MÆDICA - a Journal of Clinical Medicine

ORIGINAL PAPERS

The contribution of chronic hepatitis C virus infection and iron overload to hepatocellular injury in β thalassemia major patients

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BACKGROUND

The thalassemias comprise heterogenous haemoglobin disorders characterized by reduced synthesis of one or more of the globin chains. They are the commonest monogenic disorders in the world, with 270 million carriers, of which 80 million are carriers of β thalassemia (1). The major variant of β thalassemia is defined by severe β globin deficit, generated most often by reduced production of structurally normal β chains. This may be the result of any of more than 200 point mutations described to date, or much less frequently, of deletions (2). Those affected become symptomatic at about 4 to 6 months of age, when haemoglobin A becomes normally the dominant type of haemoglobin, severe anaemia being the main problem. Without intervention progressive anaemia and metabolic stress leads to heart failure and eventually death, generally before age of 5.

Modern treatment requires regular blood transfusions, associated with iron chelators to counteract secondary hemosiderosis. Accomplishing a normal iron balance is currently limited only by patient compliance (2).

In spite of universal screening of transfused blood for blood-borne pathogens and hepatitis B virus (HBV) vaccination at birth, transfusion related hepatitis C virus (HCV) infection, along with secondary hemosiderosis, continues to represent the main cause of hepatic disease in thalassemics (3), probably because of donor testing in the immunological window or increased nosocomial exposure of these patients (4). Moreover with extended survival advanced liver disease has emerged as an important long term treatment complication in β thalassemia major.

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This study tries to define the contribution of either chronic HCV infection or hepatic iron overload in determining chronic liver disease progression in Romanian thalassemic patients. Other iron-related complications in the same cohort will be presented elsewhere. ❑

MATERIALS AND METHODS

We performed the analysis of a prospective cohort of 90 patients, registered and treated at the National Institute of Transfusional Haematology in January 1999, which were followed until August 2008. All patients had unlimited access to standard treatment, with regular blood transfusions for pre-transfusion haemoglobin levels of 9-9.5 g/dl and deferoxamine, started at 20 mg/kg infused subcutaneously 5-6 days per week, and progressively increased to 40-60 mg/kg/day.

Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured every three months and, since 2001 serum ferritin was determined every four to six months. Patients were stratified in three ALT patterns: persistently normal, persistently high and intermittently high. Similarly five patterns of ferritin variation were identified: acceptable levels (close to 1000 ng/ml), progressive decrease to levels below 1000 ng/ml, gradual decrease (without reaching the target level of 1000 ng/ml), steady increase and fluctuations around values well above 1000 ng/ml. The mean ferritin was judged as ideal when below 1000 ng/ml, adequate when lower then 2500 ng/ml and unacceptably high when above 2500 ng/ml.

All patients were tested for Human Immunodeficiency Virus (HIV) and Human T-cell Lymphotropic Virus (HTLV) infections.

All data were entered into a database and analyzed using SPSS 10.0 for Windows software. The differences between continuous data were tested by parametric tests (t test) if normally distributed, or by non parametric test (Mann-Whitney, Wilcoxon or Kruskal-Wallis tests, as appropriate) if the contrary was true. For dichotomous or categorical variables chi square test of independence was used. ❑

RESULTS

Ninety patients were included, out of which 42 were females and 48 were males. The mean age was 23 years (range, 11-40 years), while mean age at first transfusion was 16 months (range, 2-72 months). Seventy-four percent of patients had undergone splenectomy at a mean age of 8 years (range, 1-28 years). In 44% of patients mean pretransfusional haemoglobin level was between 7 and 8 g/dl, while in 38% of subjects it was between 8 and 9 g/dl. Only 11% exceeded the recommended target of 9 g/dl while 7% presented values below 7 g/dl. There were no differences in these variables between the two sexes.

Eighty-one patients tested positive for anti-HCV (90%), of which only 45 (55.55%) had detectable HCV-RNA. None of the 8 patients transfused after 1996 was seropositive for HCV. Infection's age (calculated between the year of the first transfusion and 2005, when HCV-RNA testing was performed) was not different among those with detectable or undetectable HCV-RNA.

A single patient tested positive for hepatitis B surface antigen, other four having serological markers of antecedent cured HBV infection, which brings HBV infection prevalence in this population at about 5%. HIV infection was absent in all patients, whereas HTLV infection was documented in 3.

Mean ferritin for the entire cohort was 2828 ng/ml (range, 360-8225 ng/ml). Fourty-eight percent of the patients had unacceptably high levels of ferritin (above 2500 ng/ml), only 18% having ideal serum ferritins. Thirty-four percent of thalassemics were considered to have adequate ferritin values (FIGURE 1).

Eight of the patients had acceptable ferritin levels during the entire follow-up period (9%), while in other 22 (24.4%) ferritin decreased progressively bellow 1000 ng/ml. Another ten of the thalassemics (11%) showed the same declining tendency, without reaching the target level. Ferritin rose gradually in 14 patients (15.6%), 7 of them initiating from values less

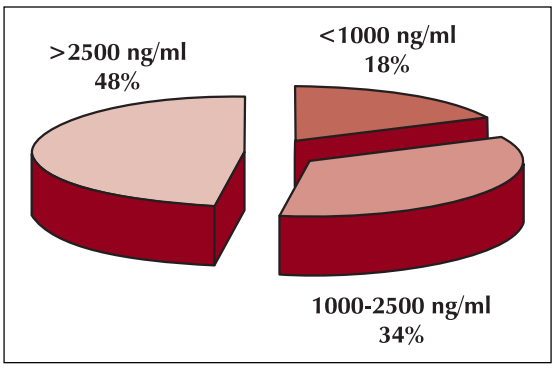

FIGURE 1. Mean ferritin

than 1000 ng/ml. The remaining 36 patients (40%) presented fluctuating levels of ferritin, only a third of them attaining transiently the desired level of 1000 ng/ml.

Only 14 subjects had mean ALT in the normal range. At univariate analysis virological status and serum ferritin were the only independent variables which influenced hepatocytolysis.

The mean ALT rise for the entire cohort was 2.3 times the upper reference limit – URL (range, 0.5-7.3 times the URL) and the mean of the highest values of ALT was 4.24 (range, 0.9-11.7 times the URL).

Patients with detectable HCV-RNA had significantly higher mean ALT ($p<0.0001$) and maximal ALT increases then the others ($p<0.0001$).

At ferritin levels inferior to 1000 ng/ml mean ALT tended to be normal, increasing significantly when this limit was exceeded ($p<0.0001$).

The majority of patients with normal ALT had ferritin levels below 1000 ng/ml and undetectable HCV-RNA. However moderate ALT increases have been identified at ferritin less than 1000 ng/ml, but always in association with detectable viraemia ($p=0.044$). On the contrary at least 80% of those with ferritin levels above 1000 ng/ml and detectable HCV/RNA (36 out of 39 thalassemics) had mean ALT increases exceeding 2 times the URL, while in those with undetectable viraemia predominated milder ALT augmentation ($p=0.001$). When mean serum ferritin got beyond 2500 ng/ml, half of the HCV-RNA negative pacients presented ALT raises in the moderate range, while for those with detectable viraemia no additional ALT increase was observed (FIGURE 2). All these observations were true also for the maximal levels of ALT.

From the entire cohort only 6 patients had persistently normal aminotransferases (6.7%), which were constantly (64.4%) or occasionally elevated (29%) in the majority (FIGURE 3). In all patients AST and ALT variations were concordant, even if with different magnitudes. At univariate analysis ferritin and HCV-RNA were predictive factors for ALT pattern ($p<0.0001$). A higher proportion of viraemic subjects were noted in the group of persistently abnormal ALT (67.24%) while those with undetectable HCV-RNA were more frequently encountered among patients with constantly normal (83.33%) or

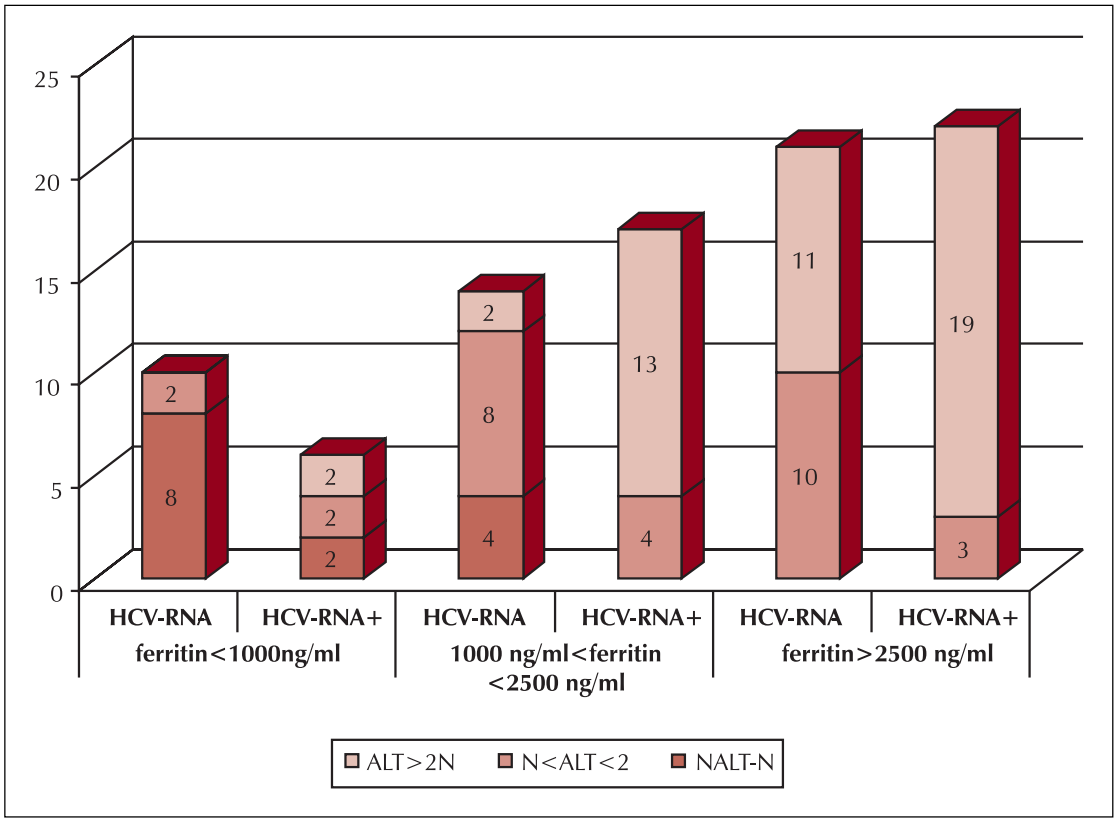

FIGURE 2

transiently increased ALT (80.77%; p=0.002). Thirty-nine out of 45 HCV-RNA detectable thalassemics had persistently high aminotransferases, only one presenting normal ALT levels.

Mean serum ferritin for patients with permanently normal ALT was 816.45 ng/ml. Five out of these 6 subjects had mean ferritin under 1000 ng/ml, along with undetectable HCV-RNA. Mean ferritin was higher, although not significantly, in the group of intermittently increased ALT (1651.6 ng/ml), while patients with persistently abnormal ALT presented much more elevated mean ferritin levels (3563.73 ng/ml; p<0.0001; FIGURE 4).

Mean ferritin was not different between those with detectable or undetectable viraemia, though in the last case it was considerably higher when ALT was persistently increased (5187.51 ng/ml) compared to patients with transiently high (1949.53 ng/ml; p<0.0001) or constantly normal ALT (636.35 ng/ml; p<0.0001). Among thalassemics with detectable HCV-RNA a single one had persistently normal ALT, mean ferritin being significantly lower in those with intermittent ALT increases (651.21 ng/ml) than in subjects with persistently high ALT (3072.6 ng/ml).

ALT pattern varied between different types of ferritin models. Only one of the patients with ideal serum ferritin during the entire follow-up period had persistently increased ALT, being also HCV-RNA positive. In the same group those with normal or intermittently high ALT were equally represented, the firsts having more often undetectable HCV-RNA while the lasts were more frequently viraemic (p<0.0001). The major part of subjects with progressive ferritin decline to levels under 1000 ng/ml demonstrated transiently increased ALT (p=0.019), many of them being also HCV-RNA negative. In the case of other ferritin variation patterns the persistent increase of aminotransferases was the most frequent model encountered, the few patients with intermittently high ALT being in almost all the cases HCV-RNA negative (FIGURE 5).

Ferritin and ALT variation analysis at individual level demonstrated a striking synchrony in the majority of patients (33 out of 36

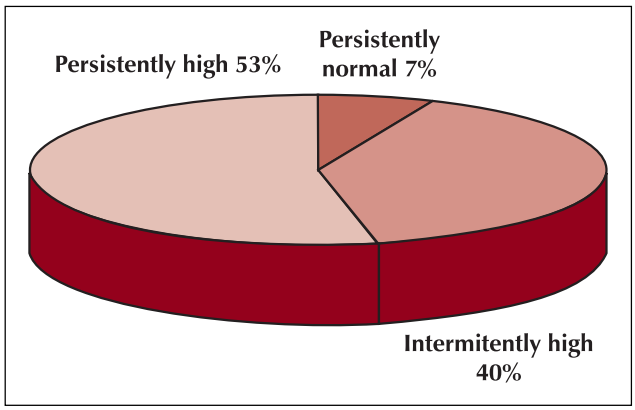

FIGURE 3. Aminotransferases variation pattern

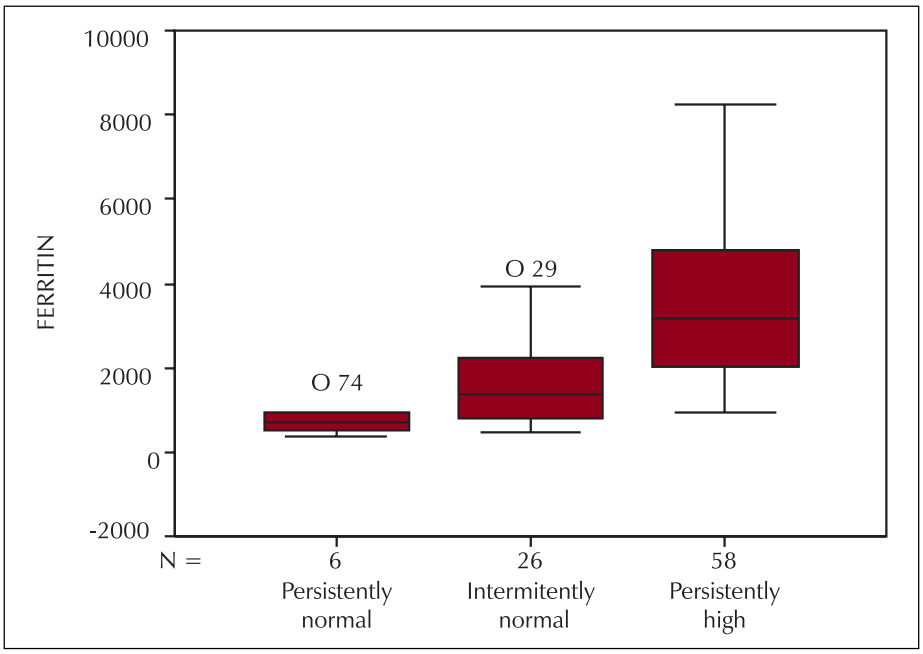

FIGURE 4. ALT pattern

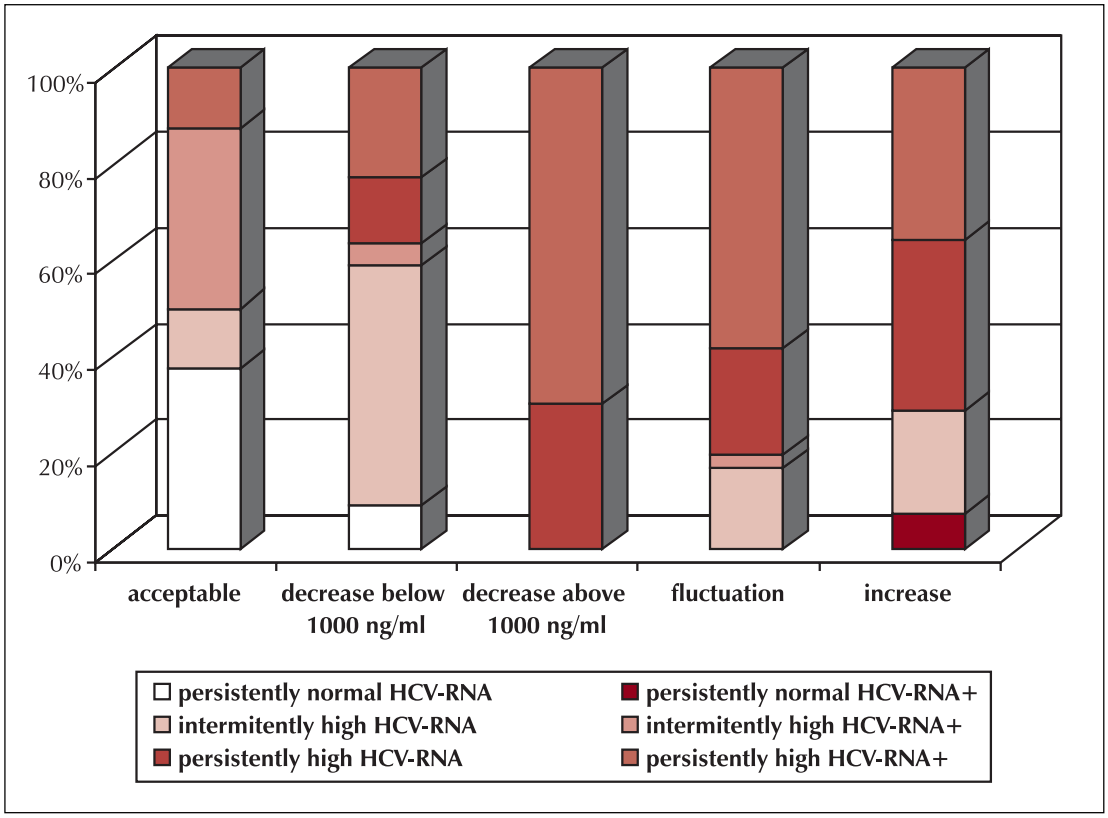

FIGURE 5. Ferritin patterns

thalassemics with transiently increased ALT, and 46 out of 48 subjects with persistently high ALT; p=0.001). Moreover 3 out of the 6 patients with persistently normal ALT showed synchronous ferritin and ALT oscillations, their mean ferritin levels being somewhat higher, although the difference did not reach statistical significance (1162 compared to 470.85 ng/ml; p=0.072).

Ten patients (11.11%) presented clinical, biochemical and ultrasonographical signs of cirrhosis, which was confirmed histologically in only one case. These subjects were significantly older than the others (28.7 versus 22.56 years; p=0.002), none being younger than 25. Seven were males, but this was not significant. After excluding from analysis the single patient with mean ferritin inferior to 2500 ng/ml, cirrhotic patients demonstrated higher mean serum ferritin than the rest of the patients (4094.5 compared to 2705 ng/ml; p=0.021). In 6 subjects ferritin fluctuated around the median value, including the case with mean ferritin close to 1000 ng/ml. In 2 patients there was a progressive increase in ferritin levels during the study period while the other 2 demonstrated a constant decrease, without reaching the target value of 1000 ng/ml. Practically 7 out of 10 thalassemics with advanced liver disease showed an unsatisfactory evolution of serum ferritin during the follow up.

All 10 cirrhotics had positive HCV serology, while 9 were also viraemic, proportion significantly higher than for the rest of the cohort (p=0.019). The only HCV-RNA undetectable patient was a women aged 28 who had a mean ferritin level of 4525 ng/ml. The mean HCV-RNA level was also higher in these subjects when compared to non-cirrhotic patients (1473778 versus 800575 UI/ml; p=0.049). Time from infection was notably longer in cirrhotics than in other viraemic patients (24.44 compared to 18.61 years; p=0.015). Only one subject had serologic evidence of cured antecedent HBV infection. ALT was persistently increased in thalassemics with advanced liver disease, although the mean and maximal ALT levels were not different from other patients. All 10 had AST/ALT over the unit (1.38 versus 1; p= 0.014).

All cirrhotic subjects demonstrated a positive correlation between aminotransferases and ferritin variation curves. AST increase constantly exceeded that of ALT. At univariate analysis se-

rum ferritin, detectable HCV-RNA, and AST/ALT ratio were predictive factors for hepatic cirrhosis, while age and sex were not. ❑

DISCUSSIONS

Apparently mean age in our cohort is comparable to that reported from countries with better access to optimal treatment (5), however we must keep in mind the fact that it was determined at the end of study period, being almost a decade lesser at the beginning of data collection. By extrapolating current data about survival in beta thalassemia major, that extends nowadays in the third decade (6), we can speculate that life expectancy in our cohort goes beyond the second decade, which represents nevertheless an improvement from previously reported data.

HCV infection prevalence is identical to that described in Italian patients (5), being almost universal in those transfused for the first time before 1995. Thereafter transmission risk diminishes dramatically, probably by efficient universal screening of transfused blood for commonly blood born pathogens. Persistent or cured HBV infection was much less frequently observed, generally after age 20, in thalassemics transfused before 1988, suggesting that Australia-antigen positive individuals were excluded from the donor pool much earlier. HIV infection was absent in every patient, perhaps because HIV epidemic in Romania took place in paediatric patients who usually do not donate blood.

Even if HCV and HIV infection no longer represent major threats in patients with beta thalassemia major, for those transfused before 1995, chronic HCV hepatitis still poses a serious problem. Forty-five out of 81 subjects with HCV positive serology had detectable HCV-RNA, which brings spontaneous viral clearance to a surprisingly high 44.45%. As compared to 10-20% reported in general population, the clearance rate is surprisingly high, yet comparable to values reported in other studies in thalassemic patients (5). It exceeds even the 25% rate observed in haemophilic individuals, being inferior only to that reported in new-borns with vertical HCV transmission, where it reaches 75% (7). This observations support the idea of very early HCV infection acquisition in thalassemic patients, most likely in the first year of transfusional treatment. Infection duration is not a predictive factor for viral clearance.

Nearly half of those with detectable HCV-RNA had low viraemia, only 20% presenting levels over 2,000,000 UI/ml.

Mean serum ferritin is similar to that published in other studies from the nineties, being significantly higher than the values reported from western countries after 2000. This reflects the management difficulties which had to be faced in thalassemic patients in our country. In spite of universal access to standard therapy nearly half of the present cohort had mean ferritin values over 2500 ng/ml, level over which cardiac mortality has been demonstrated to rise sharply (8). Moreover only a fifth of our subjects had mean ferritin under 1000 ng/ml, recently proposed as the target level for significant reduction of all causes mortality (6). These general data reflect from the beginning the very poor treatment compliance in our patients.

It comes without surprise that 84.4% of the present cohort patients demonstrated intermittent of persistent increases of aminotransferases, two third of which exceeded two times the URL. However hepatocytolysis frequency is even higher than in published cohorts with similar HCV infection prevalence but with significantly lower mean ferritin (5), which underlines the importance of iron overload in the development of hepatic lesions.

At univariate analysis the only variables that influence both mean and maximal level of ALT are ferritin and virological profile. For a given mean ferritin level viraemic patients demonstrate significantly higher aminotransferase increases, compared to those with undetectable HCV-RNA. For ferritins over 1000 ng/ml the raise goes beyond 2 times the URL. HCV-RNA undetectable patients rarely present such level of ALT increment when mean ferritin remains below 2500 ng/ml. Therefore the 2 times the URL increase can be used as a cut-of value for selecting patients with mean serum ferritin inferior to 2500 ng/ml in which qualitative HCV-RNA determination is useful in considering the initiation of antiviral treatment. If admitting that the magnitude of aminotransferases increase is a risk factor for fibrosis progression (5), the 2500 ng/ml limit acquires prognostic significance, at least for pacients with undetectable viraemia, in which hepatocytolysis rises markedly over this value. In exchange in viraemic individuals exceeding the 2500 ng/ml limit does not produce

a supplementary increase in ALT levels. Maybe it is more important to notice that, in the case of undetectable HCV-RNA, the majority o patients with mean ferritin over 1000 ng/ml have abnormal ALT, and therefore under this limit any ALT increase should raise the suspicion of chronic HCV infection. The same goes true for the maximum ALT value attained in a given patient.

In patients cured by bone marrow transplantation both ferritin and chronic HCV infection proved to be independent risk factors for hepatic disease in thalassemic patients (9). Our data supports the idea that the first option in preventing progressive liver disease should always be the optimization of chelation therapy, with the objective to reduce serum ferritin to levels below 1000 ng/ml. At this stage any ALT abnormality would need virological investigation, with HCV-RNA detectable patients being considered for antiviral therapy. When ferritin goes over 1000 ng/ml HCV-RNA should be determined at least in individuals with an ALT increase beyond 2 times the URL, with positive serology.

The more than obvious correspondence between ALT and ferritin variation curves in individual patients raises the question if ferritin changes are not the consequence rather than the cause of hepatocytolysis. Being an acute phase reactant ferritin could be increased theoretically by hepatic inflammation. However the synchrony between ALT and ferritin evolution is characteristic also to HCV-RNA undetectable patients, where iron overload is presumably the main cause of liver disease. Taking into account the oxidative mechanism of iron induced hepatic lesions (10), it seems logic that more severe iron overload produces, along with increases in serum ferritin, more extensive hepatocellular damage, translated in higher ALT values. Moreover the ALT flares noted in individual aminotransferases variation patterns of viraemic patients were not associated with similar increases in ferritin levels. In addition HCV-RNA detectable subjects have comparable mean ferritin to those which are non-viraemic. Only beyond 2500 ng/ml the magnitude of ALT augmentation is comparable between viraemic and non-viraemic patients, the last ones beginning to present even supplementary flares of hepatocytolysis. It is therefore conceivable that only above the 2500 ng/ml limit the ferritin increment is partially owed to hepatic necroinflammation. This hypothesis is supported by studies which demonstrated that the alleged poor concordance between liver iron concentration and ferritin is true only when the last one goes over 2500 ng/ml (11), at lesser values ferritin closely reflecting the degree of iron overload (5).

Reported histological prevalence of hepatic cirrhosis in patients with β thalassemia major varies between 6 and 19%, half of these patients lacking physical signs of liver deficiency or cirrhosis. The 11.11% prevalence in our cohort is therefore within the expected range, even if the diagnosis was confirmed histologically in a single case. At univariate analysis age at evaluation, detectable viraemia, mean ferritin and supraunitary AST/ALT ratio were predictive factors for liver cirrhosis.

Comparing these results with already published studies is difficult, giving the fact that our patients had more severe iron overload, owed partially to sporadic access to iron chelators. What is more they have been maintained for long periods of time at lower than recommended pretransfusional haemoglobin levels, which favors intestinal iron hyperabsorption and parenchymal iron deposition in the liver, with toxic effect on the hepatocyte. So it comes without surprise that ALT abnormalities were much more frequent in this cohort compared to others.

There was however an Italian cohort of 129 patients with comparable characteristics (5) which connected fibrosis progression to hepatocellular injury (as reflected by the ALT increase), mean serum ferritin and male gender. Detectable viraemia had no influence on the stage of fibrosis. In our set of data HCV-RNA presence and mean ferritin were both predictors of cirrhosis. Not only that 9 out of 10 patients had detectable HCV-RNA, but viraemia was significantly higher than for other subjects. Male predominance was also noted, even if it did not reach statistical significance. Cirrhotic subjects were also individualized by ALT pattern, which was persistently increased in all cases.

We can conclude that in young thalassemics free of HCV infection, iron overload accelerates fibrosis progression only if it exceeds certain limits. In the case of chronic HCV infection, secondary hemosiderosis accelerates the natural history of liver disease, the rate of fibrosis progression depending on iron overload severity. On the long term excess iron persis-

tence, especially if severe (reflected by serum ferritin levels over 2500 ng/ml), can induce both necroinflammation and liver fibrosis, irrespective of viral status.

Further support to this model is brought by the age of our cirrhotic patients, almost all between 25 and 29 years old. This suggest a much more rapid rate of fibrosis progression compared to that estimated in prospective histological studies, namely 49 years to cirrhosis for detectable HCV-RNA individuals and 67 years for HCV-RNA undetectable ones (5). ❑

CONCLUSION

1. Even if HCV infection incidence was greatly reduced by universal screening of transfused blood, it continues to represent a problem for β thalassemia major patients transfused before 1995.
2. On the contrary, efforts are still to be made to control iron overload. Improving compliance to iron chelation can prevent liver disease in patients without HCV infection, while reducing the risk of hepatic fibrosis progression in those HCV-RNA positive. If this is to fail iron excess will soon become the main cause of liver dysfunction in young thalassemic patients.
3. Iron overload and HCV-RNA are independent predictors of liver disease. At ferritin levels above 2500 ng/ml liver iron excess enhances the risk of progression to advanced liver disease even in the absence of detectable viraemia, while at ferritins below this limit, it accelerates the progression of viral induced hepatic fibrosis.
4. Liver iron overload induces hepatocellular lesions from serum ferritin levels as low as 1000 ng/ml, which has to be considered the new target of chelation therapy.
5. Thalassemic patients with ferritin over 1000 ng/ml and ALT increases above 2 times the URL have to be tested for HCV-RNA, if HCV serology is positive. In the case of detectable viraemia reducing iron overload to ferritin levels below 1000 ng/ml through intensification of iron chelation, diminishes the risk of liver fibrosis progression. Under this limit any ALT abnormality should determine virological testing, liver biopsy and antiviral treatment as indicated.
6. Under the 2500 ng/ml value, which is the minimal target for chelation therapy, serum ferritin reflects sufficiently well the severity of iron overload to permit management decisions. It offers the advantage of dynamic monitoring, which is impossible in both the case of magnetic resonance, by cause of limited availability and high cost, and liver biopsy, to invasive to be accepted by the patient as a method of periodical evaluation. ❑

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