, in parallel to fibrosis, as patients' age increased. Thus an acceleration of disease progression over time, due to advancing age and/or modifications in the host environment, cannot be ruled out.

This is the first study describing the liver pathology of thalassemics with pure iron overload, i.e. those free from infection with known hepatotropic agents. The median total iron score was 27, a figure comparable to scores observed in subjects with full-blown homozygous hereditary hemochromatosis.[27,28] All the three indicators of iron overload considered in the present study (i.e., total iron score, serum ferritin, and liver iron content) were related to fibrosis progression when separately analyzed in the multivariate model. Only one of the patients of the HCV RNA negative group (2.5%) had frank cirrhosis, whereas prevalences of 14% to 28% have been reported in subjects with hereditary hemochromatosis.[27,41,42] However, it should be taken into consideration that our patients were 15 to 25 years younger than those commonly evaluated for hereditary hemochromatosis -associated liver disease.[27,28,42,43] In addition, bridging fibrosis was a common finding (43%), which raises the concern of clinical worsening in the short-term.

With regards to the pattern of iron deposition, mesenchymal cells were markedly involved, in agreement with earlier studies on post-transfusion iron overload.[26,44,45] Unexpectedly, however, the vast majority of the patients had substantial accumulation of iron in hepatocytes, which was distributed according to a definite periportal-to-pericentral decreasing gradient (from Rappaport's zone 1 to zone 3). Based on experimental and human studies, this lobular pattern is strongly indicative of intestinal hyperabsorption.[26,27,45-47]

Our data challenge previous results on the liver pathology of transfusion-associated iron overload. Transfusional iron was previously thought to be absorbed by hepatocytes only in the more advanced stage of accumulation, as a consequence of a redistribution from the saturated reticuloendothelial system.[45] The presence of a lobular gradient was considered a distinctive feature of hereditary hemochromatosis and non-transfused ineffective erythropoiesis, being typically absent from cases of transfusional iron overload.[27,29,45,47] A possible explanation is that the reduction of the pre-transfusion hemoglobin thresholds (from 11 to 9.5 g/dL), adopted in the early 1990s to limit the transfusional iron burden in thalassemia,[1,3,48] had the simultaneous effect of stimulating ineffective erythropoiesis, leading to a relative increase in the amount of iron absorbed from the portal circulation. In fact, thalassemics with significant erythroid expansion may reach levels of iron absorption of 4 mg per day or more (up to 1.5-2 g per year, the equivalent of iron contained in 6-8 units of red blood cells necessary for an average 3 months of treatment).[48,49] However, alternative explanations should also be considered, including the possible redistribution in the hepatic parenchyma of iron mobilized from tissues following chelation therapy. Further studies will be useful to clarify these issues.

In conclusion, thalassemia-associated liver disease should now be considered a unique clinical and pathological entity, characterized by mild necroinflammatory activity, mainly attributable to chronic HCV infection, and by a variable degree of fibrosis progression. The major determinants for fibrogenesis are related to hepatic iron overload, which may be consequent not only to the catabolism of transfused red cells, but also to increased iron uptake from the gut, via the portal blood. This information will be useful for optimizing novel therapeutic strategies.

DP, MDC, and GF were primarily responsible for the design of the study protocol. DP and MDC wrote the paper. The remaining authors, who qualified for authorship according to the WAME criteria, were specifically responsible for the following parts of the content: MM and GC for histological analyses, MC and RG for data handling, SM for statistical analysis, PC, GLF, CM, and AM collected the data and contributed to their interpretation. PR, GC, and GF revised the manuscript. All authors approved the final version of the paper.The authors are indebted to Pier Luigi Brovero, MD (Genoa), Anna Crescenzi, MD (Albano Laziale, Rome), Silvia Fargion, MD (Milan), Maurizio Russello, MD (Catania) and Claudio Scisca, MD (Messina) who performed liver biopsies.

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