

## Hepatocellular carcinoma in chronic HBV-HCV co-infection is correlated to fibrosis and disease duration

Rosa Zampino,\* Maria A. Pisaturo,<sup>†</sup> Grazia Cirillo,<sup>‡</sup> Aldo Marrone,\* Margherita Macera,<sup>§</sup> Luca Rinaldi,\* Maria Stanzone,<sup>§</sup> Emanuele Durante-Mangoni,<sup>||</sup> Ivan Gentile,<sup>¶</sup> Evangelista Sagnelli,<sup>†</sup> Giuseppe Signoriello,<sup>†</sup> Emanuele Miraglia del Giudice,<sup>‡</sup> Luigi E. Adinolfi,\* Nicola Coppola<sup>†</sup>

\* Internal Medicine and Hepatology, <sup>†</sup> Department of Mental Health and Public Medicine, Section of Infectious Diseases, <sup>‡</sup> Department of Pediatrics, <sup>§</sup> Department of Clinical and Experimental Medicine and Surgery F. Magrassi e A. Lanzara, <sup>||</sup> Internal Medicine Monaldi Hospital, <sup>¶</sup> Second University of Naples; Department of Clinical Medicine and Surgery-University of Naples Federico.

### ABSTRACT

Hepatocellular carcinoma (HCC) is a development of severe liver disease frequently due to HBV and/or HCV infection. The aim of this retrospective study was to evaluate the development of HCC in patients with HBV-HCV chronic infection compared with patients with single HBV or HCV infection and the viral and host factors correlated to HCC in co-infected patients. We studied 268 patients with histology proven chronic hepatitis: 56 had HBV-HCV co-infection (HBV-HCV group), 46 had HBV infection (HBV group) and 166 had HCV infection (HCV group). Patients were followed up for at least 3 years. Viral and host factors were studied. HCC was more frequent in HBV-HCV group (14%) compared with HBV (2%,  $p = 0.006$ ) and HCV mono-infected (4%,  $p = 0.006$ ). The Mantel-Haenszel test used to investigate the relationship between HBV-HCV co-infection and development of HCC indicated an association between development of HCC and HBV-HCV co-infection ( $p < 0.001$ ). In the HBV-HCV group, patients with HCC were significantly older ( $p = 0.000$ ), had longer disease duration ( $p = 0.001$ ), higher blood glucose levels ( $p = 0.001$ ), lower levels of steatosis ( $p = 0.02$ ), higher levels of fibrosis ( $p = 0.000$ ), higher HCV RNA ( $p = 0.01$ ) than those without HCC. ALT, lipid profile, PNPLA3 variant distribution and HBV viral load did not differ among co-infected patients with or without HCC. In conclusion HCC was more frequent in our patients with HBV-HCV co-infection, than in those with HBV or HCV mono-infection; possible associated risk factors for HCC development seem a long duration of disease, high levels of fibrosis and carbohydrate intolerance.

**Key words.** HCC. Risk factors. Chronic hepatitis.

### INTRODUCTION

Hepatocellular carcinoma is a common cancer with a medium incidence in Italy (10.0 to 20.0 cases per 100,000 individuals).<sup>1</sup>

Liver cirrhosis is present in about 80-90% of patients with HCC, and viral infection by hepatitis B and/or C is frequently the cause of liver disease progression and evolution to HCC.<sup>2</sup>

In HBV infection most cases of HCC are observed in cirrhotic patients,<sup>3,4</sup> especially when a high viral

load is present,<sup>5</sup> but HBV can cause HCC in the absence of cirrhosis. The HBV genome variants have shown different behavior in different geographical areas, but it seems almost certain that HBV genotype B is associated with HCC in young people without cirrhosis.<sup>6</sup>

In HCV-infected patients, the frequency of HCC ranges from 1 to 3% over 30 years, rising to an annual rate of 1-8% when cirrhosis is present.<sup>1</sup> HCV viremia is suggested as a risk factor for HCC.<sup>7</sup> Also liver steatosis, a characteristic feature of chronic hepatitis C,<sup>8</sup> has been identified as an independent risk factor for the development of HCC in HCV-infected patients.<sup>9</sup>

Recently, a polymorphism of the patatin-like phospholipase domain-containing 3 (PNPLA3) gene, involved in the lipid metabolism, has been associated with liver steatosis in NAFLD,<sup>10</sup> chronic hepatitis B,<sup>11</sup> chronic hepatitis C<sup>12,13</sup> and also with HCC.<sup>12,14</sup>

Correspondence and reprint request: Rosa Zampino, M.D. Ph.D.  
Internal Medicine and Hepatology  
Second University of Naples  
Via Pansini, 5 - Edificio 10, 80131 Naples, Italy  
Tel.: 0039 (081) 5666225. Fax 0039 (081) 5666230.  
E-mail: rosa.zampino@unina2.it

Manuscript received: May 8, 2014.  
Manuscript accepted: August 18, 2014.

HBV-HCV chronic co-infection correlated with a more severe liver disease<sup>15-18</sup> and a more rapid progression to liver cirrhosis and HCC.<sup>19-22</sup> However, a meta-analysis of more recent studies revealed a lower oncogenic effect in co-infected patients,<sup>23</sup> perhaps related to the mutual interference between HBV and HCV.

The aim of the present retrospective study was to evaluate the development of HCC in a group of patients from Southern Italy with chronic HBV-HCV co-infection compared with patients with a single HBV or HCV infection and to identify possible viral (viral load, viral genotypes) and host (anthropometry, biochemistry, histology, PNPLA3 polymorphisms, clinical history) parameters correlated to HCC in co-infected patients.

## MATERIAL AND METHODS

This is a retrospective study involving five Liver Units in Naples, Southern Italy, which have cooperated in several clinical investigations using the same clinical approach and the same laboratory methods.

Included in the study were patients with presence of HBsAg and/or anti-HCV in serum for at least one year at the time they underwent their first liver biopsy proving chronic hepatitis and had a documented clinical, serological and virological follow-up of at least 3 years after liver biopsy. Patients positive for anti-HIV and/or anti-HDV and with other causes of liver disease were excluded.

Because we are not able to define exactly the moment of infection for all patients, the presumed time of acquisition of the disease was considered as the first time ALT elevation was recorded in the medical history of all patients. Furthermore, because the interval between the first ALT elevation and liver biopsy varied, we considered the baseline as the time of the first liver biopsy.

During their disease history patients underwent liver biopsy, if clinically indicated. Liver specimens were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin and Masson's trichrome stain. Liver biopsies were examined by a pathologist who, unaware of the clinical and laboratory data, used Ishak's scoring system to grade necroinflammation and fibrosis<sup>24</sup> and a homemade scoring system for steatosis he had been using for decades (score 1 = 1-10% of hepatocytes with fatty deposition, score 2 = 11-30%; score 3 = 31-60%; score 4  $\geq$  60%).<sup>8</sup>

In accordance with the routine admission protocol established over the years by the liver units in-

involved in the present study, at the time of the liver biopsy all patients underwent physical examination, full liver function tests, blood cell counts, HDV, HIV serum markers and liver ultrasound scan. A questionnaire on alcohol and drug consumption was submitted to patients for completion. Alcohol abuse was defined as > 30 g/die no later than 6 months before entering the study. For all patients body mass index (BMI: kg/m<sup>2</sup>), waist circumference, blood fasting glucose, triglycerides and cholesterol were also determined.

Samples of serum, plasma and whole blood were obtained for each patient on the day of the liver biopsy and stored at -80 °C. The polymorphisms of the PNPLA3 gene were tested on these stored blood samples.

All patients enrolled were followed up for at least 3 years (range 3-51) from the actual or estimated time of infection, with evaluation of liver function tests, abdominal ultrasound and alpha-fetoprotein and serological (HBsAg, anti-HCV) and virological (HBV DNA and HCV RNA) tests.

All procedures used in the study were in accordance with the international guidelines, with the standards on human experimentation of the Ethics Committee of the Second University of Naples and with the Helsinki Declaration of 1975 and revised in 1983. The study was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria of the Second University of Naples. All patients signed an informed consent for the collection and storage of plasma samples and for the collection and use of their data in clinical research.

- **Serological determinations.** Serum markers for HBV, HCV, HDV and HIV infection were sought in serum using commercially available immunoenzymatic assays (Abbott Laboratories, North Chicago, IL and Ortho Diagnostic Systems, Raritan, NJ).
- **HBV and HCV genotype and viral load.** HBV genotypes were determined by phylogenetic analysis of sequences of 400 nt of the S region, as previously described.<sup>29</sup> HCV genotypes were determined using the VERSANT HCV genotype 2 LIPA (Siemens, Erlangen, Germany), following the manufacturer's instructions.

HBV DNA and HCV RNA were sought in plasma of all patients in the study, as previously described.<sup>29,30</sup> The detection limit of HBV DNA is estimated at around 40 copies/mL and that of HCV RNA at around 40 IU/mL.

### PNPLA3 polymorphisms

Genomic DNA was extracted from whole blood by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and analyzed for PNPLA3 polymorphisms.

All patients were genotyped for the PNPLA3 rs738409 C to G variant underlying the I148M substitution. The following primers were used, F: 5'-GCCCTGCTCACTTGGAGAAA-3' and R: 5'-TGAAAGGCAGTGAGGCATGG-3'. The FokI restriction enzyme, as previously described, was used to identify the variant, since the G allele eliminates a FokI restriction site. Random samples were confirmed by direct genotyping, which provided concordant results in all cases.<sup>31</sup>

- **Statistical analysis.** Comparison between groups was made applying the Mann-Whitney U test for continuous variables and the t-test or  $\chi^2$  test for categorical data. A P value of < 0.05 was considered significant. The Mantel-Haenszel test was used to estimate the common odds ratio and to test the association of HCC and the presence of HBV-HCV co-infection in relation to the disease duration. The statistical analyses were performed using SPSS statistical software v. 17.0 (SPSS Inc., Chicago, Illinois, USA).

## RESULTS

Two hundred and sixty-eight Caucasian patients with histology proven chronic hepatitis entered the study: 56 had HBV-HCV co-infection (HBsAg/HBVDNA/anti-HCV/HCV RNA-positive; HBV-HCV group), 46 had HBV infection (HBsAg/HBVDNA-positive; HBV group) and 166 had HCV infection (anti-HCV/HCV RNA-positive; HCV group).

Table 1 shows the general characteristics of the HBV-HCV group compared with the mono-infected groups. Patients were homogeneous for age, BMI, waist circumference, cholesterol and were prevalently males in the 3 groups.

Past alcohol abuse and injection drug use were indicated as they are factors that potentially contribute to liver injury and carcinogenesis; alcohol abuse was significantly higher in the HCV group ( $p = 0.029$ ) (Table 1).

ALT and AST were significantly lower in the co-infected than in the HBV group ( $p = 0.00$  and  $p = 0.02$ , respectively) (Table 1). ALT and fasting glucose were significantly lower in the co-infected than in the HCV mono-infected group ( $p = 0.00$  and  $p = 0.056$ , respectively). Triglycerides were signifi-

cantly higher in the co-infected than in the HBV mono-infected ( $p = 0.009$ ).

In the HBV-HCV group 8/56 (14%) patients were HBeAg-positive, while in the HBV group no patient was HBeAg-positive. In the HBV-HCV co-infected group, HBV DNA and HCV RNA were significantly lower than in the HBV and HCV mono-infected groups, respectively ( $p = 0.0001$ ,  $p = 0.000$ ). No difference in the distribution of HCV and HBV genotypes was observed between the HBV-HCV group and the HCV and HBV groups.

In addition, the HBV-HCV group presented lower steatosis ( $p = 0.053$ ) than the HCV group, but significantly higher fibrosis ( $p = 0.03$ ) (Table 1). Table 2 shows the specific distribution of HAI, fibrosis and steatosis for the three groups. A higher number of HBV-HCV co-infected patients presented severe fibrosis levels ( $p = 0.002$ ) and less severe steatosis score ( $p = 0.03$ ) compared to the HCV mono-infected.

PNPLA3 polymorphisms CC and GG were more frequently present in patients with HCV infection (Table 1).

During the clinical follow-up, 199 (74%) of the 268 patients received antiviral treatment, established by the physicians in care on the basis of the plasma expression of HBV and HCV replication, liver histology and current treatment guidelines.<sup>25-27</sup> The diagnosis of HCC was made according to the AASLD management guidelines.<sup>28</sup>

### HCC development

HCC was more frequent in the HBV-HCV group (14%) compared to the HBV (2%,  $p = 0.006$ ) and HCV mono-infected groups (4%,  $p = 0.006$ ). On the basis of the previously reported data on the emergence of HCC in each single infection,<sup>1,7</sup> we evaluated the emergence of HCC in relation to the duration of disease < or  $\geq 25$  years. Excluding the confounding effect of disease duration, the Mantel-Haenszel test was used (Table 3) to investigate the relationship between HBV-HCV chronic co-infection and the development of HCC. Of the 139 patients with a disease duration less than 25 years, the development of HCC was observed in 1 of the 27 in the HBV-HCV group, in 1 of the 27 in the HBV group and in 2 of the 85 in the HCV group; of the 129 patients with a disease duration of 25 years or more, the development of HCC was observed in 7 of the 29 in the HBV-HCV group, in none of the 19 in the HBV group and in 4 of the 81 in the HCV group (Table 3), indicating an association between HBV-HCV co-infection and the development of HCC ( $p < 0.008$ ).

**Table 1.** General characteristics of the HBV-HCV group versus the single HBV and HCV groups at time of first liver biopsy

	HBV-HCV	HBV	P HBV-HCV vs. HBV	HCV	P HBV-HCV vs. HCV
Patients, n°	56	46		166	
Median age (range)	49 (25-76)	47 (23-65)	0.7	53.5 (21-80)	0.4
Males, n° (%)	34 (60)	33 (70)	0.3	95 (57)	0.7
with disease duration >30 yrs, n° (%)	10(18)	7 (15)	0.9	59 (35)	0.02
Alcohol abusers (> 30g/die), n° (%)	3 (5)	1 (2)	0.09	31 (19)	0.029
Injection drug users, n° (%)	5 (9)	1 (2)	0.3	24 (14)	0.4
BMI (mean ± SD)	25.7 ± 3	26 ± 4.5	0.68	26 ± 3.8	0.59
Waist circumference (mean ± SD)	91.7 ± 9.8	95.4 ± 12.8	0.1	91.4 ± 11	0.8
Glucose (mean ± SD) mg/dL	90 ± 13.4	85.8 ± 14.4	0.1	96 ± 22	0.056
AST (mean ± SD) IU/L	55 ± 39	83 ± 84	0.02	62 ± 50	0.03
ALT (mean ± SD), IU/L	28 ± 62.5	124.95 ± 92	0.00	89 ± 73	0.00
Cholesterol (mean ± SD) mg/dL	182 ± 34	182 ± 31	1	182 ± 41	1
Triglycerides (mean ± SD) mg/dL	109 ± 55	85 ± 29	0.009	103 ± 53	0.4
Median HCV RNA (range) IU/mL	1.15 x 10 <sup>5</sup> (120- 6.4 x 10 <sup>5</sup> )	-		8 x 10 <sup>5</sup> (2,818-7 x 10 <sup>7</sup> )	0.000
HCV genotype 1, n° (%)	41 (74)			112 (68)	0.5
HCV genotype 2, n° (%)	7 (12)			37 (22)	0.4
HCV genotype 3, n° (%)	8 (14)			17 (10)	0.5
HBV genotype A, n° (%)	1 (2)	7 (4)	0.6		
HBV genotype D, n° (%)	55 (98)	159 (96)			
Median HBV DNA (range) IU/mL	1.9 x 10 <sup>3</sup> (1,500-10 x 10 <sup>7</sup> )	2 x 10 <sup>5</sup> (3,000-1 x 10 <sup>8</sup> )	0.0001		
HAI (mean ± SD)	5.5 ± 2.8	6.1 ± 3.4	0.3	6 ± 3.6	0.3
Fibrosis (mean ± SD)	2.8 ± 1.8	2.9 ± 1.3	0.7	2.3 ± 1.4	0.03
Steatosis (mean ± SD)	0.9 ± 0.85	1.2 ± 1	0.1	1.25 ± 1.25	0.053
PNPLA3 - CC, n° (%)	33 (59)	21 (46)	0.2	65 (39)	0.01
PNPLA3 - GC, n° (%)	21 (37.5)	20 (44)	0.6	81 (49)	0.2
PNPLA3 - GG, n° (%)	1 (1.8)	5 (10)	0.1	20 (12)	0.04
HCC n° (%)	8 (14)	1 (2)	0.006	6 (4)	0.006

HAI: Histological activity index.

**Table 2.** Histology distribution in the three groups of patients.

	HBV-HCV n (%)	HBV n (%)	P HBV-HCV vs. HBV	HCV N (%)	P HBV-HCV vs. HCV
HAI					
Minimal	13 (23)	9 (20)	0.8	42 (26)	0.8
Mild	33 (59)	25 (54)	0.7	82 (49)	0.4
Moderate	10 (18)	7 (15)	0.9	33 (20)	0.8
Severe	0	5 (11)	0.039	9 (5)	0.1
Fibrosis					
Mild	30 (54)	18 (39)	0.2	96 (58)	0.6
Moderate	13 (23)	24 (52)	0.05	58 (35)	0.1
Severe (cirrhosis)	13 (23)	4 (9)	0.09	12 (7)	0.002
Steatosis					
Absent	19 (34)	11 (24)	0.3	53 (32)	0.9
Minimal	23 (41)	16 (35)	0.6	59 (36)	0.5
Mild	12 (22)	11 (24)	0.9	27 (16)	0.5
Moderate	2 (3)	7 (15)	0.08	11 (6)	0.6
Severe	0	1 (2)	0.9	16 (10)	0.03

HAI: Histological Activity Index.

**Table 3.** HCC development in relation to disease duration in the three groups of patients.

	Disease duration <25 years		Disease duration ≥ 25 years	
	HCC	No HCC	HCC	No HCC
HBV-HCV group	1/27 (3.7%)	26/27 (96.3%)	7/29 (24.1%)	22/29 (75.9%)
HBV group	1/27 (3.7%)	26/27 (96.3%)	0/19	19/19 (100%)
HCV group	2/85 (2.3%)	83/85 (97.7%)	4/81 (4.9%)	77/81 (95.1%)

P: 0.008.

**Table 4.** General characteristics of the HBV-HCV co-infected patients with and without HCC.

	HBV-HCV without HCC	HBV-HCV with HCC	P
Patients, n°	48	8	
Median age (range)	43 (25-76)	68 (59-76)	0.000
Males, n° (%)	27 (56%)	7 (88%)	0.1
Median disease duration yrs, (range)	21 (3-38)	34 (21-51)	0.001
Alcohol abusers (> 30 g/die), n° (%)	3 (6.25%)	0	0.9
Injection drug users, n° (%)	3 (6.25%)	1 (12%)	0.9
BMI (mean ± SD)	25.6 ± 3	26.5 ± 9	0.58
Waist circumference (mean ± SD)	91. ± 10	96.7 ± 5.4	0.1
Glucose (mean ± SD) mg/dL	87 ± 11	103 ± 19	0.001
AST (mean ± SD) U/L	39 ± 22	60 ± 44	0.03
ALT (mean ± SD), U/L	30 ± 65	58 ± 36	0.2
Cholesterol (mean ± SD) mg/dL	184 ± 35	171 ± 31	0.3
Triglycerides (mean ± SD) mg/dL	111 ± 59	100 ± 12	0.6
Median HCV RNA (range) IU/mL	2,900 (120-3.9 × 10 <sup>5</sup> )	3,857 (270- 2.7 × 10 <sup>3</sup> )	0.01
Median HBV DNA (range) IU/mL	2400 (1,500-1.70 × 10 <sup>7</sup> )	2,028 (1,530-1.3 × 10 <sup>3</sup> )	0.1
HAI (mean ± SD)	5.5 ± 2.9	5.7 ± 2.7	0.8
Fibrosis (mean ± SD)	2.4 ± 1.6	4.6 ± 1.9	0.000
Steatosis (mean ± SD)	0.9 ± 0.8	0.2 ± 0.5	0.02
PNPLA3 - CC, n° (%)	29 (60.5)	5 (62.5)	0.7
PNPLA3 - GC, n° (%)	18 (37.5)	3 (37.5)	0.7
PNPLA3 - GG, n° (%)	1 (2)	0	0.3
Patients with antiviral treatment	30 (62.5%)	7 (88%)	0.3

HAI: Histological activity index.

### Analysis of baseline factors associated with the development of HCC in the HBV-HCV group

Table 4 shows the analysis of the baseline factors associated with the development of HCC in the HBV-HCV group. ALT, the lipid profile, PNPLA3 variant distribution and HBV viral load did not differ between patients with HCC and those without. Patients with HCC were significantly older ( $p = 0.000$ ) than those without HCC, presented a longer disease duration ( $p = 0.001$ ) and higher levels of HCV RNA ( $p = 0.01$ ). They also showed higher blood glucose levels ( $p = 0.001$ ); one (1%) patient in the HBV-HCV group, 2 (4%) in the HBV group and 13 (8%) in the HCV group had diabetes mellitus. The histological evaluation showed significantly lower levels of steatosis ( $p = 0.02$ ) and higher levels of fibrosis

( $p = 0.000$ ) in patients with HCC compared to those without. A significantly higher number of HBV-HCV co-infected patients with HCC presented severe fibrosis levels ( $p = 0.012$ ) and an absence of steatosis ( $p = 0.04$ ) compared to HBV-HCV co-infected patients without HCC (Table 5).

During their clinical history 62% of the patients without HCC and 88% of those with HCC had received treatment in accordance with the then current guidelines.

## DISCUSSION

In the present study HCC was more frequent in HBV-HCV co-infected patients than in the mono-infected and, apparently, the longer the duration of the disease, the more severe fibrosis and the greater the development of HCC.

**Table 5.** Histology distribution in HBV-HCV infected patients with or without HCC

	HBV-HCV without HCC n (%)	HBV-HCV with HCC n (%)	p
<b>HAI</b>			
Minimal	13 (27)	1 (12)	0.6
Mild	26 (54)	5 (63)	0.9
Moderate	9 (19)	2 (25)	0.9
Severe	0	0	
<b>Fibrosis</b>			
Mild	30 (62)	1 (12)	0.017
Moderate	11 (22)	2 (25)	1.0
Severe (cirrhosis)	8 (16)	5 (63)	0.012
<b>Steatosis</b>			
Absent	15 (32)	6 (75)	0.04
Minimal	20 (42)	2 (25)	0.4
Mild	11 (22)	0 0.3	
Moderate	2 (4)	0 1.0	
Severe	0	0	-

HAI: Histological activity index.

In agreement with previous studies,<sup>14,16-21</sup> HBV-HCV co-infection correlated with a more severe liver disease, which frequently entails a more rapid evolution to cirrhosis and HCC. Unfortunately, there are no literature data from a single study on a large population to allow a better assessment of the risk of HCC development in patients with HBV-HCV co-infection. The results available emerge from meta-analyses on international studies, most of them early studies that considered patients from different parts of the world with different prevalences of viral infections.<sup>19,21</sup> Furthermore, the role of carcinogenic factors, other than viruses, have to be taken into account in people of very different cultures and habits,<sup>22</sup> and older age and a longer duration of liver disease are important factors in the development of HCC also in patients with HBV and HCV mono-infection, particularly in developed countries.<sup>32</sup>

In our HBV-HCV co-infected patients with HCC, lower steatosis and higher fibrosis levels than in patients without HCC were observed. Previous studies have implicated steatosis as a risk factor for HCC, directly in HCV<sup>8,33-34</sup> and indirectly in HBV<sup>35-37</sup> infection. Molecular mechanisms to explain the role of liver steatosis in HBV or HCV carcinogenesis have been investigated; some hypotheses have been formulated to elucidate the role of HBV-HCV co-infection in the molecular pattern involved in lipid synthesis and metabolism, which can increase HCC development.<sup>38</sup> In contrast with these data, the histological picture of our patients showed low levels of

steatosis. We may hypothesize that during the natural progression of chronic hepatitis, an evolution is possible in which steatosis is present in a first stage of the disease, followed by fibrosis<sup>8</sup> and eventually by HCC development.

The existence of a possible reciprocal interference between HBV and HCV that might control the development of liver steatosis is still unclear, but a predominant role of one virus in liver injury cannot be excluded.

During chronic viral hepatitis, factors such as alcohol abuse, treatment, etc. and genetic factors may influence the outcome of disease. Alcohol consumption is certainly the most important factor worsening chronic hepatitis progression. It is well known that alcohol is a risk factor for liver cancer<sup>39</sup> and that there is a synergistic effect of alcohol with HCV and HBV infection in promoting HCC.<sup>40,41</sup> In our population alcohol abuse was more frequent in patients with HCV infection than in the co-infected and no correlation with HCC development was observed in the HBV-HCV group. We enrolled patients who had no alcohol abuse for at least 6 months, but we can not quantify the effects of alcohol abuse in generating liver damage and predisposing to liver malignancy. Furthermore, the PNPLA3 variant showed no correlation with HCC.

Previous studies have demonstrated that viral suppression reduces, but does not eliminate, the risk of HCC both in HBV and HCV chronic infection.<sup>42-45</sup> We cannot assess the beneficial effects of antiviral

treatment in protecting our patients from the development of HCC, first because of the small number of patients, and secondly because they were treated at different times with different schedules as per the then current guidelines.

In our patients, fasting blood glucose was significantly higher in HBV-HCV co-infected patients with HCC than in those without. Previous studies have found an association between diabetes and HCC [46-48], more frequent in patients with HCV infection.<sup>49,50</sup> To our knowledge, no data are available for the HBV-HCV co-infected, but our results seem to confirm those of the literature for HCV patients. It is possible that HCV acts on the glucose metabolism, probably inducing insulin resistance also in the presence of HBV. However, this was not associated with elevated liver steatosis levels, which were not observed in our co-infected group, but it was accompanied by higher triglycerides levels.

## CONCLUSION

In our patients, HCC was more frequent in the HBV-HCV co-infected than in those with HBV or HCV mono-infection; a long duration of disease, high levels of fibrosis and carbohydrate intolerance seem to be risk factors for the development of HCC. Further prospective investigations with a higher number of cases are needed to confirm these data.

## DISCLOSURE

All authors have no conflict of interest in connection with this study.

## AUTHOR CONTRIBUTIONS

Rosa Zampino and Nicola Coppola conceived and drafted the manuscript; Grazia Cirillo carried out the laboratory work, Maria Antonietta Pisaturo, Aldo Marrone, Margherita Macera, Luca Rinaldi, Maria Stanzione, Emanuele Durante-Mangoni and Ivan Gentile cooperated in the patients' enrolment and follow-up; Giuseppe Signoriello performed the statistical analysis; Evangelista Sagnelli, Emanuele Miraglia del Giudice and Luigi Elio Adinolfi critically reviewed the manuscript. All authors approved the final version of the manuscript.

## REFERENCES

1. El-Serag HB. Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. *Gastroenterology* 2012; 142: 1264-73.

2. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004;127(5, Suppl. 1): S35-S50.
3. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol* 2008; 48: 335-52.
4. Yang JD, Kim WR, Coelho R, Mettler TA, Benson JT, Sanderson SO, Thorneau TM, et al. Cirrhosis is present in most patients with hepatitis B and hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2011; 9: 64-70.
5. Chen CJ, Yang HI, Iloeje UH. Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. *Hepatology* 2009; 49(5 Suppl.): S72-S84.
6. Ni YH, Chang MH, Wang KJ, Hsu HY, Chen HL, Kao JH, Yeh SH, et al. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 2004; 127: 1733-8.
7. Lee MH, Yang HI, Lu SN, Jen CL, Yeh SH, Liu CJ, Chen PJ, et al. Hepatitis C virus seromarkers and subsequent risk of hepatocellular carcinoma: long-term predictors from a community-based cohort study. *J Clin Oncol* 2010; 28: 4587-93.
8. Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G. Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. *Hepatology* 2001; 33: 1358-64.
9. Kurosaki M, Hosokawa T, Matsunaga K, Hirayama I, Tanaka T, Sato M, Yasui Y, et al. Hepatic steatosis in chronic hepatitis C is a significant risk factor for developing hepatocellular carcinoma independent of age, sex, obesity, fibrosis stage and response to interferon therapy. *Hepatology Res* 2010; 40: 870-7.
10. Valenti L, Nobili V, Al-Serri A, Rametta R, Leathart JB, Zappa MA, Dongiovanni P, et al. The APOC3 T-455C and C-482T promoter region polymorphisms are not associated with the severity of liver damage independently of PNPLA3 I148M genotype in patients with nonalcoholic fatty liver. *J Hepatol* 2011; 55: 1409-14.
11. Viganò M, Valenti L, Lampertico P, Facchetti F, Motta BM, D'Ambrosio R, Romagnoli S, et al. PNPLA3 I148M affects liver steatosis in patients with chronic hepatitis B. *Hepatology* 2013; 58: 1245-52.
12. Valenti L, Rumi MG, Galmozzi E, Aghemo A, Del Menico B, De Nicola S, Dongiovanni P, et al. Patatin-Like Phospholipase Domain-Containing 3 I148M Polymorphism, Steatosis, and Liver Damage in Chronic Hepatitis C. *Hepatology* 2011; 53: 791-9.
13. Zampino R, Coppola N, Cirillo G, Boemio A, Pisaturo M, Marrone A, Macera M, et al. Abdominal fat interacts with PNPLA3 I148M, but not with the APOC3 variant in the pathogenesis of liver steatosis in chronic hepatitis C. *J Viral Hep* 2013; 20: 517-23.
14. Valenti L, Dongiovanni P, Ginanni Corradini S, Burza MA, Romeo S. PNPLA3 I148M variant and hepatocellular carcinoma: A common genetic variant for a rare disease. *Dig Liver Dis* 2013; 45: 619-24.
15. Sagnelli E, Pasquale G, Coppola N, Scarano F, Marrocco C, Scolastico C, Santantonio T, et al. Influence of chronic HBV-HCV concurrent infection on liver histology. *Infection* 2004; 32: 144-8.
16. Coppola N, Pisapia R, Tonziello G, Martini S, Imperato M, Piai G, Stanzione M, et al. Virological pattern in plasma, peripheral blood mononuclear cells and liver tissue and clinical outcome in chronic hepatitis B and C virus coinfection. *Antivir Ther* 2008; 13: 307-18.

17. Sagnelli E, Coppola N, Pisaturo M, Masiello A, Tonziello G, Sagnelli C, Messina V, et al. HBV superinfection in HCV chronic carriers: a disease that is frequently severe but associated with the eradication of HCV. *Hepatology* 2009; 49: 1090-7.
18. Jamma S, Hussain G, Lau DT. Current Concepts of HBV/HCV Coinfection: Coexistence, but Not Necessarily in Harmony. *Curr Hepat Rep* 2010; 9: 260-9.
19. Benvegna L, Fattovich G, Noventa F, Tremolada F, Chemello L, Cecchetto A, Alberti A. Concurrent hepatitis B and C virus infection and risk of hepatocellular carcinoma in cirrhosis. A prospective study. *Cancer* 1994; 74: 2442-8.
20. Donato F, Boffetta P, Puoti M. A meta-analysis of epidemiological studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma. *Int J Cancer* 1998; 75: 347-54.
21. Kew MC, Yu MC, Kedda MA, Coppin A, Sarkin A, Hodgkinson J. The relative roles of hepatitis B and C viruses in the etiology of hepatocellular carcinoma in southern African blacks. *Gastroenterology* 1997; 112: 184-7.
22. Shi J, Zhu L, Liu S, Xie WF. A meta-analysis of case-control studies on the combined effect of hepatitis B and C virus infections in causing hepatocellular carcinoma in China. *Br J Cancer* 2005; 92: 607-12.
23. Cho LY, Yang JJ, Ko KP, Park B, Shin A, Lim MK, Oh JK, et al. Coinfection of hepatitis B and C viruses and risk of hepatocellular carcinoma: systematic review and meta-analysis. *Int J Cancer* 2011; 128: 176-84.
24. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, Denk H, et al. Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; 22: 696-9.
25. Carosi G, Rizzetto M. Treatment of chronic hepatitis B: recommendations from an Italian workshop. *Dig Liver Dis* 2008; 40: 603-17.
26. Prati D, Gasbarrini A, Mazzotta F, for AISF, SIMAST and SIMIT. Practice guidelines for the treatment of hepatitis C: recommendations from an AISF/SIMIT/SIMAST Expert Opinion Meeting. *Dig Liver Dis* 2010; 42: 81-91.
27. Coppola N, Pisaturo M, Tonziello G, Sagnelli C, Sagnelli E, Angelillo IF. Efficacy of Pegylated interferon a-2a and a-2b in patients with genotype 1 chronic hepatitis C: a meta-analysis. *BMC Infect Dis* 2012; 12: 357.
28. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; 53: 1020-2.
29. Coppola N, Tonziello G, Pisaturo M, Messina V, Guastafierro S, Fiore M, Iodice V, et al. Reactivation of overt and occult hepatitis B infection in various immunosuppressive settings. *J Medical Virol* 2011; 83: 1909-16.
30. Coppola N, Pisaturo M, Guastafierro S, Tonziello G, Sica A, Iodice V, Sagnelli C, et al. Increased hepatitis C viral load and reactivation of liver disease in HCV RNA-positive patients with onco-haematological disease undergoing chemotherapy. *Dig Liver Dis* 2012; 44: 49-54.
31. Miraglia del Giudice E, Grandone A, Cirillo G, Santoro N, Amato A, Brienza C, Savarese P, et al. The association of PNPLA3 variants with liver enzymes in childhood obesity is driven by the interaction with abdominal fat. *PLoS One* 2011; 6: e27933.
32. Nordenstedt H, White DL, El-Serag HB. The changing pattern of epidemiology in hepatocellular carcinoma. *Dig Liver Dis* 2010; 42(Suppl. 3): S206-14.
33. Ohata K, Hamasaki K, Toriyama K, Matsumoto K, Saeki A, Yanagi K, Abiru S, et al. Hepatic steatosis is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C virus infection. *Cancer* 2003; 97: 3036-43.
34. Koike K. Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection. *J Gastroenterol* 2009; 44(Suppl. 19): 82-8.
35. Na TY, Shin YK, Roh KJ, Kang SA, Hong I, Oh SJ, Seong JK, et al. Liver X receptor mediates hepatitis B virus X protein-induced lipogenesis in hepatitis B virus-associated hepatocellular carcinoma. *Hepatology* 2009; 49: 1122-31.
36. Ha HL, Yu DY. HBx-induced reactive oxygen species activates hepatocellular carcinogenesis via dysregulation of PTEN/Akt pathway. *World J Gastroenterol* 2010; 16: 4932-7.
37. Kim KH, Shin HJ, Kim K, Choi HM, Rhee SH, Moon HB, Kim HH, et al. Hepatitis B Virus X Protein Induces Hepatic Steatosis Via Transcriptional Activation of SREBP1 and PPAR-gamma. *Gastroenterology* 2007; 132: 1955-67.
38. Wu Q, Liu Q. Do hepatitis B virus and hepatitis C virus coinfections increase hepatocellular carcinoma occurrence through synergistically modulating lipogenic gene expression? *Hepatology Research* 2012; 42: 733-40.
39. Thun MJ, Peto R, Lopez AD, Monaco JH, Henley SJ, Heath CW Jr, Doll R. Alcohol consumption and mortality among middle-aged and elderly U.S. adults. *N Engl J Med* 1997; 337: 1705-14.
40. Donato F, Tagger A, Gelatti U, Parrinello G, Boffetta P, Albertini A, Decarli A, et al. Alcohol and hepatocellular carcinoma: the effect of lifetime intake and hepatitis virus infections in men and women. *Am J Epidemiol* 2002; 155: 323-31.
41. Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, Arase Y, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998; 28: 930-8.
42. Papatheodoridis GV, Lampertico P, Manolakopoulos S, Lok A. Incidence of hepatocellular carcinoma in chronic hepatitis B patients receiving nucleos(t)ide therapy: a systematic review. *J Hepatol* 2010; 53: 348-56.
43. Thursz M, Brown A. Can antiviral therapy of chronic hepatitis B prevent the development of hepatocellular carcinoma? *Gut* 2011; 60: 1025-6.
44. Singal AK, Singh A, Jaganmohan S, Guturu P, Mummadi R, Kuo YF, Sood GK. Antiviral therapy reduces risk of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. *Clin Gastroenterol Hepatol* 2010; 8: 192-9.
45. Craxi A, Cammà C. Prevention of hepatocellular carcinoma. *Clin Liver Dis* 2005; 9: 329-46.
46. El-Serag HB, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; 4: 369-80.
47. Wang P, Kang D, Cao W, Wang Y, Liu Z. Diabetes mellitus and risk of hepatocellular carcinoma: a systematic review and meta-analysis. *Diabetes Metab Res Rev* 2012; 28: 109-22.
48. Schlesinger S, Aleksandrova K, Pischon T, Jenab M, Fedirko V, Trepo E, Overvad K, et al. Diabetes mellitus, insulin treatment, diabetes duration, and risk of biliary tract cancer and hepatocellular carcinoma in a European cohort. *Ann Oncol* 2013; 24: 2449-55.
49. Hassan MM, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, et al. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; 36: 1206-13.
50. El-Serag HB, Richardson PA, Everhart JE. The role of diabetes in hepatocellular carcinoma: a case-control study among United States veterans. *Am J Gastroenterol* 2001; 96: 2462-7.