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Short title: Iron, HFE and hepatocellular carcinoma

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No conflict of interest exist.

Abbreviations: AFP, α-fetoprotein; CHC, chronic hepatitis C; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ROS, reactive oxygen species; SVR, sustained virological response.

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ABSTRACT

Background and aims: The influence of HFE gene mutations and liver iron overload on hepatocellular carcinoma (HCC) occurrence in patients with cirrhosis is subjected to controversial results. The aim of this work was to clarify this influence in a large cohort of prospectively followed-up cirrhotic patients classified according to the cause of their liver disease. ***Methods:*** Three hundred one consecutive cirrhotic patients (162 alcoholic and 139 HCV-infected patients) were included at time of diagnosis of cirrhosis and followed-up. Liver iron overload on initial biopsy according to modified Deugnier's score and C282Y/H63D HFE gene mutations were assessed. ***Results:*** In patients with alcoholic cirrhosis (mean iron score=2.0±3.0; mean time of follow-up; 66.1±45.1 months), 40 (24.6%) developed HCC. Thirteen (8.02%) were heterozygotes for C282Y HFE gene mutation and had higher hepatic iron scores (3.6±3.8 vs 1.9±2.8, respectively, p=0.05). In univariate analysis, liver iron as a continuous variable (HR,1.23 [1.13-1.34], p<0.001) or in binary coding with an optimal threshold of iron score≥2.0 (HR, 4.1 [2.1-7.3], p<0.0001) and C282Y mutation carriage (HR, 2.7 [1.2-6.3], p=0.01) were risk factors for HCC. In multivariate analysis, liver iron and C282Y mutation carriage remained independent risk factors for HCC. In patients with HCV-related cirrhosis (C282Y mutation carriage, 17 [12.23%]; mean liver iron score, 0.9±1.9; mean time of follow-up, 85.5±42.1 months; HCC, 63 [45.32%] patients), C282Y mutation carriage and liver iron were not associated with HCC occurrence. ***Conclusions:*** Liver iron overload and C282Y mutation are associated with a higher risk of HCC in patients with alcoholic but not HCV-related cirrhosis.

INTRODUCTION

As hepatocellular carcinoma (HCC) is increasingly becoming a major cause of death in cirrhotic patients, [1] periodical ultrasonographic screening is now widely performed as routine. [2] The search for epidemiological, biological or genetic factors that could help to select patients at higher risk and thus to modulate the indications of screening procedures is necessary. [3] Moreover, identification of predictive factors could presumably lead to a better understanding of hepatocarcinogenesis and new prevention strategies in these patients. [4]

Among these factors, the influence of liver iron overload and HFE gene mutations has been debated over the past few years. The two missense mutations C282Y and H63D explain most of the cases of haemochromatosis, [5] a condition characterized by hepatic iron overload and leading to high cancer incidence. [6, 7] Eighty to 100% of these patients are homozygotes for the C282Y mutation and about 5% are compound heterozygotes. [5] The issue whether patients with other liver diseases heterozygotes for these mutations could develop liver iron overload that could enhance liver injury and carcinogenesis remains unclear.

Liver iron accumulation leads to reactive oxygen species (ROS) formation in the liver, thus causing oxidative stress, liver cell DNA mutations and the development of HCC. [8] Besides ageing and male gender which are well known risk factors for liver iron accumulation as well as for HCC, seeking for genetic markers of HCC such as HFE gene mutations that could act by increasing hepatic iron overload and thus HCC occurrence is of major interest as it would validate the carcinogenic role of liver iron and suggest preventive measures.

Whether moderate liver iron overload observed in the course of cirrhosis and the HFE mutations carriage could be associated with the risk of HCC in these patients remains unclear. [9] Some studies described higher prevalence of the C282Y mutation in patients with HCC compared with cirrhotic patients without HCC [10, 11]while other studies displayed a lack of association between this mutation and HCC. [12, 13] Moreover, the influence of the HFE

mutations on hepatic iron accumulation is also debated. Finally, the influence of liver iron overload on the risk of HCC, requiring prospective cohort studies, has never been clearly shown. Unfortunately, all previous works were case-control studies including small cohorts of cirrhotic patients and mixing various causes of liver diseases, thus limitating the confidence in the different conclusions drawn. Indeed, several limitations are obvious when studying these parameters: the different importance of iron metabolism in hepatocarcinogenesis pathways according to the aetiology of liver disease, the strong correlation between liver iron overload and ageing or male gender leading to methodological bias in multivariate analysis and the weak prevalence of the C282Y mutation in the studied populations.

To our knowledge, the only prospective study dealing with the influence of liver iron overload in cirrhotic patients was the one by Ganne-Carrie et al. [14] Conducted in 229 cirrhotic patients before the era of routine HFE gene mutations determination, this work displayed a lack of influence of liver iron overload on survival in patients with HCV-related cirrhosis but a strong influence in alcoholic cirrhotic patients. This study failed to demonstrate an association between liver iron overload and HCC incidence in these patients, possibly due to an insufficient number of patients with alcoholic cirrhosis and a low number of events.

The aim of the present study was therefore to assess on a large cohort of aetiologically defined and prospectively followed-up cirrhotic patients the influence of liver iron overload and HFE gene mutations on the risk of HCC according to the cause of liver disease (alcoholic or HCV-related) and the possible role of HFE gene mutations in this setting.

PATIENTS AND METHODS

Patients

The present study was part of an ongoing prospective study, aiming to assess the rates of HCC development in the course of various liver diseases.

In the present study, we compiled all new patients who were consecutively referred to our liver unit for diagnosis and management of cirrhosis between January 1, 1990 and December 31, 2000, and who fulfilled the following inclusion criteria: 1) biopsy-proven cirrhosis; 2) no infection by the human immunodeficiency virus, or hepatitis B virus; 3) no evidence of HCC at the time of inclusion, as judged by negative ultrasonographic findings and serum α-fetoprotein (AFP) less than 50 ng/mL; 4) residence in France 5) Caucasian origin; 6) acceptance of a regular follow-up and periodical HCC screening; and 7) written informed consent for the use of frozen DNA. A total of 301 patients met all these inclusion criteria. All had a known outcome at least until August 31, 2005.

These patients were divided in two distinct cohorts according to aetiology of liver disease. Patients with alcoholic cirrhosis (n=162) were defined as 1) daily alcohol intake > 80 grams per day and 2) no infection by HCV defined by negative serum HCV antibodies. Patients with HCV-related cirrhosis (n=139) were defined as 1) daily alcohol intake < 20 grams per day and 2) chronic infection by HCV defined by positive serum HCV-RNA. In this study, no patients with mixed alcoholic and HCV-related cirrhosis referred in our unit during this period were enrolled (n=28).

For each patient, the date of inclusion was the date of the first liver biopsy showing cirrhosis. Gender, age, presence of ascites or hepatic encephalopathy, serum bilirubin, albumin and prothrombin levels, serum alanine aminotransferase (ALT) activity, serum aspartate aminotransferase (AST) activity, serum gamma glutamyl transferase (GGT) activity, platelet

count, serum ferritin levels, body mass index (BMI), past history of diabetes were recorded at inclusion. Daily alcohol intake was recorded by interviewing all patients. In patients with HCV-related cirrhosis, virus genotype was precised.

All patients were prospectively evaluated at least every 6 months by physical examination, liver ultrasonography and serum AFP measurements. When these investigations suggested a possible diagnosis of HCC, computed tomodensitometry and/or magnetic resonance imaging and/or a guided liver biopsy were performed according to the recommendation of the Barcelona Conference. [3] HCC was diagnosed on either one of the following criteria: histological evidence or convergent demonstration of a focal lesion more than 2 cm in size and with arterial hypervascularization by two different imaging techniques, or the combination of one imaging technique showing this morphological aspect with a serum AFP level of 400 ng/mL or more.

The two main end-points were the occurrence of HCC, and the occurrence of death or liver transplantation. Follow-up ended at the date of death or liver transplantation, or at the last recorded visit (or information) within the last 6 months before August 31, 2005, which was set as the final time limit for upgrading the patients' file, using our computerized data-base, departmental certificates for patients who died outside our liver unit or by reaching patients, their relatives or their usual physicians.

In patients with HCV-related cirrhosis, anti-viral treatment and sustained virological response (SVR) were recorded.

DNA Extraction, Amplification and HFE Genotyping.

DNA samples were prepared from blood (n=228) or frozen liver samples (n=73, without any unsuccessful extraction reported). All patients gave written consent for blood sampling and genotyping. The use of left over liver specimens (no longer used for diagnostic purposes) for

research purposes had been approved by the Comité Consultatif d'Éthique Médicale du Centre Hospitalier Bichat-Beaujon.

For the determination of the Cys282Tyr and His63Asp mutations of the HFE gene, the DNA of each patient was amplified in real-time PCR accordingly with the manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). [15]

Histological Assessment of Hepatic Iron.

Hepatic iron was assessed on the initial liver biopsy showing cirrhosis. Liver specimens were fixed in formalin and routinely processed. Four-µm thick paraffin embeded liver biopsy sections were stained with the Perls' stain, and examined on a multipipe microscope by two observers unaware of genotypic and clinical data. To semi-quantitatively evaluate hepatic iron deposits, we used the Deugnier's histological hepatic iron score, adapted to cirrhotic samples as described previously (ranging from 0 to 33). [14]

Statistical Analysis.

Qualitative variables were compared using the Fischer exact Chi-2 test or Chi-2 trend test with 1 degree of freedom, while quantitative variables were compared using the non-parametric Wilcoxon test. Pearson correlation coefficient was calculated to measure the relationship between variables. Multivariate analysis (analysis of variance) was also conducted to compare more than two means. The Kaplan-Meier method was used to estimate the occurrence of HCC for each parameter noted at enrolment, death was considered as an outcome in the experiment. The distribution of death and HCC were compared with the Log-rank test. Different thresholds of liver iron scores for the risks of death and HCC occurrence were step by step tested. A significant level below 0.10 was used to select the variables in the Cox's proportional hazards model, using a stepwise backward procedure with a threshold of α=0.05. Variables (iron score,

age, sex) associated with risks of HCC based on knowledge and findings from previous studies were also selected. Statistical analysis used the SAS System Package version 8.02 (SAS Institute, Cary, NC). All reported *P* values are two-tailed. Associations were first considered statistically significant at a two-tailed α of 0.05. Bonferroni adjustment was also applied to correct for the number of primary outcomes tested (i.e., for 10 primary outcomes, α= 0.005). All reported *P* values are not corrected.

RESULTS

Baseline characteristics of patients and follow-up

Clinical and biochemical features of the 301 patients recorded at inclusion are summarized in Table 1 according to the aetiology of cirrhosis. Along with parameters reflecting iron metabolism, parameters estimating the severity of liver disease such as Child-Pugh score or known risk factors for HCC occurrence are displayed in this table.

Regarding HFE gene mutations, none of the studied patients were homozygotes for the C282Y mutation, 3 patients with alcoholic cirrhosis were homozygotes for the H63D mutation and 4 patients were compound heterozygotes (1 with alcoholic cirrhosis and 3 with HCV-related cirrhosis).

Mean duration of follow-up in patients with alcoholic cirrhosis was 66.5±44.1 months. Twelve patients were lost to follow-up during this time. Mean duration of follow-up in patients with HCV-related cirrhosis was 85.5±42.1 months. Two patients were lost to follow-up during this time.

Liver iron in patients with alcoholic cirrhosis

During follow-up, 40/162 (24.6%) patients developed HCC (Table 1). According to Kaplan Meier method (Figure 1), the presence of stainable liver iron was associated with the risk of HCC occurrence (first quartile time to occurrence: 54.0 months vs 180.0 months, HR=3.3 [1.6-6.7], LogRank=0.0006). An optimal threshold value ≥ 2 for liver iron score was found associated with HCC occurrence in this cohort (first quartile time to occurrence: 49.0 months vs 180.0 months, HR=4.2 [2.1-8.5], LogRank<0.0001) (Figure 2). In univariate analysis according to Cox's proportional hazards model, liver iron score was associated with the risk of HCC when considered as a continuous variable (1.23 [1.13-1.34], $p<0.0001$) or in

binary coding (4.1 [2.1-7.3], p<0.0001) along with age, male gender, and C282Y mutation carriage (Table 2). In multivariate analysis taking in account all these parameters, liver iron overload remained an independent risk factor for HCC along with age and C282Y mutation carriage, when considered as a continuous variable (1.14 [1.04-1.25], p=0.003) or in binary coding (2.8 [1.4-5.4], p=0.002).

During follow-up, 49/162 (30.2%) patients died (n=45) or underwent liver transplantation (n=4) (Table 1). Death was attributable to liver disease in all cases, due to advanced HCC in 19 cases or due to variceal bleeding and/or liver failure in the 30 other cases. An optimal threshold value ≥ 5 for liver iron score was found associated with death in this cohort (first quartile time of survival: 49.0 months vs 70.0 months, HR=2.3 [1.2-4.4], LogRank=0.01).

The amount of daily alcohol intake recorded at inclusion was not significatively correlated with liver iron score (r=0.15, p=0.07) and the risk of HCC occurrence (r=0.17, p=0.06).

Liver iron score when considered as a continuous variable was associated in this cohort with old age, male gender, hyperferritinemia, and C282Y mutation carriage (Table 3).

Liver iron in patients with HCV-related cirrhosis

During follow-up, 63/139 (45.3%) patients developed HCC (Table 1). According to Kaplan Meier method (Figure 1), the presence of stainable liver iron was not associated with the risk of HCC occurrence in this cohort (first quartile time to occurrence: 48.0 months vs 50.0 months, HR=0.9 [0.5-1.7], LogRank=0.9). Conversely to patients with alcoholic cirrhosis, no optimal liver iron cut-off value was found associated with the risk of HCC (Figure 2). In univariate analysis according to Cox's proportional hazards model, liver iron score was not associated with the risk of HCC; only old age, male gender, the absence of anti-

viral treatment or of SVR were associated with the risk of HCC occurrence in this cohort (Table 2). In multivariate analysis taking in account all these parameters, only anti-viral treatment and male gender remained independent risk factors for HCC.

During follow-up, 28/139 (20.1%) patients died (n=24) or underwent liver transplantation (n=4) (Table 1). Death was attributable to liver disease in all cases, due to advanced HCC in 25 cases or due to variceal bleeding and/or liver failure in the 3 other cases. The presence of stainable liver iron was not associated with the risk of death in this cohort (first quartile time of survival: 48.0 months vs 50.0 months, HR=0.5 [0.1-1.5] LogRank=0.2). Conversely to patients with alcoholic cirrhosis, no optimal liver iron cut-off value was found associated with the risk of death.

Eighty/139 (57.5%) patients underwent curative anti-viral treatment, which led to SVR in 32 of them. Twenty-eight/80 (35%) treated patients had stainable liver iron. In patients with SVR, 14/32 (43.7%) had stainable liver iron (median=0 [Q1=0 – Q3=2.5]) vs 14/48 (29.1%) (median=0 [Q1=0 – Q3=1]) in patients without SVR (p=0.1).

In the subset of the 59 untreated patients, 14 (23.7%) had stainable liver iron. This stainable liver iron was not associated with the risk of HCC occurrence (HR=1.1 [0.5-2.3] LogRank=0.7).

Liver iron score when considered as a continuous variable was associated in this cohort with male gender, hyperferritinemia, and Child-Pugh score (Table 3).

HFE gene mutations and risk of HCC

Patients with alcoholic cirrhosis

Thirteen/162 patients (8.0%) were carriers of the C282Y mutation (table 1). Among them 7/13 (53.8%) developed HCC vs 33/149 non carriers (22.5%) (Chi2 test=0.01) According to Kaplan-Meier method (Figure 3), C282Y mutation carriage was found associated with HCC occurrence (first quartile time to occurrence of HCC: 50.0 vs 79.0

months, HR=2.7 [1.2-6.3] LogRank=0.01). In univariate analysis according to Cox's proportional hazards model, C282Y mutation carriage was associated with the risk of HCC (2.7 [1.2-6.3], p=0.01) along with age, male gender, and liver iron score (Table 2). In multivariate analysis taking in account all these parameters, C282Y mutation carriage remained an independent risk factor for HCC along with age and liver iron score (4.6 [1.8-11.5], p=0.001).

During follow-up, 3/13 (23.0%) of the C282Y mutation carriers died vs 46/149 (30.8%) non carriers (Chi2 test=0.5). C282Y mutation carriage was not associated with the risk of death (first quartile time of survival: 64.0 vs 64.0 months, HR=0.7 [0.2-2.5] LogRank=0.1).

C282Y mutation carriers had higher liver iron scores (3.6±3.8 vs 1.9±2.8, p=0.05) (Table 3).

Fourty-four/162 patients (27.1%) were carriers of the H63D mutation. This carriage was not associated neither with liver iron overload (1.9±2.9 vs 1.8±3.1, p=0.8) nor with the risks of HCC (HR=1.3 [0.7-2.5] LogRank=0.3) or death (HR=1.6 [0.7-3.6] LogRank=0.1).

Patients with HCV-related cirrhosis

Seventeen/139 patients (12.2%) were carriers of the C282Y mutation (table 1). Among them 5/17 (29.4%) developed HCC vs 58/122 non carriers (47.5%) (Chi2 test=0.1) According to Kaplan-Meier method (Figure 3), C282Y mutation carriage was not associated with the risk of HCC occurrence (first quartile time to occurrence of HCC: 60.0 vs 48.0 months, HR=0.2 [0.5-1.2], LogRank=0.1).

During follow-up, 3/17 (17.6%) of the C282Y mutation carriers died vs 25/122 (20.4%) non carriers (Chi2 test=0.7). C282Y mutation carriage was not associated with the

risk of death (first quartile time of survival: 114.0 vs 112.0 months, HR=0.7 [0.2-2.24], LogRank=0.6).

Thirty-five/139 patients (25.1%) were carriers of the H63D mutation. This carriage was not associated neither with liver iron overload (0.8±1.6 vs 0.9±2.0, p=0.6) nor with the risks of HCC (HR=0.7 [0.4-1.4] LogRank=0.4) or death (HR=0.9 [0.5-1.6] LogRank=0.3).

Finally neither C282Y nor H63D mutations influenced SVR in this cohort (data not shown).

Comparison of the two cohorts

Patients with HCV-related cirrhosis were older, more often females, had a less severe liver dysfunction and lower liver iron scores than patients with alcoholic cirrhosis: mean iron score 0.9±1.9 (ranging from 0 to 11, median=0 [Q1=0 – Q3=1]) *versus* mean iron score 2.0±3.0 (ranging from 0 to 14, median=0 [Q1=0 – Q3=3]) respectively (p=0.0007). Similarly, the number of HCV-infected patients with stainable liver iron (liver iron score>0) was lower compared with alcoholics (28.0% *versus* 45.6%, p=0.006).

Patients with HCV-related cirrhosis had a higher incidence of HCC for a longer follow-up (first quartile time to occurrence of HCC: 50.0 vs 72.0 months, HR=1.51 [1.01-2.26], LogRank=0.04), but this difference did not remain significant in multivariate analysis including age, gender and Child-Pugh score.

Although both cohorts were independent, we compared the influence of liver iron overload on the risk of HCC by calculating the likelihood ratio taking in account the results of multivariate analysis. Using this method, the likelihood ratio for patients with HCV-related cirrhosis was 0.22 (p=0.6) compared with 5.21 (p=0.02) in patients with alcoholic cirrhosis.

DISCUSSION

By independently analyzing iron metabolism parameters in two distinct cohorts of cirrhotic patients according to the cause of their liver disease, our results suggest that liver iron overload and HFE gene mutations may not participate equally in alcohol- or HCV-induced hepatocarcinogenesis. In our study, patients with HCV-related cirrhosis had lower hepatic iron scores than patients with alcoholic cirrhosis even if older. As a possible consequence, this lower iron accumulation did not influence the risk of HCC occurrence or death in these patients and was not associated with the presence of HFE gene mutations. The mechanisms of iron accumulation in CHC are not clearly understood: several works reported a possible relationship with HFE gene mutations, [16, 17] while others as in our study did not. [18, 19] These conflicting results suggest that other genetic or environmental factors may participate in hepatic iron accumulation; it has been suggested that the mild liver iron overload observed in the course of CHC could be a consequence of hepatocyte necrosis leading to release of ferritin and subsequent uptake by macrophages. [20, 21] Indeed, iron overload seems to be associated with increased histological activity and enhanced fibrosis [20, 21] and a recent study focused on liver iron accumulation in patients with CHC concluded that the observed mild iron overload in these patients may contribute to liver fibrosis progression but not aggravation towards end-stage liver disease. [22] Finally, the lack of influence of HFE gene mutations on HCC occurrence in our work is consistent with all previous case-control studies conducted in HCV-related cirrhotic patients. [11-13] Taken together, these reports suggest that moderate liver iron accumulation in the course of CHC is not clearly related to the presence of HFE gene mutations and does not lead to a higher incidence of HCC in patients with HCV-related cirrhosis, either because it is limited or because hepatocarcinogenesis in the course of this disease is not mainly related to oxidative stress but to an oncogenic although probably indirect role of the virus. Along with the assessment of

clinical and biochemical risks factors for HCC occurrence in large cohorts of HCV-infected patients without cirrhosis, the influence of the virus itself on hepatocarcinogenesis needs further investigations.

Conversely to HCV infection, increased hepatic iron accumulation is a contributing factor to liver injury in the course of alcoholic liver disease; mechanisms by which ethanol leads to liver iron overload have been explored. [23] If liver iron overload is a well-recognized carcinogen in experimental studies, clinical studies failed up to now to clearly assess its role as a risk factor for HCC, except in patients with haemochromatosis [6] or patients with HCC developped on non-cirrhotic liver. [24] The present work describes a strong association between this histological parameter and the risk of subsequent HCC occurrence in patients with alcoholic cirrhosis, either when considering liver iron overload as a continuous variable or in a binary coding. One of the major challenge for admitting a physiopathological role of liver iron deposition in hepatocarcinogenesis is the powerful relationship between hepatic iron overload, ageing and male gender which are also well-established epidemiological risk factors for HCC in cirrhotic patients. Thus, a multivariate analysis including these factors is required to demonstrate an independant role of hepatic iron overload: such an analysis was made possible in our study by the large number of events due to a long follow-up of our cohort. Additionally, the predictive value of C282Y mutation provides a confirmation that liver iron is not the surrogate marker of other risk factors. Furthermore, the observed influence of liver iron on death in these patients (confirming data from Ganne-Carrie et al with the same optimal threshold value) [14] suggests a possible underestimation of this carcinogenic effect as patients with high liver iron overload died early from liver failure before developing HCC.

The influence of the C282Y mutation carriage on the risk of HCC as well as on hepatic iron overload is still debated. Some but not all case-control studies found a higher prevalence of this mutation in patients with HCC developped on alcoholic cirrhosis along

with higher liver iron accumulation, [10, 11] as in the present work. As the prevalence of this mutation is low in the general population, all these studies, including ours, suffer from a lack of statistical power. Despite this limitation, our results display the influence of this mutation on the risk of HCC in alcoholic cirrhotic patients; furthermore, this association remains statistically significant in multivariate analysis taking in account age and gender. Although the association between the C282Y mutation carriage with higher liver iron scores just reached statistical significance, we hypothesize that the observed influence of this mutation on hepatocarcinogenesis in alcoholic patients is related to hepatic iron overload. Still, HFE C282Y mutation is not the exclusive factor explaining why some alcoholic patients develop hepatic iron overload; environmental factors such as the type of alcohol consumption or other nutritional parameters could be involved. As well, seeking for genetic factors other than HFE gene mutations influencing this accumulation is of major interest. Recent reports studying the influence of mitochondrial antioxidant enzymes polymorphisms displayed a possible role of the imbalance in antioxidant systems caused by alcohol consumption and differences in antioxidant enzymes activity in liver iron accumulation and HCC developpment, [25, 26] possibly through enhanced mitochondrial hydrogen peroxide production. These findings highlight the fact that hepatic iron accumulation could be also a consequence and not only a cause of enhanced oxidative stress in the course of alcoholic liver disease, establishing the basis of a vicious circle.

Our suggestion that liver iron overload on initial biopsy and C282Y mutation carriage may influence the outcome of alcoholic cirrhotic patients is consistent with previous studies conducted in patients with end-stage liver disease. Indeed, it has been shown that iron overload in patients undergoing liver transplantation was associated with an increased prevalence of HCC. [27] Moreover, carriage of the C282Y mutation, presumably by favoring iron overload, was also found associated with a poorer prognosis in patients undergoing

resection for HCC. [28] Our findings demonstrate that these parameters if evaluated at the time of diagnosis of alcoholic cirrhosis predict a poor outcome and that liver iron overload could be therefore a therapeutic target before the onset of end-stage liver disease.

Taken together, these results stress the need to prospectively assess HCC risk factors in well-defined cohorts of cirrhotic patients. Our data suggest that in patients with alcoholic cirrhosis, liver iron overload (whatever its cause) is a major risk factor of HCC occurrence. Prevention strategies aiming to lower liver iron load in selected patients might help to clarify its pathophysiological role. Trying to prevent HCC occurrence by iron depletion or chelation seems reasonable and should be evaluated in the future in alcoholic cirrhotic patients with liver iron overload and/or carriers of the C282Y mutation.

	Patients with alcoholic cirrhosis (n=162)	Patients with HCV-related cirrhosis (n=139)	*p*
Age (years)[a]	53.3±9.6	59.2±13.0	<0.0001
Male gender[b]	115 (70.9)	73 (52.6)	<0.0001
Alcohol consumption (g/day)[a]	112.3±30.6	<20	-
BMI (kg/m^2)[a]	25.8±4.5	26.9±6.8	0.09
Diabetes[b]	25 (15.4)	31 (22.3)	0.1
Child Pugh score[a]	8.0±2.5	5.2±1.0	<0.0001
Prothrombin activity (%)[a]	59.8±18.8	85.3±14.8	<0.0001
Bilirubin (mol/L)[a]	69.3±90.1	17.8±8.4	<0.0001
Albumin (g/L)[a]	34.8±7.0	41.3±4.7	<0.0001
Platelet count ($10^3/mm^3$)[a]	144.8±72.7	140.0±59.4	0.8
AST (ULN)[a]	2.3±1.5	2.1±0.8	0.8
ALT (ULN)[a]	1.3±0.8	2.7±1.7	<0.0001
GGT (ULN)[a]	7.1±9.5	2.0±1.1	<0.0001
HCV genotype 1[b]	-	109 (78.4)	-
Ferritin (mg/L)[a]	452.3±552.6	290.2±288.9	0.01
Liver iron score[a]	2.0±3.0	0.9±1.9	0.0007
>0[b]	74 (45.6)	39 (28.0)	0.006
>2[b]	52 (32.0)	22 (15.8)	0.0006
>5[b]	22 (13.5)	6 (4.3)	0.006
C282Y mutation[b]	13 (8.0)	17 (12.2)	0.2

H63D mutation [b]	44 (27.1)	35 (25.1)	0.7
Follow-up (months) [a]	66.1±45.1	85.5±42.1	<0.0001
Anti-viral treatment [b]	-	80 (57.5)	-
Sustained virological response [b]	-	32 (23.0)	-
HCC [b]	40 (24.6)	63 (45.3)	0.0002
Death [b]	49 (30.2)	28 (20.1)	0.04
HCC-related	19	25	
Liver-related	30	3	
Transplantation	4	4	

NOTE: aMean ± SD. bNumber (percentage) of patients.

Table 1- Baseline characteristics and outcome of 162 patients with alcoholic cirrhosis and 139 patients with HCV-related cirrhosis.

	Alcoholic cirrhosis			HCV-related cirrhosis	
	Cox univariate analysis	**Cox multivariate analysis (including liver iron score as a continuous variable)**	**Cox multivariate analysis (including liver iron score with threshold ≥ 2)**	**Cox univariate analysis**	**Cox multivariate analysis (including liver iron score as a continuous variable)**
Age	1.084 [1.048-1.121] p<0.0001	1.082 [1.042-1.124] p<0.0001	1.086 [1.046-1.128] p<0.0001	1.026 [1.003-1.049] p=0.02	1.019 [0.987-1.079] p=0.2
Male gender	3.1 [1.1-8.8] p=0.03	2.6 [0.8-6.8] p=0.1	2.8 [0.9-6.7] p=0.08	1.7 [1.0-2.8] p=0.03	1.6 [1.0-2.7] p=0.04
Liver iron score (continuous variable)	1.2 [1.1-1.3] p<0.0001	1.1 [1.0-1.2] p=0.003	_	1.0 [0.9-1.2] p=0.3	0.9 [0.7-1.3] p=0.8
Liver iron score (Threshold ≥ 2)	4.1 [2.1-7.3] p<0.0001	_	2.8 [1.4-5.4] p=0.002	1.0 [0.5-1.8] p=0.8	_
C282Y mutation	2.7 [1.2-6.3] p=0.01	4.6 [1.8-11.5] p=0.001	4.7 [1.9-11.7] p=0.0007	0.5 [0.2-1.2] p=0.1	0.6 [0.3-1.4] p=0.2
Anti-viral treatment	_	_	_	0.2 [0.1-0.4] p<0.0001	0.3 [0.1-0.5] p<0.0001
Sustained virological Response	_	_	_	0.4 [0.2-0.9] p=0.03	0.6 [0.3-1.4] p=0.6

Table 2- Clinical and biochemical features associated with the risk of HCC occurrence in patients with alcoholic or HCV-related cirrhosis according to Cox's proportional hazards model.

	Alcoholic cirrhosis	HCV-related cirrhosis
Age (Correlation)	r=0.26 (p=0.0008)	r=0.09 (p=0.2)
Male gender (males vs females)	2.4±3.2 vs 1.4±1.9 (p=0.02)	1.4±2.2 vs 0.8±1.7 (p=0.008)
Child-Pugh score (Correlation)	r=0.9 (p=0.08)	r=0.18 (p=0.03)
Ferritin (Correlation)	r=0.22 (p=0.006)	r=0.30 (p=0.006)
C282Y mutation (carriers vs non carriers)	3.6±3.8 vs 1.9±2.8 (p=0.05)	1.1±1.9 vs 0.9±1.9 (p=0.2)

Table 3- Clinical and biochemical features associated with liver iron overload in patients with alcoholic or HCV-related cirrhosis

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FIGURE LEGENDS

Figure 1- Influence of stainable liver iron on the risk of HCC occurrence in patients with alcoholic or HCV-related cirrhosis according to Kaplan-Meier method.

Figure 1a- 162 patients with alcoholic cirrhosis (first quartile time to occurrence: 54.0 months vs 180.0 months, HR=3.3 [1.6-6.7], LogRank=0.0006).

Figure 1b- 139 patients with HCV-related cirrhosis (first quartile time to occurrence: 48.0 months vs 50.0 months, HR=0.9 [0.5-1.7], LogRank=0.9)

Figure 2- Influence of liver iron score≥2 on the risk of HCC occurrence in patients with alcoholic or HCV-related cirrhosis according to Kaplan-Meier method.

Figure 2a-162 patients with alcoholic cirrhosis (first quartile time to occurrence: 49.0 months vs 180.0 months, HR=4.2 [2.1-8.5], LogRank<0.0001).

Figure 2b- 139 patients with HCV-related cirrhosis (first quartile time to occurrence: 48.0 months vs 55.0 months, HR=1.1 [0.5-2.3], LogRank=0.7).

Figure 3- C282Y mutation carriage and risk of HCC occurrence according to Kaplan-Meier method.

Figure 3a- 162 patients with alcoholic cirrhosis (first quartile time to occurrence of HCC: 50.0 vs 79.0 months, HR=2.7 [1.2-6.3], LogRank=0.01).

Figure 3b- 139 patients with HCV-related cirrhosis (first quartile time to occurrence of HCC: 60.0 vs 48.0 months, HR=0.2 [0.5-1.2], LogRank=0.1).