UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Università degli Studi di Padova
DIPARTIMENTO DI MEDICINA – DIMED
DOTTORATO DI RICERCA IN SCIENZE CLINICHE E SPERIMENTALI
CURRICOLO: SCIENZE EPATOLOGICHE E TRAPIANTOLOGICHE
XXXII CICLO

"Hepatocarcinoma in chronic viral hepatitis: from epidemiological data, through new pathophysiological findings, up to new possible clinical tools."

Coordinatore: Ch.mo Prof. (Paolo Angeli)
Supervisore: Ch.mo Prof. (Paolo Angeli)

Dottorando: (Antonietta Romano)

INDEX

ABSTRACT

Background and aim: Hepatocellular carcinoma (HCC) is the first cause of liver tumor and the leading cause of death among patients with liver cirrhosis. HCC is a major public health concern, with an incidence and mortality of HCC in increasing in North America and in some European regions[1]. In this contest, is strategically relevant to determine HCC epidemiology, physiopathological alterations and its potential early biomarkers. The aim of the three studies presented in this thesis is to define HCC incidence in HCV patients treated with direct acting antiviral drugs (DAAs), and investigate the role of novel targets and circulating biomarker in HCC related to chronic viral hepatitis. The main aim of study 1 is to evaluate incidence of newly diagnosed HCC in patients with advanced hepatitis C treated with DAAs. Aim of study 2 is to evaluate the expression of mi-RNAs in patients with HCC, compared with patients without HCC. The main aims of study 3 is to better define the role of OPN in HCC and elucidate the immunoregulatory functions of OPN in Hepatocellular Carcinoma

Methods: *Study1*. The study is based on the NAVIGATORE platform, a prospectively recording database of all patients with hepatitis C receiving DAAs in Veneto region (Italy). Inclusion criteria: fibrosis stage ≥ F3. Exclusion criteria: Child-Pugh C, liver transplantation before DAAs, history or presence of HCC, follow-up <4 weeks after starting DAAs. *Study 2.* A total of 68 patients with cirrhosis HCV related who achieved SVR after therapy with DAAs have bee enrolled. 20 patients with HCC within the 24 weeks after the end of therapy with DAAs and 48 patients without HCC after DAAs. In the two groups selected micro-RNAs have been analyzed at the start and at the end of DAAs therapy. *Study 3.* This study includes: measurement of Osteopontin (OPN) in the plasma of patients with HCC, analysis of OPN on supernatant of Peripheral blood mononuclear cells (PBMCs) culture in HCC patients

and evaluation of the impact of recombinant OPN on modulating the phenotype of PBMCs in patients with HCC.

Results: *Study 1***.** During the first year, HCC incidence was 0.46% (95% CI: 0.12-1.17) in F3, 1.49% (1.03-2.08) in Child-Pugh-A and 3.61% (1.86-6.31) in Child-Pugh-B cirrhotics. In the second year HCC incidences were: 0%, 0.2%, and 0.69%, respectively. *Study 2.* When we have compared mi-RNAs expression between the two groups (patients with HCC or without HCC after DAAs) at baseline, miR28-5p is resulted significantly increased (p=0.029) in HCC group compared to not HCC group. *Study 3.* In patients with HCC hepatitis B (HCC-CHB) related and with HCC non-viral related, the levels of OPN are higher than patients with chronic hepatitis without HCC (p=0.0016 and p=0.0063 respectively). In the analysis of OPN on supernatant of PBMCs cultured with Glypican 3, following PD1 blockade, OPN production was reduced (p=0.023) in HCC-non viral but not HCC-CHB or Healthy controls. We also observed a reduction in OPN in both HCC-non viral (p=0.0078) and HCC-CHB (p=0.0010) in PBMCs cultured with p53.

Conclusions: *Study 1* These data, obtained in a large, prospective, population-based study, indicate that in patients with advanced hepatitis C receiving DAAs, the risk of "de novo" hepatocarcinoma during the first year is not higher, and might be lower, than that of untreated patients, and further declines thereafter. *Study 2.* In conclusion the expression of miR 28-5p is altered in patients treated with DAAs who develop HCC, suggesting its potential prognostic role in HCC related to HCV. *Study 3.* These results suggest its use as biomarker of HCC and reveal an immunomodulatory role for Osteopontin in HCC and warrant its further investigation as an immunotherapeutic target for HCC.

BACKGROUND

Hepatocellular carcinoma (HCC) is the sixth common neoplasm and the third cause of death related to cancer in the world. HCC is directly correlated with the spread of liver disease with the most common causes being hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD). The worldwide prevalence varies according to the etiology and the prevalence of liver diseases in the different areas of the world. In some parts of the world a reduction of incidence of HCC is contemplated, due to an improvement in HBV and HCV therapy. Nevertheless, the risk of HCC is not completely eliminated in HBV-HCV treated patients and with the increasing burden of NAFLD; the worldwide incidence of HCC is expected to be growing in the coming years. In addition, the screening program for HCC does not ensure adequate diagnostic accuracy. The gold standard for HCC screening, ultrasound alone or in combination with alpha-fetoprotein (AFP), has a suboptimal sensitivity and specificity in the diagnosis of the early stage of HCC and no other circulating markers are validated for the early diagnosis of HCC. Therefore, HCC therapy is still a challenge with most of the treatment options characterized by unsatisfactory patient survival[1].

Aim of the Studies: HCC incidence has a great impact on global public health. It is strategically relevant to define its new epidemiology, risk factors, pathophysiological alterations and new potential early biomarkers to improve the patient survival, with the aim to improve an early diagnosis and to develop novel therapeutic strategies. The aim of the three studies presented in this thesis is to define HCC incidence in HCV patients treated with

Directly acting antivirals (DAAs), and clarify the role of novel targets and prognostic predictors in HCC related to chronic hepatitis.

Hepatocellular carcinoma (HCC): worldwide epidemiology

Incidence

HCC is the first cause of liver tumor and still a major public health concern, the incidence and mortality of HCC have been increasing in North America and in some European regions[1].In limited areas, such as Japan and parts of China HCC is declining, with the reduction and control of HBV infection[2]. HCC has been recognised as a leading cause of death among patients with cirrhosis. The age-adjusted of worldwide incidence of HCC results of 10.1 cases per 100 000 person-years, with 746000 deaths in 2012, resulting the sixth most common neoplasm and the third cause of cancer related death[3]. The development of HCC is in greatest part related to the presence of chronic liver disease, so the worldwide incidence is heterogeneous due to the different prevalence of the risk factors. The 80% of HCC cases occur in sub-Saharan Africa and eastern Asia, where the main risk factors are chronic hepatitis B and aflatoxin B1 exposure. In the USA, Europe, and Japan the main risk factors for HCC are chronic HCV or HBV, heavy alcohol drinking, diabetes, and nonalcoholic fatty liver disease (NAFLD)[4,5]. The estimate for HCC of the age-standardised incidence rates (ASIR) per 100, in 2018 was highest in Eastern Asia (17.7) and Micronesia (15.2), followed by other regions in Northern Africa (14.1) and South Eastern Asia (13.3). The lowest rates were registered in South Central Asia (2.5), followed by Central and Eastern Europe and Western Asia (about 4.0) [6]. In an analysis of all fifty US states, HCC age-adjusted incidence increased from 4.4/100,000 in 2000 to 6.7/100,000 in 2012, with a growth of 4.5% annually

between 2000 and 2009 and a lower annual increase of 0.7% from 2010 through 2012 (Figure 1)[2]. The increase of HCC incidence is greater in males aged 55 to 64 years, a range that includes the peak era of HCV infection and in some ethnic/racial groups, including Hispanics, African Americans, and Caucasians. The global age-adjusted incidence of HCC in Hispanics is currently above that among Asians, with a higher incidence in Hispanics born in the USAcompared to Hispanics not born in the USA. In this group of patients, this should be related to higher rates of HCV, alcoholic liver disease, NAFLD and metabolic syndrome[7-9]. Besides, Hispanics with chronic HCV or NAFLD have a higher risk of HCC due to genetic (eg, PNPLA3) predisposition. While Asian males still have the highest HCC age-adjusted incidence rates attributed to chronic HBV[2]. The higher and increasing HCC incidence rates among individuals born in the peak-HCV cohort (1945– 1965), has not decreased yet, but it should potentially decrease in the next years, due to the availability of Directly acting antivirals (DAAs) that have completely changed the HCV therapy.

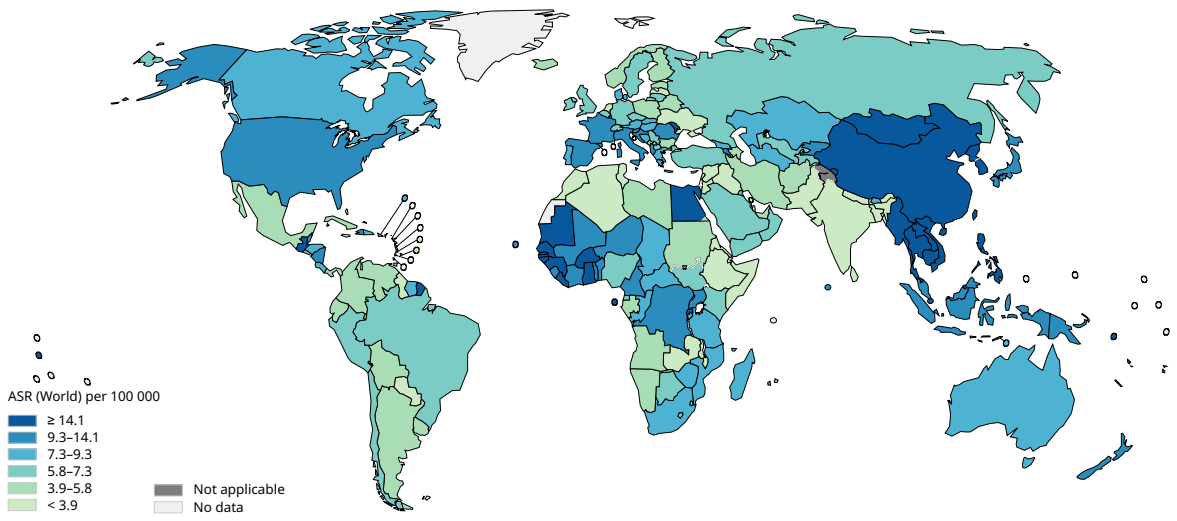


Fig. 1. Estimated age-standardised rates of incident cases, both sexes, liver cancer, worldwide in 2018[6].

HCC and risk factors

More than 80% of patients with HCC have cirrhosis, but also any chronic liver disease that causes cirrhosis could represent a risk factor for HCC. Therefore, the risk factors for HCC are closely related to the presence of cirrhosis and to any other cause of liver disease. For these reasons the most common risk factors for HCC are: cirrhosis (every ethiology), chronic infections with HBV or HCV, alcoholic liver disease, and nonalcoholic fatty liver disease (NAFLD)[10-13]. HBV is known to cause HCC without established cirrhosis and in some cases patients with NAFLD have been reported developing HCC in absence of cirrhosis. Italian data report only less than 2% of HCC in non cirrhotic patients and almost never in patients with a normal liver[14]. The risk of HCC in cirrhotic patients changes with the underlying etiology. The highest 5-year cumulative risks are reported in HCV cirrhosis (17% in the West), hemochromatosis (21%), HBV cirrhosis (10% in the West and 15% in Asia), alcoholic cirrhosis (8%–12%), and biliary cirrhosis (4%)[15].

Until today, HCV has been the most common cause of HCC in the USA, while HBV has been the most common etiological agent worldwide. Today with the epidemy of obesity-related NAFLD, NAFLD is becoming a leading cause of HCC, especially in the USA and other Western countries[10].

In the last two decades, the large expansion of HBV therapy with nucleoside/nucleotide analogues (NAs), has determineted a reduction of incidence of HCC, especially in the east area. NAs and the HBV viral suppression are directly correlated with the reduction of HCC incidence, altough not associated with a completely elimination of the risk of HCC[1].

Morover, in the recent period the new and high effective therapy for HCV with DAAs is rapidly changing the epidemiology of HCV. The high number of patients with sustained virological response (SVR) and the improvememt and reduction of liver disease related to HCV should be associated with a reduction of HCC incidence. An initial trend of reduction of

HCC incidence has been reported in patients with cirrhosis HCV-related and with a virological response after DAAs, but data on large population and on long term follow-up are still lacking[16, 17].

On the other hand, recent data on prevalence of NAFLD has shown a rapid increase not only in the west part of the world, but also in the east part, where the dietary and lifestyle changes are fueling the obesity epidemic. Therefore, an increase of HCC related to NAFLD is expected in the west and east of world. The risk of HCC in cirrhotic patients with NASH is lower compared to cirrhotics with HBV and HCV, in a large study of United States is reported a cumulative incidence of HCC in cirrhotic patients with NASH of 2.3% and of 4% in patients with HCV cirrhosis. Despite the relatively lower incidence of HCC in NAFLD compared to other liver diseases, such as HBV and HCV, the high and increasing prevalence of NAFLD is making NAFLD a major contributor to new-onset HCC[18]. In addition to etiologic agents associated with HCC, there are gender differences in the prevalence of HCC, as men have a higher risk to develop HCC compared to women. In the USA, this ratio is about 2.5–3 to 1, while in other parts of the world, this ratio can reach 6 to 1. These differences should be multifactorial, including environmental exposures as well as hormonal differences, considering that the estrogens should have potentially protective effects and androgens should have potentially trophic effects[19].

Pathophysiology of HCC development

Development of HCC is a complex multistep process that involves inflammatory damage, hepatocyte necrosis and regeneration, associated with fibrotic deposition. The process is induced by viral hepatitis or other etiologic factors and produces as first thing a chronic

inflammation that is the first passage of the multistep process of HCC development.[10] Risk of hepatocellular carcinoma increases in parallel with the progression of liver damage. The liver fibrosis is the main risk factor in the HCC process , with about 90 % of cases of HCC occurring in cirrhotic livers[20] .

Fibrosis develops after inflammation and repeated chronic damage. Damage of hepatocytes triggers the release of reactive oxygen species (ROS) and mediators of fibrosis that induce activation of hepatic stellate cells (HSCs). These together with Kupffer cells (KCs) and liver sinusoidal endothelial cells (HSCs) are central for the the establishment of liver fibrosis[21]. Paracrine signals from damaged hepatocytes and KCs play an important role in the activation of HSCs and in the collagen synthesis, leading to liver fibrosis. In addition to the several cell types involved in the development of fibrosis and cirrhosis, many cytokines have also been identified to be crucial,including platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α), interferon and interleukins (ILs). The release of these cytokines determines the activation of HSCs, the activation of the synthesis of extracellular matrix (ECM) and the induction of hepatocyte apoptosis [22]. In this setting the inception and progression of HCC is widely influenced by the liver microenviroment that includes not only cell activation and cytokine release, but also changes in the genetic and cellular signalling. Chronic inflammation increases production of ROS leading to an increased hepatocellular oxidative stress which induces DNA mutations and DNA damage, all factors that contribute to HCC development[23]. This chronic inflamed state determines also a greater proliferation of hepatocytes, the shortening of telomeres and consequently chromosomal instability and a predisposition to malignant transformation[24]. This can progress to fibrosis and then cirrhosis inducing further changes in the liver microenvironment. In cirrhotic liver, the areas of hypoxia lead to up-regulation of angiogenic factors such as vascular endothelial growth factor (VEGF), facilitating angiogenesis and

tumour growth. Cytokine expression within the liver, ECM production, TNF-α receptors and the mitogenic cytokine IL6 are significantly increased in advanced cirrhosis leading to a higher risk of cancer. The effects of IL6 are regulated by nuclear factor-κB signalling, both pathways are altered in liver inflammation and hepatocarcinogenesis [25].

In this multistep process, HCC is then characterized by the accumulation of somatic genomic alterations and epigenetic modifications, which explains its huge molecular heterogeneity.

Molecular alterations associated with this stepwise pathological sequence have been evaluated, and at present, key cancer-associated pathways, such as Notch, Toll-like receptor, MYC, TGF-β, WNT, and epithelial–mesenchymal-transition (EMT)-related signaling, have been demonstrated as active during hepatocarcinogenesis. Additionally, genome-wide studies have revealed genomic aberrations that occur during stepwise HCC development, including DNA methylation, DNA copy number changes, mutations [e.g., TP53, CTNNB1, or TERT promoter], and transcriptional deregulation[26]. The most recent multiomic studies have given a large view of genomic aberration associated with liver cancer. These studies are now also focused on precancerous lesions with the idea of creating a molecular, structural, and functional maps that provide information regarding tumor start and progression[27].

HCC and immune system

The immune system plays a critical role both during the early stages and progression of HCC. The tumour microenvironment decreases the efficiency of the immune system determining the immune escape of the tumour. In this process of HCC development, tumour infiltration with immune competent cells is strongly associated with the prognosis[28]. In liver cancer

tumour associated macrophages (TAMs), monocytes, lymphocytes (TILs) and Natural Killer cells (NK cells) play an important role in the cancer related inflammation, tumour escape mechanisms, progression and response to therapy[29].

TILs are the hallmark of the anti-cancer immunity in human cancer, in relation to their ability to specifically interact and neutralize tumour-related neoantigens. TILs' immune activity has an action in the adaptive immune response and release of cytokines but also in the neoangiogenesis and innate immune response, through the interaction with monocytes and macrophages[30]. CD8+ cytotoxic T cells play a primary role in anti-tumour mechanisms and CD4+ helper cells have a role in generating CD8+memory T cells involved in the attack of cancer cells[31]. T cell surface molecules are very important in controlling tumour development, these molecules inhibit or stimulate the immune system, and for this reason are defined as immune checkpoints. Programmed cell death protein 1(PD-1) is one of the best characterised immune co-inhibitory receptor expressed mainly on Tcells, an increased expression of PD-1 on CD8+ T cells and its ligand PD-L1 on cancer cells have been reported in patients with HCC, which results in the inhibition of antitumour T cell responses[32]. HCC uses several mechanisms to evade the control of T cells. One of these mechanisms is the changing in the expression of inhibitory immune checkpoint molecules, alteration of dendritic cell function, increase in number of regulatory T cells (Tregs), and release of immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor-β (TGF-β). Moreover, the continue exposure to HCC antigens leads to the overexpression of inhibitory immune checkpoint molecules on T cells, determining energy or cell exhaustion[33].

Hepatic NK cells play a critical role in immune surveillance in the normal liver, and its exhaustion and dysfunction are involved in the progression of HCC. NK cells are characterized by CD56+CD3− cytotoxic lymphocytes and they are capable to modulate the activity of other immune cells, including dendritic cells and macrophages, *via* cytokine

release. They are crucial into the innate immune system due to their rapid response, the production of cytokines and ability of releasing cytotoxic granules that directly kill target cells. The tumour induces escape from NK cell-mediated cytotoxicity through two main mechanisms: imbalance between killing activity receptors and killing inhibitory receptors on NK cells; and tumour selection/editing of poorly immunogenic antigens to evade NK cells detection or destruction. Several studies have demonstrated that the percentage and types of NK cells in the liver are transformed significantly in the development and progression of HCC. A significant reduction of the total proportion of peripheral NK cell compartments have been found in HCC patients with different disease stages compared to healthy subjects[34].

Surveillance and diagnosis of HCC

HCC surveillance aims to reduce disease-related mortality. Several non randomised studies have shown that patients included in a surveillance programme have an earlier diagnosis of HCC with a potential more effective cure and better survival[35]. In these studies, α-fetoprotein test or ultrasonography were evaluated as surveillance tests, and usually repeated every 6 months. Clinical recommendation to start a surveillance programme in each patient is essentially determined by the risk of HCC, but no experimental data are available to indicate surveillance based on HCC risk, so the decision is based on cost-effectiveness models. Thus surveillance is recommended for patients with cirrhosis, irrespective of the aetiology, and for patients with hepatitis B but no cirrhosis, in whom the annual incidence of HCC is more than 0-2%[36]. The most used test for surveillance is ultrasonography. This diagnostic examination is tolerated, not-invasive and widely available, with a sensitivity of 60–80% and a specificity of more than 90% when it is done by an expert staff[37]. Circulating tumour markers are an interesting alternative for surveillance and early diagnosis of HCC since they allow a non-

invasive, objective, and reproducible evaluation. The most used blood test is α-fetoprotein, but in retrospective case-control studies which evaluated its accuracy it has been reported a non adequate sensivity and specificity (60 and 80% respectively). Combining the use of α-fetoprotein and ultrasonography increases the disgnostic accuracy, but also increases the number of false-positive and costs[38,39]. Ultrasonography is the first examination for the surveillance and diagnosis of HCC and allows to identify suspected hepatic nodules. When nodules are identified, those with a diameter of less than 1 cm are infrequently diagnosed as HCC, and further investigation should determine overdiagnosis, in these cases the guidelines suggest only a follow-up at three months intervals. In the setting of liver cirrhosis with the presence of nodules larger than 1 cm, diagnosis can be reliably estabilished using an imaging technique if the nodule shows a specific imaging pattern[40].

This pattern is characterized by an intense contrast uptake during the arterial phase followed by contrast washout during the venous phase in contrast-enhanced CT or MRI. The value of these non-invasive criteria for HCC in cirrhosis has been prospectively validated. When nodule size is between 1 cm and 2 cm, the finding of typical imaging characteristics has a specificity of approx. 100%, and a sensitivity that can reach 71%[41-43]. Recently, the American College of Radiology has proposed a system for standardized performance, interpretation, and data collection for CT and MRI examinations of the liver in patients at risk of HCC. This system, is named Liver Imaging Reporting and Data System (LI-RADS), and stratifies the lesions in five main categories from lesions that are plain benign (LR 1) to those that are certainly hepatocellular carcinoma (LR 5). Prospective evaluation of LI-RADS accuracy in the diagnosis of HCC has shown a specifity of 25% for LR 2, 69% for LR 3 lesions and of 98.2% for LR 4 and 5[44]. These non-invasive diagnostic criteria are valid in patients with cirrhosis. In other clinical settings or when imaging fails to display a specific vascular profile, a diagnostic biopsy should be required. A negative biopsy does not exclude HCC,

considering that the false-negative rate of biopsies can reach 30%[45].

Staging of HCC

Prognostic assessment is a crucial step in the management of patients with HCC. Several prognostic systems have been evaluated to stratify patients according to their outcome. The most exstensive systems are the Cancer of the Liver Italian Program; Groupe d'Etude et de Traitement du Carcinome Hépato- cellulaire; Tumour, Node, Metastasis (TNM); Japanese Integrated Staging; the Taipei Integrated Scoring system and the Barcelona Clinic Liver Cancer (BCLC)[46].

The Barcelona Clinic Liver Cancer (BCLC) is the most commonly used staging system for HCC. Since its original version in 1999, it has been updated according to the results of more recent clinical evidences. Patients with very early-stage (BCLC 0) and early-stage (BCLC A) hepatocellular carcinoma have a solitary lesion or up to three nodules that are less than 3 cm in diameter (without macrovascular invasion or extrahepatic spread) and preserved liver function. These patients can benefit from resection, transplantation, or ablation. Patients with intermediate-stage hepatocellular carcinoma (BCLC B) do not have symptoms, but they have large, multifocal tumours without vascular invasion or spread beyond the liver. If liver function is preserved, these patients could be candidates for transarterial chemoembolisation. Patients with advanced-stage disease (BCLC C) have one or more of the following features: tumours that have spread beyond the liver, vascular invasion, and mild cancer-related symptoms. Finally, patients with end-stage disease (BCLC D) have poor liver function or marked cancer-related symptoms. These patients are not candidates for transplantation because of their poor prognosis and require only supportive care[47].

Diagnostic biomarkers of HCC

Early diagnosis of HCC is crucial to improve patient survival, for this purpose, tests every six months including ultrasound alone or in combination with AFP serum level are considered the gold standard. Despite this, both techniques have a suboptimal sensitivity and specificity in the diagnosis of the early stage of HCC. For this reason, alternative circulating or histological markers to predict or diagnose HCC are urgently needed[37-39].

Recent advances in the understanidng of the biology of HCChave led to the identification of several new promising circulating biomarkers, but evidence for their clinical utility remains low.

AFP is one of the first biomarkers of HCC, is an oncofetal glycoprotein produced by the fetal yolk sac and liver, usually detected by enzyme-like immunosorbent assays (ELISA). AFP has been widely used for the screening of HCC, with or without abdominal ultrasound, from the early 1970s until to the last few years. In the last few years the use of AFP has been limited by the scientific evidences that underline a low diagnostic accuracy. Indeed AFP is characterized by a low sensivity and specificity in the monitoring of HCC development, with only the 70% of HCCs that are characterized by significantly high level of AFP at the time of diagnosis. Rising interest has been dedicated to the different isoforms of AFP (AFP-I1, AFP-I2 and AFP-I3), and particularly to AFP-I3, that can be detected using liquid phase binding assays. Over 15 studies have evaluated the clinical potential use of AFP-I3, identyfing a wide range of sensitivity and specificity from 21% to 84% and from 89% to 94% respectively. AFP-I3 has been proposed in the diagnosis of HCC without elevated levels of AFP, but more data are needed for the validation of this preliminary results[48-50]. Other potential biomarkers

have been evaluated in several studies, like: Des-gamma carboxy prothrombin (DCP), Golgi protein-73 (GP73), Glypican-3 (GPC3), Squamous cell carcinoma antigen (SCCA), Dickkopf-1 protein (DKK1), osteopontin and micro-RNAs (mi-RNAs).

In addition to micro-RNAs also the long non-coding RNAs (lncRNAs) belonging to the family of non-coding RNAs have been evaluated as biomarkers of HCC. Genomic aberrations and transcriptional deregulation that occure early in the HCC development have been proposed as early markers of HCC[27]. Des-gamma carboxy prothrombin (DCP) is a non-carboxylated form of prothrombin, produced by the hepatocytes, released into the circulation and measured using electrochemoluminiscence assays. In several studies, DCP has shown a diagnostic performance only slightly higher compared to AFP[50]. Golgi protein-73 (GP73) is a membrane protein located on the Golgi, and can be detected using either ELISA assays, immunoblotting or Western blot. A meta-analysis has pointed out a diagnostic performace of GP73 higher then AFP[51]. Glypican-3 is a proteoglypican involved in the embryonal development and in the promoting HCC growth trough Wnt signalling pathway. The protein is preferably measured using ELISA assays. The diagnostic accuracy of GPC3 is moderate, with a pooled sensivity and specificity of 59.0% and 76.0% respectively[50].

Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor, physiologically located in the squamous epithelial cells, SCCA serum levels can be detected using ELISA. Its diagnostic accuracy for HCC has been investigated in several studies and resulted moderate with a pooled sensitivity of 59% and a specificity of 76%[52]. Dickkopf-1 protein (DKK1) is a glycoprotein secreted by human hepatocytes, its exact function is not known but has been suggested to be an inhibitor of the Wnt/β catenin signaling pathway. This protein appears to have a diagnostic accuracy comparable to AFP with a pooled sensivity and specificity of 65 % and 94% respectively[53].

Osteopontin

Osteopontin (OPN) is a highly modified integrin-binding extracellular matrix protein, produced by the immune system, the epithelial tissue, smooth muscle cells, osteoblasts, and tumour cells. OPN is produced in different tissues: brain, liver, gastrointestinal tract, lung, bone, cardiac tissue, joints, and kidney; and can be found in a variety of biologic fluids including blood, urine, milk, and seminal fluid. OPN function is also closely related to the immune system[54]. It has been demonstrated the involvement of OPN in neutrophil chemotaxis and OPN is known to be secreted by activated T-lymphocytes in the sites of inflammation[55]. Several works have highlighted the role of OPN in liver inflammation and liver fibrosis and in the development of HCC. The role of OPN in the development of HCC and as a prognostic factor for HCC is of great interest. OPN has been identified to be highly over expressed in HCC, and its overexpression appears to be correlated with tumour grade and tumour stage[56]. When the gene expression as absolute values have been measured, OPN gene resulted highly upregulated in tumour tissue [57]. OPN protein has been immunohistochemically detected in 40% to 55% of HCC and OPN mRNA results overexpressed in 55% of HCC[58]. The plasma OPN levels have been found significantly higher in patients with HCC than in patients with chronic liver diseases without HCC. As biomarker of HCC in patients with cirrhosis, plasma OPN level results to have a greater area under curve (AUC) value than AFP by receiver operating characteristic (ROC) analysis.

Furthermore, the combination of OPN and AFP levels enhance sensitivity and specificity in detecting HCC[59-60].

The mechanism by which OPN is involved in the HCC development is still poorly explained. The molecular mechanisms of tumour development and progression, influenced by OPN, have been investigated mostly in breast cancer, but have not been clarified in HCC[61].

miRNAs: promising biomarkers for HCC detection

mi-RNAs are a class of small, endogenous, non-encoding, circulating RNAs, detectable and stable in different body fluids, that regulate the gene expression through the bound to messenger-RNA (mRNA) or through post-transcriptional regulation. Mi-RNAs regulate about 1/3 of total gene expression aside from control a wide range of biological processes, including cellular development, apoptosis, proliferation, differentiation and tumorigenesis. As mentioned mi-RNAs are stable in fluids as well as in blood, with the help of their various forms existing in the blood, such as microvescicles and others[62-63]. In the early stage of HCC, abnormal mi-RNA expression should be detected in the blood, and these characteristics give to circulating mi-RNAs a great potential as new kind of HCC biomarker. In recent years, several studies have suggested that the alteration of circulating micro-RNAs can be detected and used as biomarkers for HCC diagnosis. However, many types of mi-RNAs HCC associated have been identified with a different diagnostic accuracy among the different studies[64-66]. In a recent meta-analysis including 59 studies, the diagnostic value of circulating miRNAs, single miRNAs, and miRNA panels has been tested trying to differentiate HCC patients from non-HCC controls, healthy controls, and patients with chronic liver disease (including HBV/HCV infected, chronic hepatitis, cirrhosis, etc). The results have shown that the sensitivity and specificity of using single miRNAs to distinguish between HCC and non-

HCC controls were 0.80 (95% CI: 0.78–0.82) and 0.73 (95% CI: 0.71–0.76) respectively. When multiple miRNAs were combined, the sensitivity and specificity rose to 0.83 (95% CI: 0.79–0.87) and 0.87 (95% CI: 0.83–0.91) respectively[67].

HCC Therapy

Despite the improvement of surgical and transplant techniques and availability of new systemic treatments, HCC therapy is still a challenge and most treatment options are characterized by unsatisfactory patient survival.

The management of HCC patients is complex due to the huge molecular heterogeneity of liver cancer, the impact of the stage of liver disease on patient prognosis, and different available treatments. Therefore, patients with HCC should be referred to multidisciplinary teams involving hepatologists, surgeons, radiologists, pathologists, and oncologists[45]. The choice of treatment has to take into account the strength of scientific evidence for any treatment approach, to select the most appropriate option for each patient and each tumour stage. Treatments with a proven survival benefit are: surgical resection, transplantation, ablation, transarterial chemoembolisation and the sistemic therapy with tyrosine-kinase inhibitors such as sorafenib, lenvatinib, and regorafenib. Arterial embolisation and radioembolisation have shown antitumour activity, but lack of survival benefit has been found. Systemic chemotherapy has only a limited use, because is associated with toxicity and poor survival benefit [45,68-72].

A new promising treatment of great interest is immunotherapy. that is actually under

investigation. For example, nivolumab is a programmed cell death protein-1 (PD-1) immune checkpoint inhibitor and it has shown antitumoural activity with a 15–20% rate of objective responses and with an appropriate safety profile. Survival data in single arm phase 2 studies are promising, but these data should be confirmed in the ongoing phase 3 trial[73,74].

Hepatic resection is the HCC treatment of choice in patients without cirrhosis, because this group is characterized by low surgical complications. In patients with compensated cirrhosis the stage of liver disease has to be carefully evaluated before resection in order to avoid severe treatment-related complications. In patients with decompensated cirrhosis, hepatic resection is contraindicated and liver transplantation should be considered. Indications for resection are usually restricted to patients with a single tumour, as multifocality is associated with a higher recurrence rate and reduced survival[45]. Even if multifocality might not be taken as a contraindication, a careful evaluation is needed to estimate the survival expectancy with other options, such as transplantation, ablation, or chemoembolisation. After hepatic resection, the tumour recurrence, including true recurrence and de novo tumours, complicates 70% of cases at 5 years and currently there is a lack of valid neoadjuvant or adjuvant therapeutic options to reduce the risk of recurrence[75].

Liver transplantation is the best treatment option considering the possibility of a simultaneous cure both of the tumour and the underlying cirrhosis. Indications to liver transplantation in HCC have to take into account several factors including the patient survival with other treatments, the patient survival after liver tranplatation and the donor availability in each area. The patient survival after transplantation remains the essential criterion to indicate this treatment for HCC. The Milan criteria (a single nodule ≤5 cm or up to three nodules ≤3 cm) are the benchmark to offer the best post liver transplantation survival in HCC (>70% 5-year survival with a recurrence rate of <10–15%) [76-77].

Tumour ablation is a widely used treatment option for patients with early-stage HCC. Ablation induces tumour necrosis thanks to temperature modification (radiofrequency, microwave, laser, or cryo- ablation) or injection of chemical agents (most frequently ethanol). Radiofrequency ablation is the first-line ablation technique, since it has been demonstrated to have a better disease control and better outcomes than percutaneous ethanol injection. Ablation has almost 100% efficacy in HCC nodules that are less than 2 cm in diameter (very early stage), and survival is almost identical after resection or ablation[78]. Transarterial chemoembolisation has shown a survival benefit. The tecnique is based on the injection of chemotherapy with blockade of the arterial blood supply. The median survival in old studies was around 20 months, but in recent studies with appropriate patient selection and optimal treatment, the median survival has been increased to approx.30–40 months[79-80].

Several agents have been tested or are under development for systemic therapy, sorafenib and regorafenib have demonstrated to improve survival versus placebo. Both drugs are oral multikinase inhibitors that block RAF signalling as well as vascular endothelial growth factor, platelet-derived growth factor, and KIT; the mechanism of action is still not well known, but these drugs are characterised by antiproliferative and antiangiogenic effects[81,82].

Study 1

"Newly diagnosed Hepatocellular Carcinoma in patients with advanced hepatitis C treated with DAAs: a prospective population study "

Introduction

Therapy of hepatitis C (HCV) has been revolutionized by the direct-acting antiviral drugs (DAAs)[83-84]. DAAs are safe and highly effective in eradicating HCV in patients with different stages of HCV infection, including decompensated cirrhosis[85-90]. Indeed, evidence has been provided that DAAs allows to achieve a sustained virological response (SVR) in more than 90% of patients with compensated cirrhosis (Child-Pugh A) and in above 80% of those with decompensated cirrhosis (Child-Pugh B or C). Eradication of HCV has created great expectations in terms of clinical outcomes in these patients. Indeed, SVR obtained by DAAs was found to be associated, on a short-term basis, with significant improvement of liver function, and of prognostic scores such as Child-Pugh and MELD scores[89-93]. In addition, a reduction in hospitalization and mortality has been observed in HCV cirrhotics achieving SVR with DAAs[94-95] In this exciting contest, controversial data have been reported on the rate of development of hepatocellular carcinoma (HCC) in patients treated with DAAs[96-100] In the Interferon era, the risk of developing HCC in patients with HCV-related cirrhosis, was reported to be equal to 2-3% yearly in those who were not treated, and reduced to 0.66% in those who achieved HCV eradication with IFN-based therapy[100-106]. It was therefore surprising and at the same time alarming the report of "an unexpected high rate" of HCC early recurrence during or after DAAs in patients with a previous history of cured HCC[96]. Since than, there have been several publications on this topic, reporting different rates of

recurrence as well as of *"de novo"* occurrence in HCV patients treated with DAAs. More recently, a meta-analysis of 26 studies of interferon-based or DAA-based therapies, suggested that DAAs are not associated with increased risk of HCC occurrence[107]. Accordingly, two recent large retrospective studies did not find any evidence that DAAs might favor development of HCC[108,109]. Nevertheless, the issue is still debated, continuing to represent a reason of concern for treating physicians in the absence of large prospective studies. Aim of this study was therefore to assess rate of newly diagnosed HCC and associated risk factors in a large prospective, population-based study of patients with HCV and advanced liver fibrosis treated with DAAs.

Patients and Methods

Study Design

This study is based on the analysis of the Web-based platform "NAVIGATORE", on which relevant data of all HCV patients treated with DAAs in the Veneto region (Italy), are recorded prospectively. All the 26 Clinical Centers authorized to prescribe DAAs by the Veneto Regional Governing Board are involved in the project. The analysis described in this study relates to patients initiating DAA treatment from January 1st 2015 to September 1st 2017.

The study is conducted according to the ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Local Ethics Committee.

The "NAVIGATORE" database

Individual data at baseline and during periodical follow-up are prospectively recorded on the NAVIGATORE platform, which is based on REDCap (Research Electronic Data Capture: a secure, distributed, web-based application designed to support data capture for research studies, providing an intuitive interface for validated data entry and audit trails for tracking data manipulation; http://project-redcap.org/.

Baseline data include: Age, gender, BMI, any associated disease and medication, any previous antiviral therapy for HCV, blood cell counts and hemoglobin levels, ALT, AST and liver function tests, alfa-fetoprotein levels, HCV-RNA levels and genotype, HBsAg and anti-HIV results, staging of liver disease by invasive (liver biopsy) or non-invasive (APRI-score and FIBROSCAN) methods. Follow-up data include: a) clinical assessment with liver function tests, ALT, AST and HCV-RNA measurements at monthly intervals during DAA treatment and at 3 and 6 months after end of therapy and b) long-term follow-up data with biannual clinical assessment and liver imaging surveillance by adequate ultrasound or computed tomography (TC) scan or magnetic resonance imaging (MRI). Patients developing any suspicious liver lesions by these techniques during or after therapy were further assessed with a complementary imaging method to confirm or exclude HCC according to EASL Guidelines on HCC[106]. In addition, a guided liver biopsy was performed when indicated according to the EASL recommendations. Thus, HCC diagnosis was established either by histological examination or by validated non-invasive imaging techniques[110].

Data entry in NAVIGATORE is optimized through periodic meetings, at quarterly frequency, to homogenize behaviors and to strengthen common goals. In addition, the data quality is assured through interactive controls generating triggered alerts and queries.

Patient Selection

Inclusion criterion was baseline fibrosis stage ≥ F3 according to METAVIR classification[111] as assessed by transient elastography or liver biopsy.

Exclusion criteria were: a) Child-Turcotte-Pugh (CTP) class C b) liver transplantation (LT) before the start of DAAs, c) history or presence of HCC before the start of DAAs, d) follow-up < 4 weeks after the start of DAAs.

Assessment of HCC at baseline

All patients underwent a complete clinical and laboratory evaluation including an abdomen ultrasonography during the 3 months preceding the initiation of DAA therapy. If a suspected focal lesion was identified in the liver, a full diagnostic work-up was completed with another complementary imaging technique and, when indicated by liver biopsy[110]. Patients diagnosed with HCC were not eligible to initiate DAAs therapy according to the National reimbursement rules.

Antiviral therapy

All patients received DAAs treatment based on available DAAs and according to National and Regional guidelines[112, 113]. From January 2015 to the end of February 2015 patients were treated with the combination of sofosbuvir (SOF) and ribavirin (RBV), and since the last week of February the combination of sofosbuvir plus simeprevir (SMV) also became available for patients with HCV genotype 1 (HCV-1) or HCV genotype 4 (HCV-4). In May 2015, sofosbuvir and ledipasvir (LDV), sofosbuvir and daclatasvir (DCV) and then

ombitasvir (OBV) plus paritaprevir (PTV) plus ritonavir (RTV) and dasabuvir (DSV) also became available. Patients were treated for 12 or 24 weeks, with or without addition of ribavirin, according to guidelines and medical decision. The assigned schedule and any modifications during treatment were recorded on the NAVIGATORE platform. Patients were seen monthly during therapy, and 12 and 24 weeks after termination of treatment and then at 6 months intervals.

Sustained Virological response (SVR) to therapy was defined as undetectable HCV-RNA by a quantitative real-time PCR with a limit of detection of 12 IU/ml and was assessed at week 12 after therapy (SVR_{12}) and confirmed at week 24.

Statistical methods

Sample size

Since our study is prospectively enrolling patients on a consecutive basis, we decided to estimate an adequate sample size to meet our primary objective, that was to compare the incidence of HCC in our DAA treated patients with the expected incidence in untreated patients. Based on the historical findings of an annual incidence of HCC of 2.0-3.0% in untreated patients with HCV related cirrhosis form our geographic region[22,23], a cohort size of about 4000 was found to produce a two-sided 90% confidence interval with a width equal to 0.009 when the proportion to estimate is 0.028.

Statistical analysis

We have evaluated development of HCC and estimated the incidence as cases per 100 patients-years, with its 95% confidence limits, for all patients and by stratifying for stage of liver disease and by baseline factors. Their Hazard Ratios, with 95% confidence limits, were estimated by univariate Cox's regression. Patients not developing HCC were censored at the last visit or at the date of death, including those who had prematurely discontinued DAA therapy.

We have also developed a proportional hazards multivariate Cox's model by entering the baseline factors that lead the criterion of $p < 0.10$ in univariate Cox's analysis, corrected for potential interaction.

The Kaplan-Meier curves of the incidence of HCC were plotted for the 3 fibrosis strata (F3, CTP-A and CTP-B cirrhosis) and the statistical differences were tested by the Mantel-Cox method. Another Kaplan-Meier plot was created to show the effect of the different combinations between factors entered into Cox's model. For reporting activity and statistical analysis we used IBM-SPSS Statistics v23 (Chicago, IL, USA) and STATISTICA v12 (StatSoft Inc. Tulsa, OK, USA).

Results

Patients and baseline features

From January 1st 2015 to September 1st 2017, 4234 patients with F3 or with cirrhosis were entered in the database. 317 of them were excluded from further analysis, including: 11 patients with CTP-C, 111 patients with a previous HCC, 154 patients who had received a liver transplant before starting DAAs, and 41 patients with <4 weeks of follow up after

starting DAAs. The baseline characteristics of the remaining 3917 patients are described in Table 1. Patients were predominantly males (62.2%) with a mean age of 58.1±11.9 years and a range from 21 to 90 years. The majority was infected with HCV-1 (61.8%), mainly with HCV genotype 1b (HCV-1b), and 41.6% were treatment-experienced. Nine hundred and fifty-nine patients had F3 fibrosis according to the METAVIR classification, while 2388 had CTP A cirrhosis (CTP-A), and 352 CTP B cirrhosis (CTP-B). As regard to comorbidities: 11.2 % had type 2 diabetes, 4.9 % were obese and 15.8% had cardiovascular disease. Coinfection with HIV and with HBV was present in 9.7 % and in 1.3 % of the cases, respectively. Patients were treated with different DAAs regimens including or not ribavirin (Table 1).

Follow-up and Virological response

At the time of this analysis, patients had a mean follow-up of 536.2±197.6 days (median 523 days, IQR 381-699 days) after the start of DAAs. SVR_{12} rate was of 97.2% in F3 patients, 92.7% in patients with CTP-A cirrhosis and 80% in CTP-B cirrhosis. SVR_{12} was observed in 92.9% of patients with genotype 1a, 95.1% of those with genotype 1b, in 92.4% of those with genotype 2, in 90,1% of those with genotype 3 and in 95% of those with genotype 4. Therapy with DAAs was discontinued prematurely in 53 patients including: 21 patients who died (11 for liver-related causes and 10 for non-liver-related causes), 4 patients who developed HCC and 21 patients who developed drug-related severe side effects.

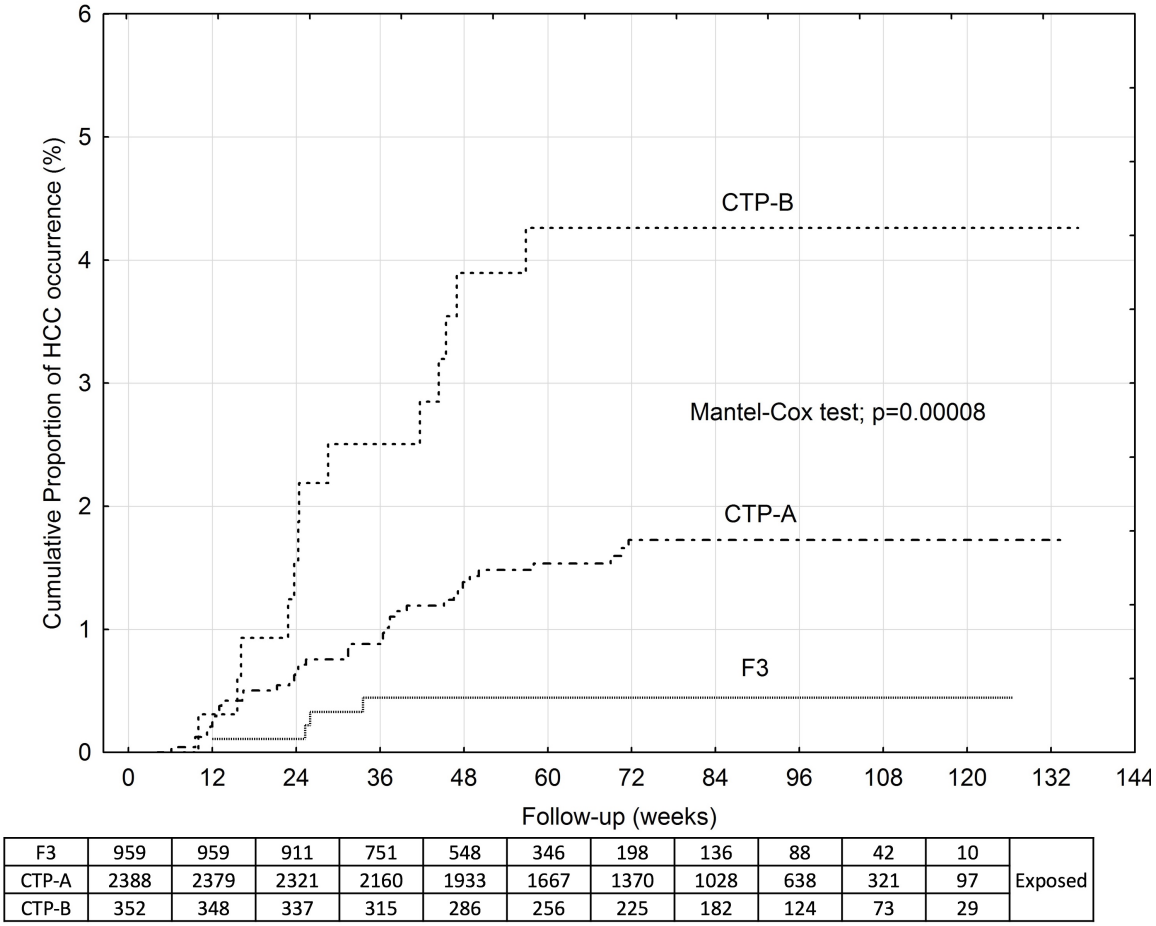
Table 1. **Baseline characteristics of 3917 patients included.**

	All Patients n=3917	No HCC Occurrence n=3862	HCC occurrence n=55 (%)*
Males/Females n(%)	2437/1480 (62.2/37.8)	2401/1461 (62.2/37.8)	36/19 (65.5/34.5)
Age years, mean±SD [range]	58.1±11.9 [21-90]	58±11.2 [21-90]	60.9±10.6 [40-82]
Age >65 years n(%)	1097 (28)	1076 (27.9)	21 (38.2)
Genotype n (%)			
Genotype 1**	79 (2.0)	79 (2.0)	0
Genotype 1a	740 (18.9)	734 (19.0)	6 (10.9)
Genotype 1b	1601 (40.9)	1575 (40.8)	26 (47.3)
Genotype 2	498 (12.7)	492 (12.7)	6 (10.9)
Genotype 3	657 (16.8)	645 (16.7)	12 (21.8)
Genotype 4	293 (7.5)	288 (7.5)	5 (9.1)
Others	12 (0.3)	12 (0.3)	0
Stage of liver disease n(%)			
Fibrosis F3	959 (24.5)	955 (26.2)	4 (7.3)
Cirrhosis CTP-A***	2388 (61)	2350 (64.5)	38 (69.1)
Cirrhosis CTP-B***	352 (9.0)	339 (9.3)	13 (23.6)
APRI score**, mean±SD[range]**	1.8±2 [0.1-43.1]	1.8±1.9 [0.1-25.6]	3.8±6.3 [0.5-43.1]
APRI score** >2.5 n(%)***	791 (23.45)	767 (19.9)	24 (43.6)
Fibroscan***, mean±SD[range]**	18.8±11.9 [1.1-75]	18.7±11.5 [1.1-75]	24.5±16.4 [10.1-75.0]
Fibroscan*** > 25 KPa(%)**	563 (21.0)	550 (20.9)	13 (23.6)
Comorbidities n(%)			
Diabetes	436 (11.2)	413 (10.7)	13 (23.6)
Obesity	194 (4.9)	193 (4.9)	1 (1.8)
CVD	636 (15.8)	622 (16.1)	14 (25.4)
Coinfections n (%)			
HIV	381 (9.7)	377 (9.7)	4 (7.3)
HBV	51 (1.3)	48 (1.2)	3 (5.8)
Previous therapy n (%)			
Naive	2452 (62.6)	2428 (62.9)	24 (43.6)
Experienced	1465 (41.6)	1434 (37.1)	31 (56.4)
DAA regimens n (%)			
SOF/RBV 12-24 wks	671 (17.1)	653 (16.9)	18 (32.7)
SOF/SMV±RBV 12 wks	334 (8.5)	329 (8.5)	5 (9.1)
SOF/DCV±RBV 12 wks	617 (15.8)	611 (15.8)	6 (10.9)
SOF/LDV±RBV 12-24 wks	1353 (34.5)	1336 (34.6)	17 (30.9)
OMV/PTV/RTV±DSV±RBV 12-24 wks	942 (24.0)	933 (24.1)	9 (16.4)
Virological response n (%)			
SVR12******	2637/2807 (93.9)	2606 (94.7)	31 (56.4)

Legend: CVD: cardiovascular diseases, HIV: patients anti-HIV positive; HBV: patients HBsAg positive. * % among all cases in column. ** 2 % of patients had a genotype 1 not defined as a or b, ***Child Pugh class was available in 2640/2958 ****APRI-score reported when available.***** Fibroscan was available in 2685 of 3917, ******SVR12 was available in 2807 of 3917

Incidence of Hepatocellular carcinoma

During follow-up, 55 patients developed HCC, including 4/959 (0.42%) with F3 at baseline and 51/2710 (1.88%) with cirrhosis at baseline. The overall HCC incidence was of 0.97x100patients/year (95% CI: 0.73-1.26). The HCC incidence in the whole cohort of cirrhotic patients was of 1.18% patients/year (95% CI: 0.92-1.49). When patients were stratified according to the stage of liver disease at baseline, HCC incidence rates during the first year of follow-up were 0.46x100patients/year (95% CI: 0.12-1.17) in patients with fibrosis F3, 1.49x100patients/year (95% CI: 1.03-2.08) in CTP-A cirrhosis and 3.61x100 patients/year (95% CI: 1.86-6.31) in CTP-B cirrhosis. The HCC incidence rate in the second year of follow-up declined to 0% in F3, to 0.20x100patients/year (95% CI: 0.05-0.51) in CTP-A cirrhosis and to 0.69x100 patients/year (95% CI: 0.08-2.49) in CTP-B cirrhosis and these differences were statistically significant (Mantel-Cox test, p=0.00008). The cumulative incidence of HCC in the 3 subgroups of patients (F3, cirrhosis CTP-A and cirrhosis CTP Kaplan-Meier curves is shown in figure 1.



F3	959	959	911	751	548	346	198	136	88	42	10	Exposed
CTP-A	2388	2379	2321	2160	1933	1667	1370	1028	638	321	97	
CTP-B	352	348	337	315	286	256	225	182	124	73	29	

Fig. 1: The Kaplan Meier curves of cumulative HCC occurrence in patients with cirrhosis according to the Child-Turcotte-Pugh class (CTP) and in those with severe fibrosis (F3) according the Metavir. *Patients not developing HCC were censored at last visit or at the date of death, including those who discontinued prematurely antiviral therapy.*

Risk factors for the development of HCC in cirrhotics treated with DAAs

Several variables at baseline as well as the virological outcome (SVR) were investigated as potential risk factors for the development of HCC at univariate Cox regression analysis in cirrhotic patients (Table 2). Stage of liver disease was associated with the risk of developing HCC (CTP-B vs CTP-A: HR: 2.55, 95% CI: 1.36-4.78, p= 0.004) as well as stage of liver fibrosis by APRI ≥ 2.5 (HR: 2.23, 95% CI: 1.28-3.91, p= 0.005) but not by transient Elastography.

Hepatitis B (HBV) coinfection was also significantly associated with HCC development (HR: 3.93; p=0.021) while HIV coinfection was not. Among comorbidities, presence of diabetes was almost significant in its association with HCC development (HR: 1.9, p=0.059). On the other hand, patient's age, gender, the HCV-genotype and the type of DAA regimen received was not related to the risk of developing HCC.

Finally, DAA treatment failure was strongly associated with development of HCC during/after treatment (HR: 9.09, 95% CI:5.2-16.1, p=0.0001).

Multivariate analysis of baseline variables confirmed an APRI score value > 2.5 (HR: 2.03, 95% CI: 1.14-3.61; p=0.016) and HBV coinfection (HR: 3.99, 95% CI : 1.24-12.91; p=0.021) as independent risk factors for HCC (Table 3). Figure 2 describes the cumulative incidence of HCC derived by Cox's modelling in relation to the presence of one or more risk factors, including APRI score value ≥ 2.5, CTP-B, HBV coinfection and Diabetes.

Table 2. **Risk factors for the development of HCC in cirrhotic patients by univariate Cox regression analysis.**

Cirrhotic patients (risk factors)		HCC (n)	HCC incidence (%person-years)	95% CI	HR	95% CI	P value
Gender	Female	19	1.27	0.83-1.87	0.90	0.51-1.59	0.717
	Male	32	1.12	0.82-1.51			
Age	< 65 years	31	0.98	0.71-1.32	1.66	0.95-2.92	0.076
	>= 65 years	20	1.70	1.13-2.47			
Diabetes	no	40	1.05	0.79-1.37	1.90	0.98-3.71	0.059
	yes	11	2.03	1.14-3.36			
Obesity	no	50	1.23	0.96-1.55	0.32	0.04-2.30	0.257
	yes	1	0.37	0.02-1.77			
CVD	no	38	1.05	0.78-1.37	1.7	0.091-3.19	0.099
	yes	13	1.85	1.10-2.95			
HIV	no	48	1.30	1.01-1.65	0.52	0.16-1.67	0.271
	yes	3	0.67	0.18-1.73			
HBsAg	no	48	1.17	0.91-1.49	3.93	1.22-12.61	0.021
	yes	3	4.57	1.25-11.82			
HCV genotype	1	31	1.18	0.86-1.60	1		0.883
	2	5	0.97	0.30-2.03	0.81	0.31-2.07	
	3	12	1.45	0.84-2.35	1.25	0.64-2.43	
	4	3	0.90	0.25-2.32	0.71	0.22-2.32	
Fibroscan stiffness>25KPa *	<= 25	38	1.12	0.84-1.47	1.28	0.68-2.41	0.444
	> 25	13	1.38	0.81-2.19			
APRI score*	< 2.5	25	0.93	0.65-1.30	2.23	1.28-3.91	0.005
	>= 2.5	24	2,03	1.40-2.85			
Child –Pugh score*	A	38	1.00	0.75-1.31	2.55	1.36-4.78	0.004
	B	13	2.40	1.42-3.81			
DAA regimens°	OMV/PTV/RTV± DSV	9	0.96	0.50-1.68	1		0.102
	DCV/SOF	6	0.93	0.41-1.84	0.91	0.23-2.55	
	LDV/SOF	15	1.18	0.73-1.82	1.15	0.50-2.63	
	SOF/RBV	17	1.93	1.23-2.89	2.42	1.08-5.43	
	SMV/SOF	4	0.66	0.22-1.50	0.91	0.28-2.96	
SVR-12*	no	20	7.73	5.12-11.23	9.09	5.2-16.1	0.0001
	yes	31	0.95	0.69-1.48			

Legend: CI: confidence interval, HR: hazard ratio by univariate Cox regression analysis. * Reported data of fibroscan, APRI score, Child-Pugh, SVR-12 only when available. ° Therapy: Combinations of DAA reported (OMV/PTV/RTV±DSV, DCV/SOF, LDV/SOF,SMV/SOF) could be with or without RBV.

***Table 3. Baseline* risk factors for the development of HCC at multivariate analysis**

Risk factors	Multivariate Cox's regression		
	HR	95% CL	p value
CTP-B vs CTP-A	1.73	0.88-3.39	0.109
APRI score >2.5	2.03	1.14-3.61	0.016
Haig+	3.99	1.24-12.91	0.021
Diabetes	1.89	0.96-3.71	0.065

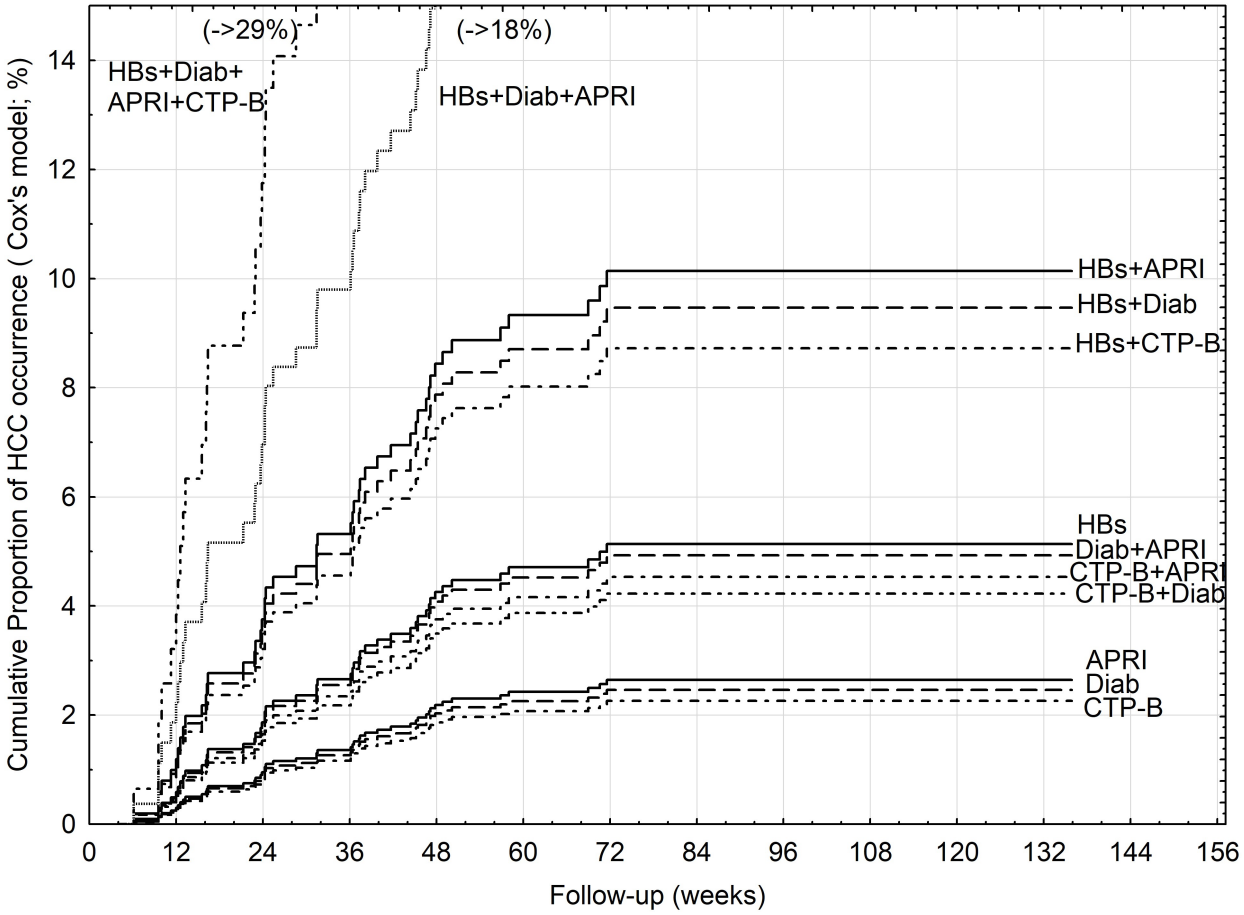


Fig.2: The Kaplan Meier of HCC occurrence, in relation to the presence of one or more risk factors. *APRI=APRI score≥2.5, HBs=HBsAg positive, Diab=Diabetes. ->18%=HCC incidence equal to 18%. ->29%= HCC incidence equal to 29%.*

Timing and pattern of HCC occurrence during/after DAA

The individual data of 55 patients who developed HCC includ 37 male and 18 female patients with a mean age of 59,15 ± 9.7 years (range 40 to 82 years) All major HCV genotypes were represented (6 HCV-1a 10.9%, 25 HCV-1b 45.4%, 6 HCV-2 10.9%, 13 HCV-3 23.6%, 5 HCV-4 9.0%). SVR12 was obtained in 33 patients while the remaining 22 either interrupted treatment prematurely or did not achieved SVR12. Time from initiation of DAAs and HCC diagnosis ranged from 4 to 75 weeks (mean 31.8 ± 20.1 weeks). Twenty-two patients developed HCC while treated with DAAs while in the remaining 33 the tumor was diagnosed after the end of treatment.

When the pattern of HCC developed in the 55 patients was considered, 30 patients (54.5%) presented with a single HCC lesion, and 9 (16.3%) with 2-3 small HCC nodules < 3 cm in diameter. A more aggressive type of tumor was seen in the remaining 16 patients (29.1%) with multiple nodules of variable size (8 cases: 14.5%) or an infiltrative diffuse HCC (8 cases: 14.5%) with portal thrombosis in 6 (10.9%) and extrahepatic metastasis in 4 (7.2%).

The frequency of these different HCC patterns in relation to virological outcome is reported in Fig. 3. The more aggressive pattern of HCC was seen somehow more frequently (54.6%) in patients without SVR compared to those with SVR (12.1%) in which the single nodule pattern prevailed (69.7%).

The distribution of different patterns of HCC in relation to timing after initiation of DAA is reported in Fig.4. The more aggressive pattern prevailed in the early period of observation and often occurred during therapy, while the single HCC nodular pattern was predominant at longer follow-up.

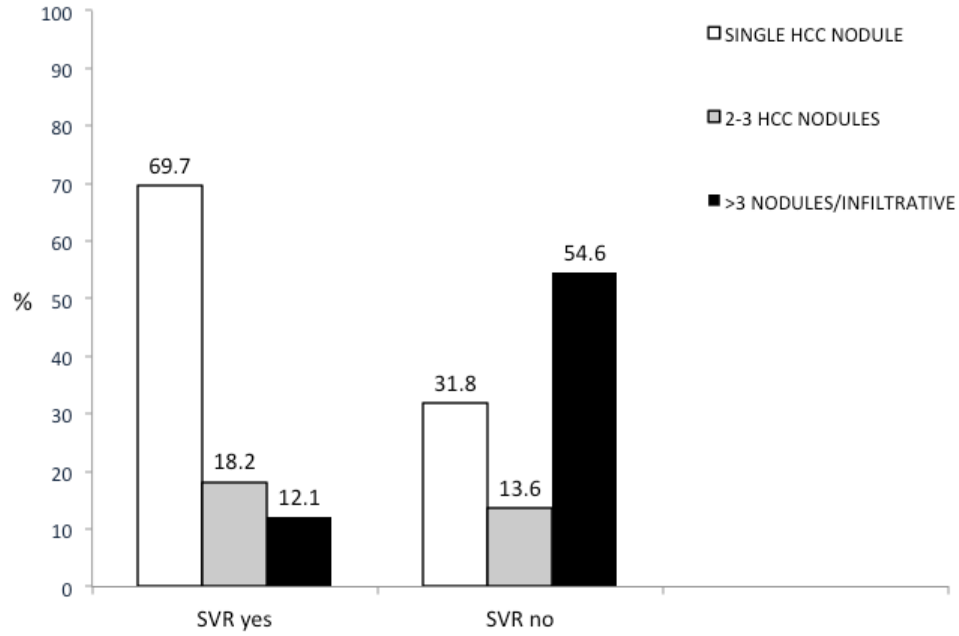


Fig. 3: Frequency of these different HCC patterns according to virological outcome.

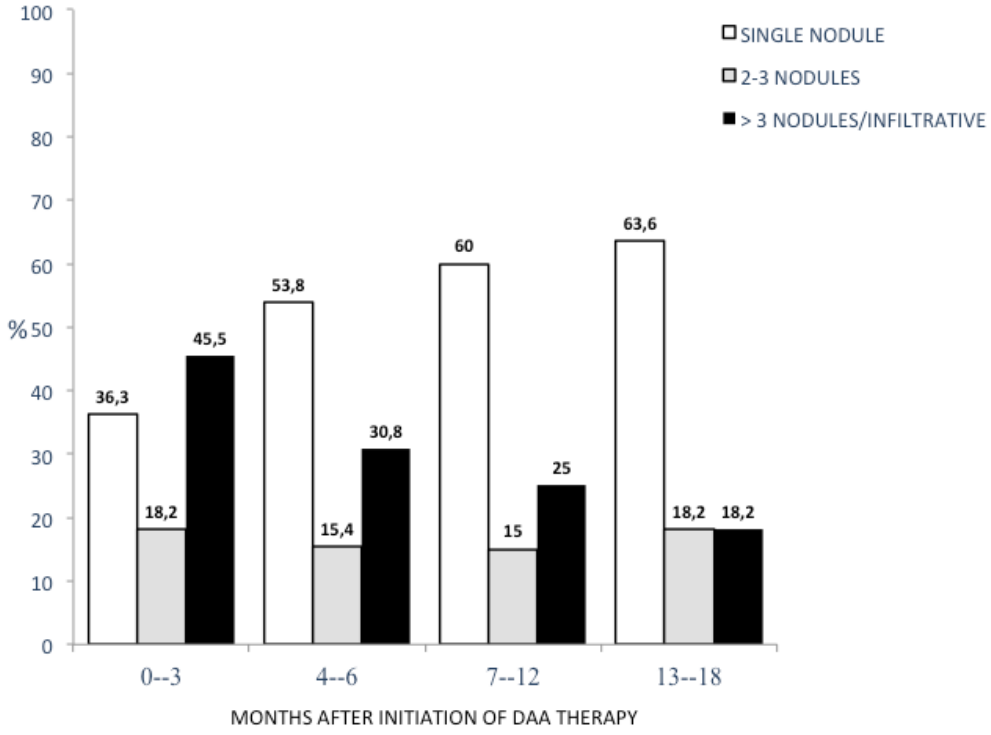


Fig. 4: Distribution over time of different patterns of HCC after initiation of DAAs.

Discussion and Conclusions

The risk of developing HCC during and after DAAs therapy in HCV patients with advanced liver disease has been recently at the center of a vibrant and controversial debate, after the observation by Reig et al of an "unexpected" high rate of HCC recurrence in HCV patients treated with DAAs after their original HCC had been successfully cured[96]. Afterwards, several studies have not put an end to the debate since they have produced controversial results for both the incidence of HCC after DAAs therapy (reporting different data on HCC occurrence from 6.6% to 1.79%) and the relationship between the occurrence of HCC and response to DAAs[101,106,108]. The main limitation of most of these studies lies in both their retrospective nature and the heterogeneity of the patients included. Our study has been conducted prospectively on a population basis in a large consecutive series of HCV patients with advanced liver disease treated with various DAA regimens. It provides evidence that in these patients the risk of developing HCC during the first year after initiation of DAAs is similar, or even reduced, compared to that expected without antiviral therapy, main risk factors being the same previously described in the natural history of HCV-related cirrhosis, i.e. the stage of disease, HBV coinfection and type 2 diabetes[114]. Indeed, in CTP-A cirrhotic patients treated with DAAs, the incidence of HCC (1.00 x100 patients/year) was lower that observed in two historical cohorts of untreated patients from the same geographic region (2.8% and 3.9% patients/year)[104, 105]. Likewise, in CTP-B cirrhotics, the incidence of HCC after DAAs was lower than that observed in an historical untreated cohort followed-up in our institution between 2006 and 2013 (2.40 versus 6.4 patients/year, respectively) (unpublished data, shown in Table S2). Our results clearly indicate that the risk of developing HCC is higher during the first year after initiation of antiviral therapy and declines significantly thereafter. A striking difference in our study was in the risk of HCC in relation to the response to antiviral therapy. Although development of an HCC was also observed in several

patients with SVR, the tumor incidence was significantly higher in patients without HCV eradication. Considering that most of our patients developed HCC early in the course of antiviral therapy, a relation of cause and effect between HCC and virological failure rather than the opposite appears more likely. Other studies have reported higher rates of HCC development in patients without SVR compared to those achieving SVR[94, 108, 109]. In a recent study the presence of an active HCC before the start of DAAs has been reported to be a negative predictor of SVR[115]. The biological explanation for the lower rate of SVR in patients developing HCC is not entirely clear. Nevertheless, it might be that HCC cells serve as a viral reservoir for HCV less permissive to DAAs, with the consequent relapse of HCV infection after antiviral therapy. On these bases, our patients who did not reach SVR and developed HCC could already have microscopic, undetectable foci of HCC before initiation of DAAs. In a subgroup of these patients, growth and spread of HCC could even be boosted by the profound immunological and molecular changes that are known to occur in the liver microenvironment following the abrupt reduction of HCV replication by DAAs[116-121], thus explaining the development of an aggressive tumor in some of them[40], although we cannot exclude that what we observed in these patients merely reflects the natural history of their liver disease. Presence of HCC at baseline was in our study entrusted to an imaging test that could be dated up to 3 months before initiation of antiviral therapy. So we may hypothesize that an adequate imaging assessment just before the start of DAAs could be important to exclude an active HCC, particularly in patients at higher risk, and DAAs should not be started before the nature of any hepatic focal lesions has been properly defined. In addition, patients should continue to be closely monitored with adequate liver imaging during and after therapy independently of the virological outcome. It could be hypothesized an imaging assessment every three months during and after therapy in patients with decompensated liver disease and

in those with significant cofactors such as HBV coinfection or diabetes, and every six months for all other patients with advanced liver disease.

In conclusion, our study provides solid evidence that treatment with DAAs of HCV patients with advanced liver disease doesn't increase the risk of developing HCC. In these patients the residual HCC risk appears dependent on variables well known to affect HCC development in untreated patients, such as stage of liver disease and comorbidities such as HBV and diabetes, being higher in patients without virological response and decreasing progressively over time in patients with SVR. Further follow-up of our cohort, currently ongoing, will clarify whether the HCC risk could become insignificant at any time in any patient's subgroup.

Study 2

"Alteration of miRNA$_S$ expression in patients with HCV-related cirrhosis who developed HCC after therapy with DAAs."

Introduction

Hepatitis C virus (HCV) is still one of the leading causes of end-stage liver disease, hepatocellular carcinoma (HCC) and death related to liver disease[122]. Recently new direct acting antiviral drugs (DAAs) infection have revolutionized the treatment of HCV. These have proven to be effective and well tolerated also in patients with decompensated cirrhosis[123, 124]. In cirrhotic patients with HCV the risk of developing HCC is estimated above 5% per year[125, 126]. In the interferon era the risk of HCC appeared to be reduced but not completely eliminated after anti-HCV therapy. Data on long-term risk of HCC after therapy with DAAs are not available, although the short-term risk of HCC does not appear to be increased[127-130]. Consequently, the residual risk of HCC in HCV cirrhotics treated with DAAs is already a relevant clinical problem. Therefore, despite improvement of treatments for HCV and hepatis B virus (HBV), the global burden of HCC related to all causes of chronic liver disease is increasing with an expected growth in the next years[131]

In this scenario the early diagnosis of HCC is crucial to improve its treatment and prognosis. Actually, clinical diagnosis of HCC is difficult due to the lack of characteristic clinical signs and symptoms. Alpha-fetoprotein (AFP) and ultrasound, considered the gold standard for the surveillance of HCC in cirrhotic patients, have a suboptimal sensibility and specificity in the early stage of HCC. Indeed, they have a sensibility ranging from 59 to 89% and specificity above 90 % when used as surveillance test, but in the early HCC the sensibility is reduced to 63%[132,133]. Micro-RNAs (mi-RNAs) have been investigated as potential biomarkers in

patients with viral hepatitis, liver fibrosis and HCC[133-136]. Mi-RNAs are a class of non-encoding RNAs, circulating, detectable and stable in different body fluids, which negatively regulate gene expression by binding to complementary sequences of specific m-RNAs targets or through post-transcriptional regulation[137-141]. Some studies have shown a correlation between serum and tissue levels of specific micro-RNAs and the development of HCC in patients with chronic hepatitis C, so this could suggest their use as markers for the early and non-invasive diagnosis of HCC[134-137].

Aim of the study

The main aim of the study is to evaluate the expression of mi-RNAs in patients who develop HCC, compared to patients who don't develop HCC, in a group of HCV cirrhotic patients treated with DAAs.

Patients and Methods

In this study we have enrolled a total of 68 patients with cirrhosis HCV related who achieved SVR after therapy with DAAs. They were outpatients followed in our hospital unit from January 2015 to March 2016. Among these patients, 20 patients have developed HCC (HCC group) within the 24 weeks after the end of therapy and 48 patients have not developed HCC (not HCC group) (figure 1). In the two groups (HCC and not HCC) there were patients treated with DAAs for 12 or 24 weeks, we have analyzed these patients separately. Samples for the analysis of mi-RNAs, have been collected at the start of therapy (baseline) and at the end of therapy with DAAs. Clinical data, liver imaging data and laboratory results have been evaluated at baseline at the end of therapy and 24 weeks after the end of therapy. Long-term data on HCC outcomes and patient's survival have been recorded. During long term follow-up, patients have been evaluated every six months with a clinical assessment and liver

imaging, including ultrasound or computed tomography (TC) scan or magnetic resonance imaging (MRI). Patients received different DAAs, choosing available DAAs and according to National and Regional guidelines[142].

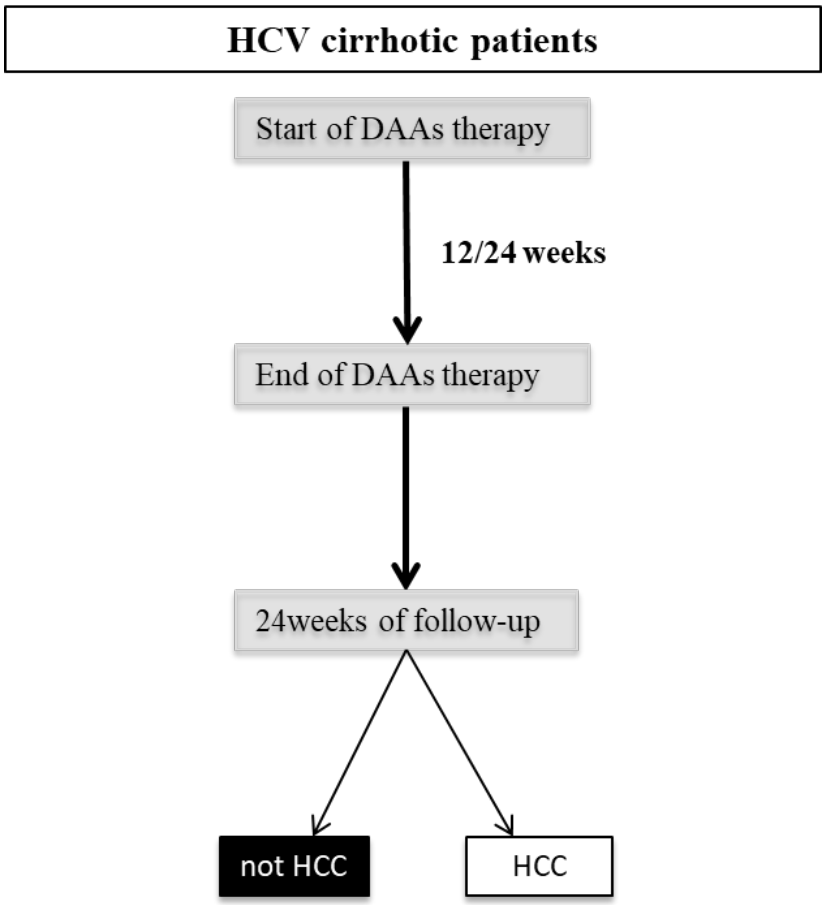


Figure 1. HCV cirrhotic patient's enrolment*: patients were assesed at the start of DAAs therapy, treated for 12 or 24 weeks. Patients with SVR have been included and after 24 weeks of follow-up divided in HCC or not HCC group.*

Sustained Virological response (SVR) to therapy is defined as undetectable HCV-RNA by a quantitative real-time PCR with a limit of detection of 12 IU/ml and evaluated at week 12 after therapy (SVR_{12}) and confirmed at week 24. The study is conducted according to the to

the ethical guidelines of the 2013 Declaration of Helsinki and was approved by the Local Ethics Committee.

In the preliminary part of the study, 16 patients with the same features of enrolled patients were selected for the screening phase, 8 cirrhotic patients treated with DAAs without HCC at 24 weeks after the end of therapy (HCC group) and 8 treated cirrhotic patients with HCC after 24 weeks. In the screening phase, 172 mi-RNAs were detected in the baseline samples in microarray plates. The mi-RNAs with a significantly different expression between the 2 groups have been selected and validated in the whole cohort at baseline by RT-q-PCR (validation phase, figure 2). The same mi-RNAs have been evaluated at the end of therapy, in the HCC and not HCC group.

Inclusion and exclusion criteria:

Inclusion criteria: a) Patients with HCV related cirrhosis evaluated by clinical, radiological, elastographic or histological criteria, treated with DAAs and cured (with SVR) b) HCC development in the HCC group occurred between the start of therapy and 24 weeks after the end of therapy**.** Exclusion criteria: HCC at enrolment, liver transplantation, HBV and HIV co-infections, < 18 years old.

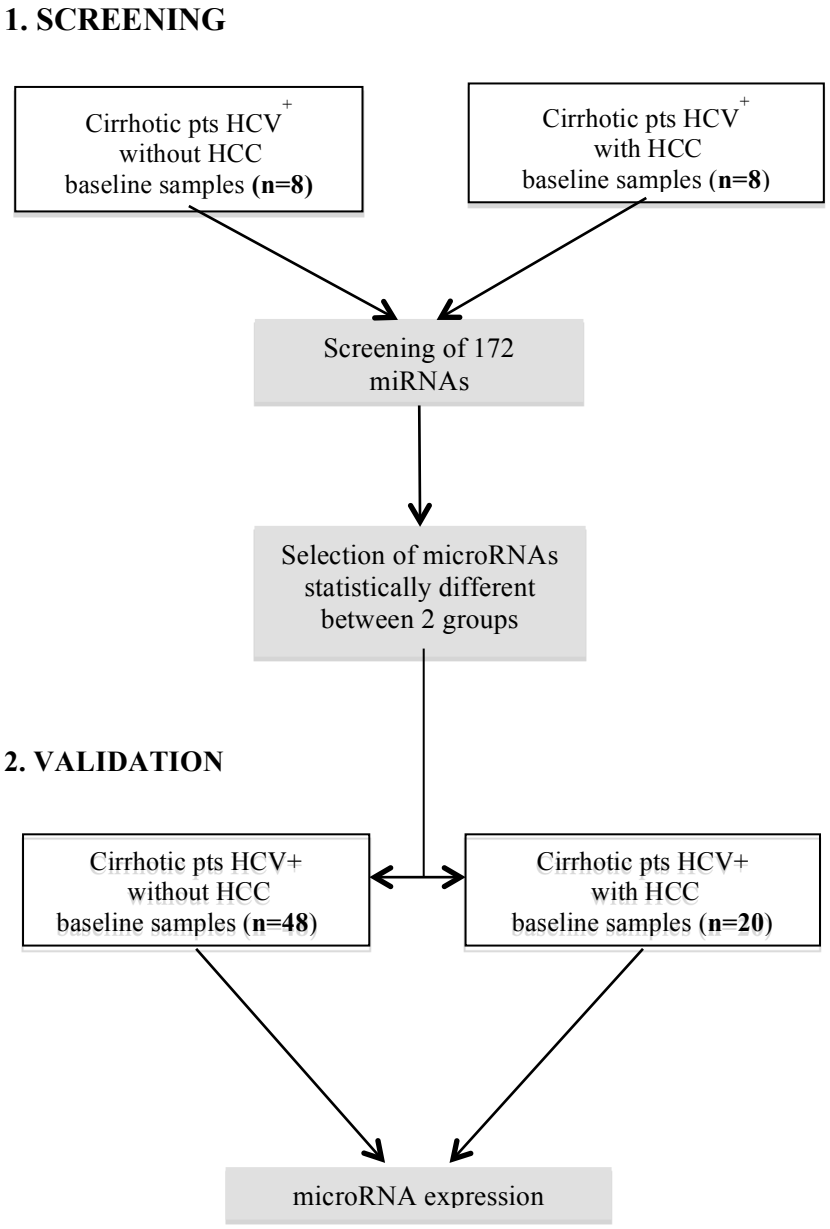


Figure 2: Flowchart of the Study, with screening phase and validation phase.

micro-RNAs analysis

Analysis is done on 200 µl of plasma obtained by centrifuge at 2000 X G and then frozen at -80°C. Isolation and purification of mi-RNAs from plasma is done using the Exiqon's miRCURYTM RNA Isolation Kit. The purification is based on spin column chromatography using a proprietary resin as the separation matrix. The kit can be used to isolate high quality RNA in amounts sufficient for qPCR analysis using the miRCURY LNATM Universal RT microRNA PCR system. The analysis of mi-RNAs is based on the software Bio-Rad CFX Manager 3.0. RNA. Extraction: microRNA from plasma samples is extracted using the miRCURYTM RNA isolation kit-Biofluids (Exiqon, Denmark), according to the manufacturer's protocol. All purified RNA is eluted in 40 µl RNase-free water and stored at -80 ˚C. To monitor the RNA isolation, is used the RNA Spike-in kit, UniRT (UniSp2, UniSP4, UniSp5 and UniSp6, Exiqon, Denmark). mi-RNA profiling: microRNA expression profiling is performed using reagents from Exiqon. RNA is reverse transcribed using the miRCURY LNA™ Universal RT mi-RNA PCR and Universal cDNA Synthesis Kit II. cDNA is diluted 40 times and assayed in 10-µl PCR reactions. Each mi-RNA is assayed once by quantitative polymerase chain reactions (qPCR) on the mi-RNA ready-to-Use Serum/Plasma Focus microRNA PCR panels V.3. Each set of panels comprises two 96-well PCR plates with 179 LNA mi-RNA primer sets, 5 spike-in control primer sets and candidate reference mi-RNAs. Negative controls are performed and profiled like the samples. Amplification is performed in a Bio-Rad CFX96 Real Time PCR detection system and ExiLENT SYBR® Green master mix. Amplification curves are analyzed using the CFX Manager software for determination of cycle (Cq) values. qPCR data processing and analysis are performed by GeneEX (Exiqon). Individual qPCR Assays and normalization: individual mi-RNAs are detected by qPCR for validation. qPCR is performed by ExiLENT

SYBR®Green master mix using LNA™ primers (Exiqon) and hsa-miR-92a-3p as miRNA reference for plasma samples, according to the manufacturer's protocol[143].

Statistics

Comparisons between more than two groups have been performed with Kruskal-Wallis test. In case of positive test ($p<0.05$), comparison analysis between couple of groups has been made using Mann-Whitney test (non parametric samples). Mann-Whitney test has been used also for comparison between two indipendent groups. For paired samples comparison, has been used paired t-test. Statistical significance accepted corresponds to a p value<0.05. Statistical analyses have been performed with the MedCalc™ software (vers. 15.6 MedCalc Software, Mariakerke, Belgium) and GraphPad Prism 5.01.

Results

The study has been divided in two different phases, a preliminary phase corresponding to the screening phase and a second phase with an analysis of mi-RNAs on the whole population, corresponding to the validation phase.

In the validation phase, only mi-RNAs with a significantly different expression in the two screening groups have been evaluated in the whole population of the study, comparing patients who have developed HCC to those without HCC. In the analysis of results, we have considered two different groups of cirrhotic patients, patients treated with DAAs for 12 weeks and patients treated for 24 weeks.

Screening phase

At baseline, 172 miRNAs have been analyzed in 8 patients of each group (not HCC or HCC group) to identify candidate miRNAs with a different expression between the groups (Table 1). We have performed a Volcano plot filtering between the two groups using as selection criterion a p-value<0.05 (Figure 3 and Table 2). We have identified 10 mi-RNAs differently expressed in the two groups at baseline: nine miRNAs have resulted upregulated in the HCC group compared to not HCC group (miR-382-5p, hsa-miR-28-5p, hsa-miR-132-3p, hsa-miR-122-5p, hsa-miR-130b-3p, hsa-miR-27a-3p, hsa-miR-324-3p, hsa-miR-584-5p, hsa-miR-221-3p), while hsa-miR133b has resulted downregulated in HCC group compared to the not HCC group.

Table 1. Baseline characteristics of patients included in the screening phase.

	Not HCC (n=8)	**HCC (n=8)**	**p value**
Age (SD)	62,25 (10,86)	57,25 (12,67)	0.5737
Sex (M/F)	4/4	6/2	0.3173
Genotipe HCV n (%)			0.2712
Genotype 1	8 (100)	6 (75)	-
Genotype 2	0	1 (12,5)	-
Genotype 3	0	1 (12,5)	-
HCV-RNA (U/ml) median (min-max)	1.286.453 (2.305-3.463.656)	342.787 (28.549-1.617.930)	**0.0207**
CTP class % (n)			0.2636
CTP-A	6 (75)	6 (75)	-
CTP-B	2 (25)	2 (25)	-
MELD median (SD)	8,7 (1,9)	9,3 (2,4)	0.7897
Cirrhosis complications n (%)	4 (50)	2 (25)	0.1432
AFP median (min-max)	9,1 (3,7-68,7)	11,7 (4,1-162,6)	0.6126
Previous Therapy n (%)	3 (37,5)	5 (62,5)	0.4936
IFN+/-RIBA	0	1 (20)	-
IFN + DAA 1°generation	3 (100)	4 (80)	-
DAA 2° generation	0	0	-
Type of DAAs therapy n (%)			0.0695
SOF+RBV	0	2 (20)	-
SOF+LDV ±RBV	8 (100)	4 (50)	-
SOF+DCV ±RBV	0	1 (12,5)	-
IFN+RBV+SOF	0	1 (12,5)	-
Ribavirin n (%)	6 (75)	7 (87)	0.5351

***Legend**: CTP: Chlid-Turcotte-Pugh score. IFN: interferon, SOF: sofosbuvir, LDV: ledipasvir, DCV: daclatasvir, RBV: ribavirin.*

Table 2. miRNAs expression data in the screening phase.

miRNAs	*fold change ($2^{-\Delta\Delta Ct}$)*	*Log fold change*	*p-value*
hsa-miR-382-5p	*1.59*	*0.669*	*0.011*
hsa-miR-28-5p	*1.74*	*0.802*	*0.037*
hsa-miR-132-3p	*1.89*	*0.920*	*0.014*
hsa-miR-122-5p	*1.89*	*0.922*	*0.030*
hsa-miR-130b-3p	*4.22*	*2.07*	*0.016*
hsa-miR-27a-3p	*2.73*	*1.45*	*0.039*
hsa-miR-324-3p	*2.51*	*1.32*	*0.001*
hsa-miR-133b	*0.096*	*-3.38*	*0.020*
hsa-miR-584-5p	*2.79*	*1.48*	*0.002*
hsa-miR-221-3p	*3.82*	*1.94*	*0.003*

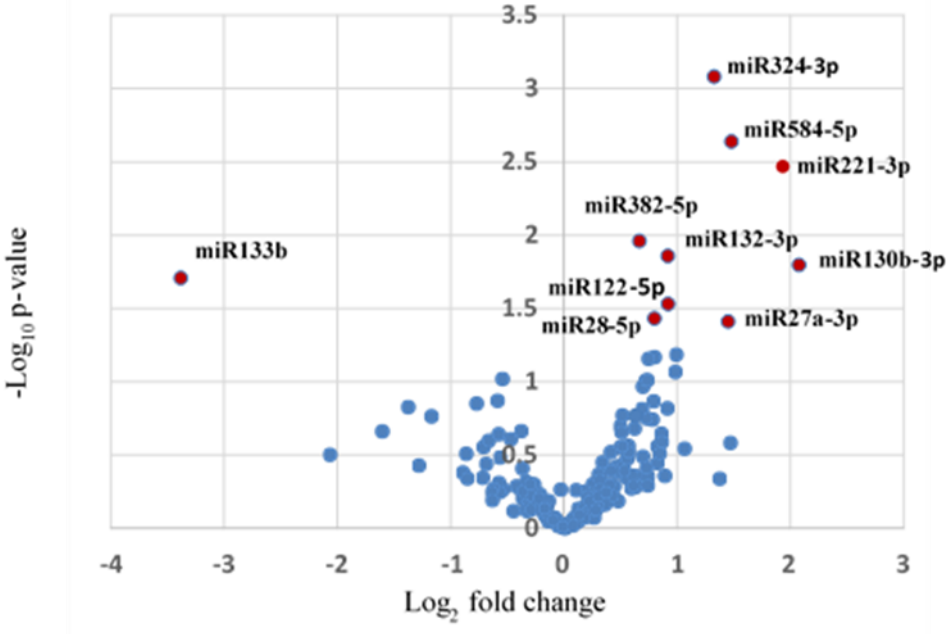


Figure 3. Vulcano Plot of mi-RNAs expression data in the screening phase.

Fold change and Log fold change of mi-RNAs in the HCC group vs not HCC.

Baseline validation phase in patients treated with DAAs for 12 weeks

mi-RNAs selected in the screening phase have been validated in 37 patients using qRT-PCR at baseline. Comparing the not HCC group (26 patients) with the HCC group (11 patients). The characteristics of patients of each group are reported in Table 3 and 4. The expression levels of mi-RNAs in the two groups are reported in Table 5.

Table 3. Baseline characteristics of patients included in validation phase and treated for 12 weeks (not HCC group vs. HCC group).

	not HCC group (n=26)	HCC group (n=11)	p-value
Age median (Q_1-Q_3)	67 (58-75)	73 (69-78)	0,06
Gender M/F (%)	15/11 (58-42)	6/5 (55/45)	0,86
BMI kg/m^2 n (%)			
X ≤ 25	10 (38)	4 (36)	0,91
25 < x < 30	9 (35)	6 (55)	0,27
X ≥ 30	7 (27)	1 (9)	0,23
Genotipe n (%)			
Genotipe 1a	1 (4)	0	0,52
Genotipe 1b	23 (88)	11 (100)	0,25
Others	2 (8)	0	0,35
HCV-RNA UI/L x10^6 median (Q_1-Q_3)	0,6 (0,3-2,2)	1,7 (0,7-2,0)	0,31
CTP Class n (%)			
CTP- A	23 (88)	9 (82)	0,59
CTP-B	3 (12)	2 (18)	0,59
MELD median (min-max)	7 (6-13)	7 (6-11)	0,80
APRI- score median (min-max)	1,8 (0,4-8,1)	3,9 (0,7-6,8)	**0,05**
Hepatic complications (%)	4 (15)	3 (27)	0,83
Previous therapy n (%)	14 (54)	6 (55)	0,97
INF +/- RBV	13 (93)	5 (83)	0,80
INF +/- RBV + DAAs	1 (7)	1 (17)	0,52
Type of DAAs therapy n (%)			
SOF+LDV ±RBV	9 (35)	3 (27)	0,67
OMV+PTV+DSV± RBV	14 (54)	4 (36)	0,34
SOF+SMV±RBV	3 (12)	4 (36)	0,08

***Legend**: CTP: Child-Turcotte-Pugh score. IFN: interferon, SOF: sofosbuvir, LDV: ledipasvir, OMV: ombitasvir, PTV: paritaprevir, DSV: dasabuvir, RBV: ribavirin, SMV: Simeprevir.*

The two groups of patients are not different for the entire baseline characteristics evaluated (age, gender, genotype, liver prognostic scores, hepatic complications, DAA regimens). Patients who developed HCC have a higher value of APRI-score compared to not HCC group, with a p-value close to a statistical significance. In the HCC group, regarding the timing of occurrence of HCC, 91% of patients resulted to have an occurrence between the end of therapy and the 24 weeks after the end, while only one patient (9%) resulted to have an occurrence during therapy. Patients who developed HCC had an early stage HCC or a very early stage HCC (BCLC stage 0-A) in 90%, and only one patient (9%) had an intermediate stage (BCLC stage B). When we have compared mi-RNAs expression between the two groups at baseline, only miR28-5p is resulted significantly increased (p=0.029) in HCC group compared to not HCC group, while the others mi-RNAs are not significantly different (Table 5, figure 4 and 5).

Table 4: HCC characteristics in HCC group treated for 12 weeks.

HCC group n=11	
Imaging screening pre-therapy n (%)	
Ultrasound	10 (91)
CEUS	1 (9)
HCC timing n (%)	
HCC occurrence during therapy	1 (9)
HCC occurrence after the end of therapy	10 (91)
BCLC staging n (%)	
Very early stage (0)	5 (45)
Early stage (A)	5 (45)
Intermediate stage (B)	1 (9)
Advanced stage (C or D)	0

Legend: *CEUS: Contrast-Enhanced Ultrasound, BCLC: Barcelona Clinic Liver Cancer.*

Table 5: baseline mi-RNAs expression in Validation phase in patients treated with DAAs for 12 weeks (not HCC group vs. HCC group).

mi-RNAs	not HCC group (n =26)	HCC group (n=11)	Fold change	p-value
	Relative miRNA expression (median)	Relative miRNA expression (median)		
miR122-5p	0,09958	0,1177	1,182	0,816
miR130b-3p	0,009706	0,01618	1,667	0,219
miR132-3p	0,005700	0,005634	0,988	0,425
miR133b	0,002397	0,001114	0,465	0,550
miR221-3p	0,06209	0,1168	1,881	0,163
miR27a-3p	0,02894	0,02882	0,996	0,947
miR28-5p	0,005239	0,01010	1,928	**0,029**
miR324-3p	0,01791	0,01930	1,078	0,506
miR382-5p	0,003033	0,006570	2,166	0,135
miR584-5p	0,001530	0,002057	1,344	0,361

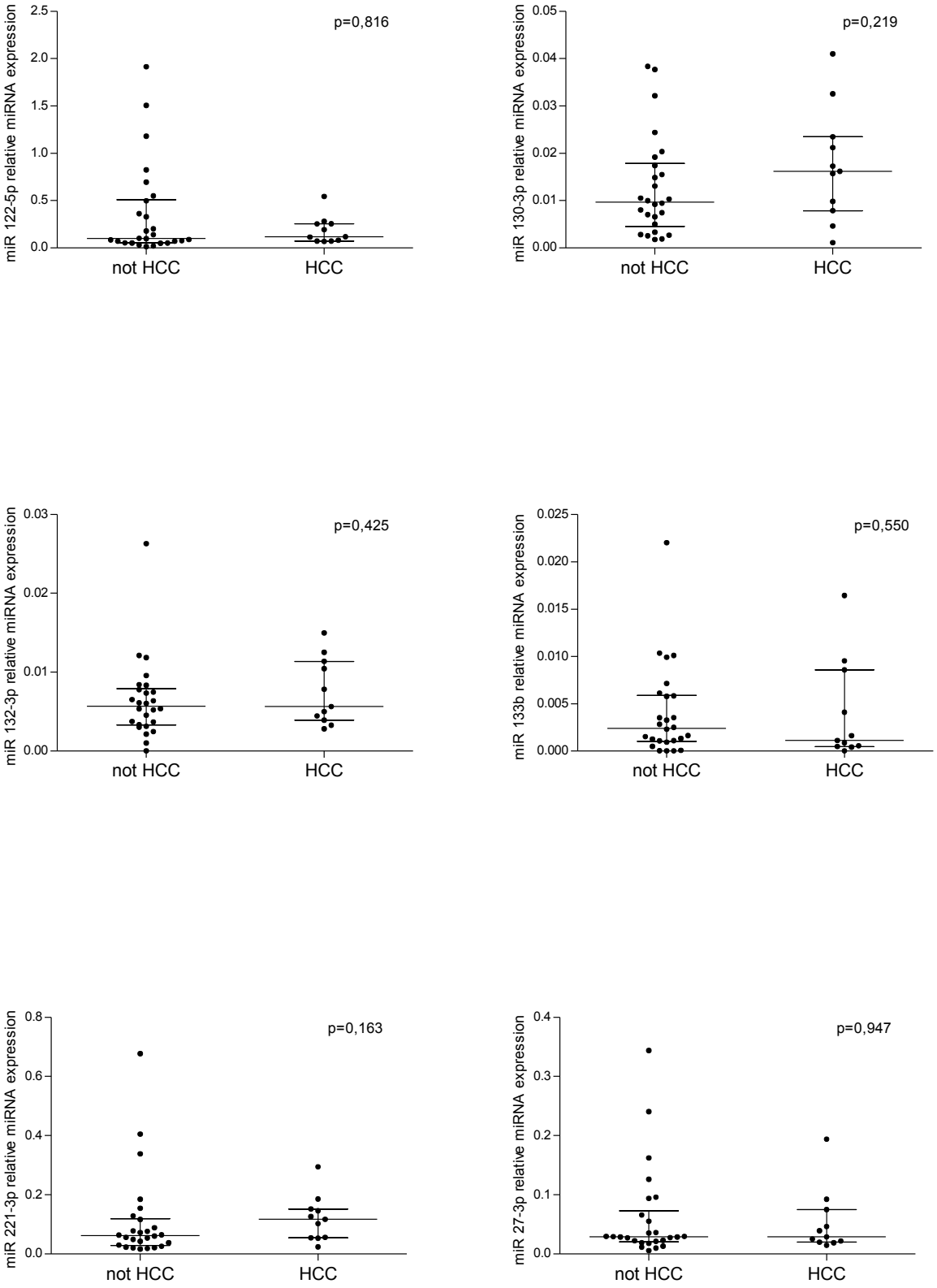


Figure 4. Baseline mi-RNAs expression in not HCC group compared to HCC group, patients treated for 12 weeks. mi-RNAs: miR122-5p, miR130b-3p, miR132-3p, miR133b, miR221-3p, miR27a3p.

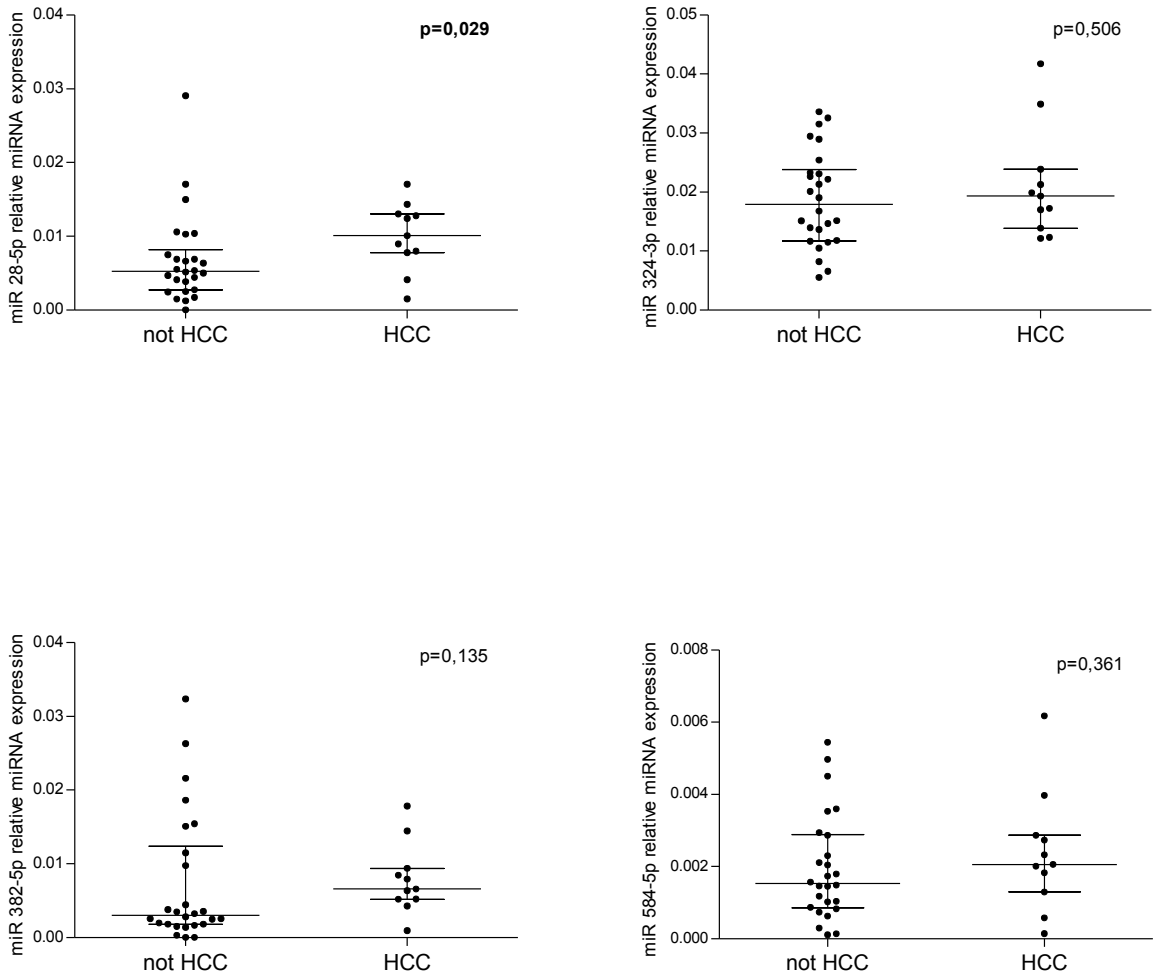


Figure 5. Baseline mi-RNAs expression in not HCC group compared to HCC group patients treated for 12 weeks. mi-RNAs: miR28-5p, miR324-3p, miR382-5p, miR584-5p.

miR28-5p predictive value in HCC group treated for 12 weeks

When we have used a Receiving Operating Characteristics (ROC) curve to evaluate the baseline prognostic value of miR-28-5p, the relation between sensibility and specificity is resulted in a p value of 0,018. The area under the curve (AUC) is resulted of 72.9% (figure 6).

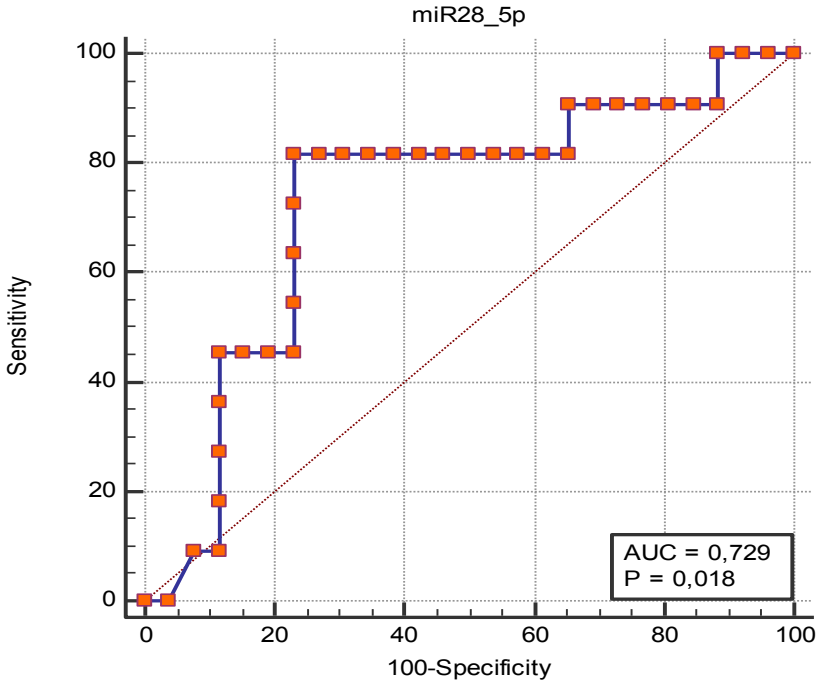


Figure 6. ROC curve relative to miR285p, in HCC group (12 weeks)

End of therapy validation phase in patients treated for 12 weeks with DAAs

mi-RNAs expression on samples collected at the end of DAAs therapy has been compared with the mi-RNAs expression at the start of the therapy (Table 6). In not HCC group six of the mi-RNAs evaluated, have been found significant lower after therapy. In details: miR122-5p, miR132-3p, miR133b, miR221-3p, miR324-3p, miR382-5p have resulted significant lower after DAAs. In the

HCC group a reduction in the same six mi-RNAs has been found. Comparing with the not HCC group, in the HCC group has also been highlighted a reduction of miR-28-5p (p=0.0391) (figure 7 and 8).

Table 6: mi-RNAs expression baseline vs. end of therapy in patients treated with DAAs for 12 weeks (not HCC group and HCC group).

not HCC group (n=26)				
	Relative miRNA expression **at baseline** (median)	Relative miRNA expression at the **end of therapy** (median)	Fold change	p-value
miR122-5p	0,09289	0,01688	0,18172	**0,0001**
miR130b-3p	0,01014	0,01760	1,7357	0,0824
miR132-3p	0,005864	0,003279	0,559175	**0,0051**
miR133b	0,002003	0,0004567	0,228008	**0,0017**
miR221-3p	0,06400	0,03850	0,601563	**0,0019**
miR27a-3p	0,02894	0,03127	1,080511	0,3720
miR28-5p	0,005239	0,005078	0,969269	0,9612
miR324-3p	0,01957	0,008679	0,443485	**0,0001**
miR382-5p	0,003151	0,001743	0,553158	**0,0099**
miR584-5p	0,001530	0,0003747	0,244902	0,2491
HCC group (n=11)				
	Relative miRNA expression **at baseline** (median)	Relative miRNA expression at the **end of therapy** (median)	Fold change	p-value
miR122-5p	0,1177	0,01471	0,124979	**0,0039**
miR130b-3p	0,01618	0,01548	0,956737	0,7344
miR132-3p	0,007849	0,003188	0,406166	**0,0195**
miR133b	0,001114	0,0003831	0,343896	**0,0391**
miR221-3p	0,1266	0,05219	0,412243	**0,0117**
miR27a-3p	0,03928	0,04039	1,028259	0,3594
miR28-5p	0,01243	0,006539	0,526066	**0,0391**
miR324-3p	0,01987	0,009663	0,486311	**0,0039**
miR382-5p	0,007922	0,004110	0,518808	**0,0039**
miR584-5p	0,002323	0,0002347	0,101033	0,1289

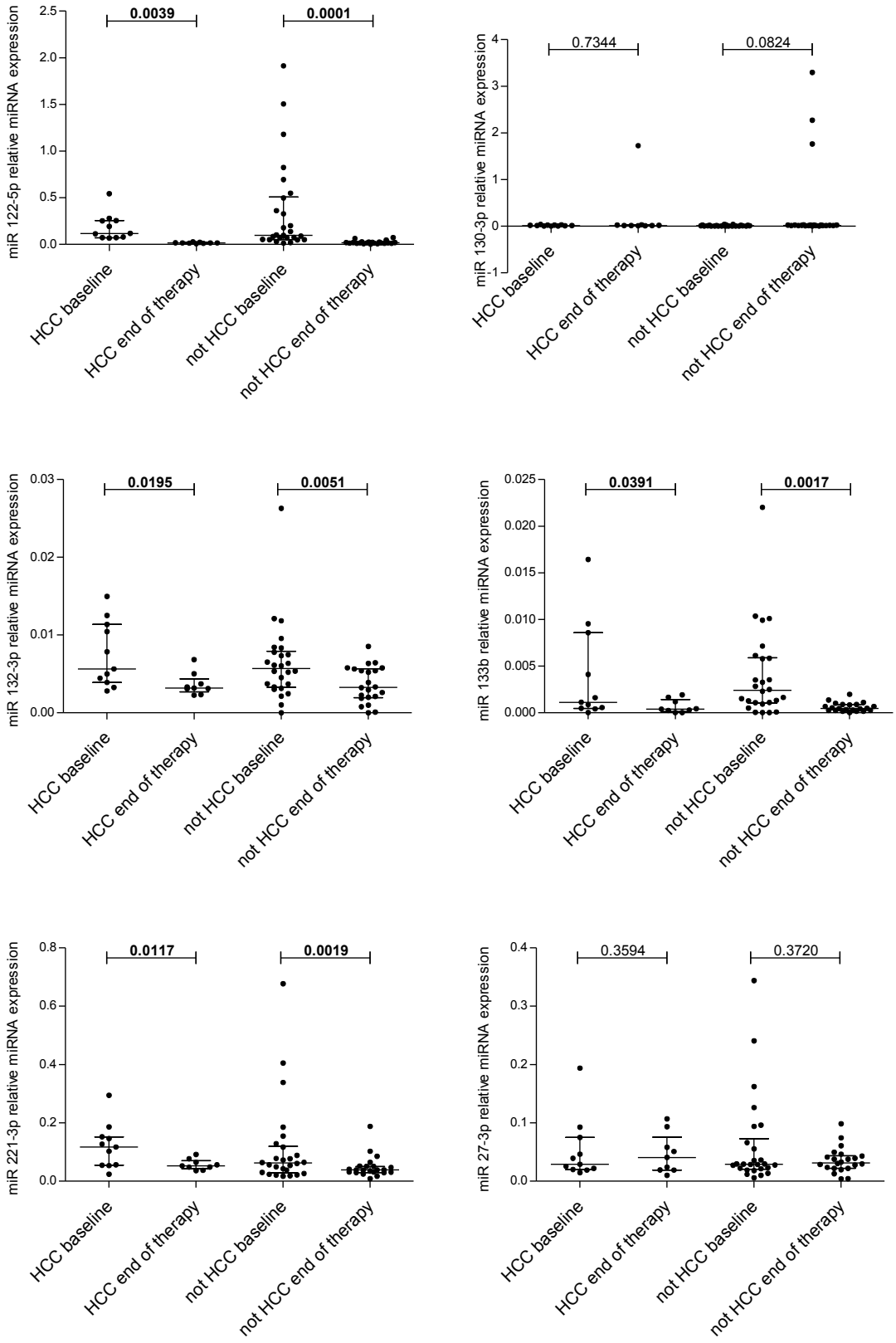


Figure 7. mi-RNAs expression in HCC and not HCC group: end of therapy and baseline (patients treated with DAAs for 12 weeks). mi-RNAs: miR122-5p, miR130b-3p, miR132-3p, miR133b, miR221-3p, miR27a3p.

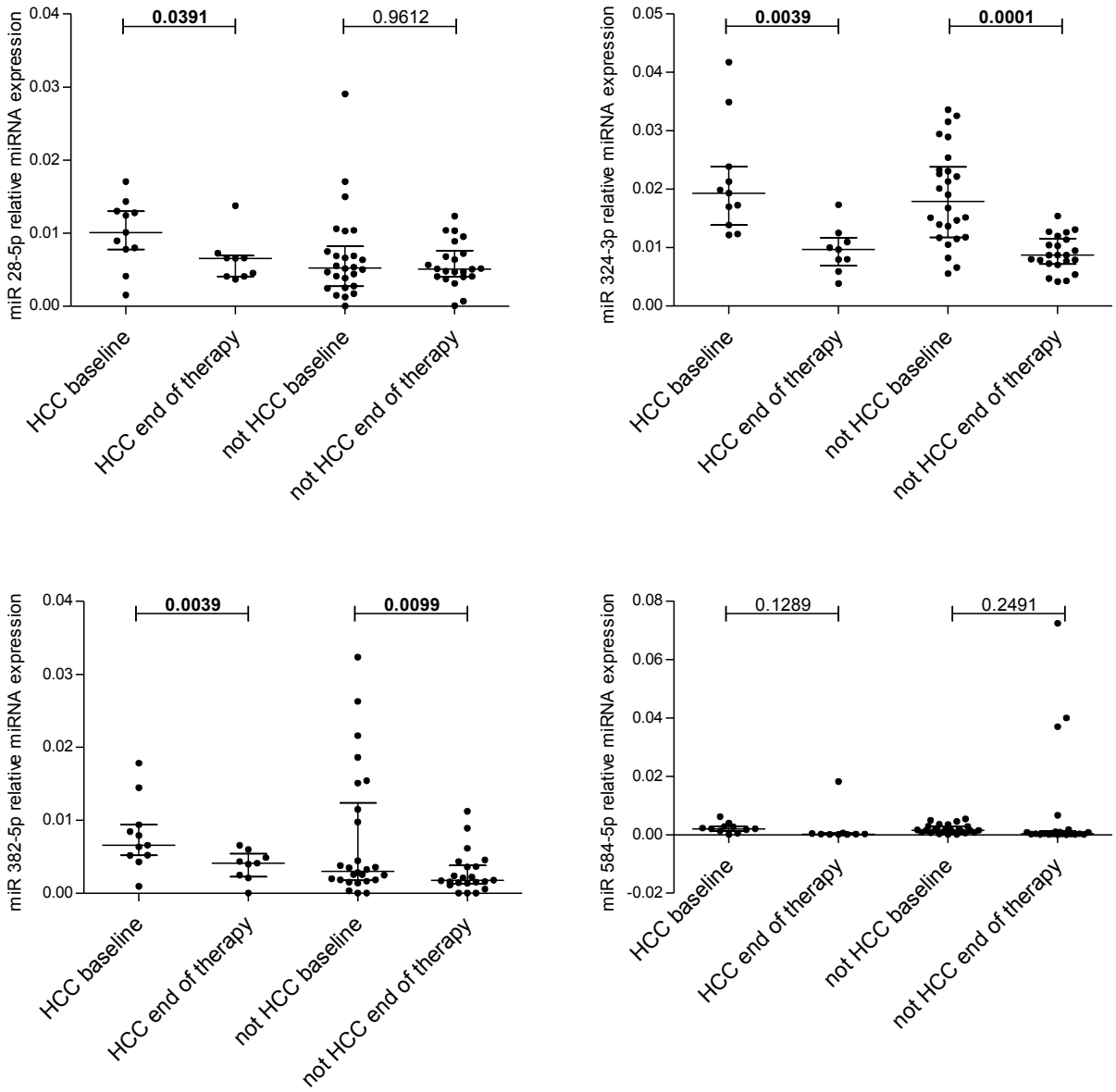


Figure 8. mi-RNAs expression in HCC and not HCC group: end of therapy and baseline (patients treated with DAAs for 12 weeks). mi-RNAs: miR28-5p, miR324-3p, miR382-5p, miR584-5p.

Baseline validation phase in patients treated for 24 weeks with DAAs

Table 7. Baseline characteristics of patients treated with DAAs for 24weeks (not HCC group vs HCC group)

	not HCC group (n=12)	HCC group (n=9)	p-value
Age median (Q_1-Q_3)	62 (56-74)	63 (60-80)	0,36
Gender M/F (%)	9/3 (75/25)	6/3 (67/33)	0,09
BMI kg/m^2 n (%)			
X ≤ 25	6 (50)	5 (56)	0,81
25 < x < 30	5 (42)	4 (44)	0,90
X ≥ 30	1 (8)	0	0,39
Genotipe n (%)			
Genotipe 1a	2 (17)	0	0,73
Genotipe 1b	5 (42)	6 (67)	0,90
Others	2 (17)	3 (33)	0,73
HCV-RNA UI/L x10^6 median (Q_1-Q_3)	0,5 (0,04-0.9)	1,1 (0,4-2,6)	0,16
CTP Class n (%)			0,46
CTP- A	6 (50)	6 (67)	-
CTP-B	6 (50)	3 (33)	-
MELD median (min-max)	10 (6-15)	7 (4-15)	0,17
APRI- score median (min-max)	1,4 (0,7-4,2)	3,4 (0,2-8,4)	0,39
Hepatic complications (%)	6 (50)	1 (11)	0,07
Previous therapy n (%)	6 (50)	3 (33)	0,59
INF +/- RBV	4 (67)	3 (100)	0,85
INF +/- RBV + DAAs	2 (33)	0	0,24
Type of DAAs therapy n (%)*			
SOF+RBV	3 (25)	2 (25)	1
SOF+LDV ± RBV	5 (42)	3 (42)	0,86
SOF+DCV	2 (17)	2 (22)	0,66
OMV+PTV+DSV± RBV	2 (17)	1 (11)	0,80
SOF+SMV±RBV	0	0	-
Range from last liver imaging and start of DAAs (m.) median (min-max)	2,6 (0,0-5,3)	3,0 (0,0-6,3)	0,52

Legend: ***** *1 patient HCC group not available type of therapy. m.: months. CTP: Chlid-Turcotte-Pugh score. IFN: interferon, SOF sofosbuvir, LDV: ledipasvir, OMV: ombitasvir, PTV: paritaprevir, DSV: dasabuvir, RBV: ribavirin*

The baseline characteristics of the two groups of cirrhotic patients with or without HCC (HCC, not HCC) are reported in Table 7. The two groups of patients don't differ for the main baseline characteristics. Patients who do not develop HCC compared to the HCC group, have comparable age, sex, BMI, genotypes. The two groups don't differ regarding the prognostic scores of liver disease and presence of complications of liver disease. DAAs regimens are not significantly different in the two groups.

In the HCC group, 67% of patients resulted to have an occurrence between the end of therapy and the 24 weeks after the end, while six patients (33%) resulted to have an occurrence during therapy. Patients who developed HCC had an early stage HCC or a very early stage HCC (BCLC stage 0-A) in 88%, and only one patient (11%) had an advance stage (BCLC stage C) (Table 8).

Table 8. HCC characteristics in HCC group treated for 24 weeks.

Pazienti HCC (n =9)	
Imaging screening pre-therapy n (%)	
Ultrasound	6 (66)
CEUS	2 (22)
MRI	1 (11)
HCC Timing n (%)	
HCC occurrence during therapy	3 (33)
HCC occurrence after the end of therapy	6 (67)
BCLC staging	
Very early stage (0)	4 (44)
Early stage (A)	4 (44)
Intermediate stage (B)	0
Advanced stage (C)	1 (11)

Legend: *CEUS: Contrast-Enhanced Ultrasound, MRI: magnetic resonance imaging, BCLC: Barcelona. Clinic Liver Cancer.*

Selected mi-RNAs of the screening phase have been tested in HCC and in not HCC group. The baseline expression of mi-RNAs doesn't show a significant difference between the two groups (Table 9, Figure 9 and 10).

Table 9. baseline mi-RNAs expression in patients treated with DAAs for 24 weeks (not HCC group vs HCC group).

miRNAs	**Not HCC (n=12)**	**HCC (n=9)**	**Fold change**	**p-value**
	relative miRNA expresssion (median)	relative miRNA expresssion (median)		
miR122-5p	0,2339	0,3860	1,650278	0,2864
miR130b-3p	0,004475	0,009618	2,149274	0,6698
miR132-3p	0,006658	0,006479	0,973115	0,9433
miR133b	0,000002381	0,004260	1789,164	0,8184
miR221-3p	0,04462	0,07911	1,772972	0,1021
miR27a-3p	0,02335	0,04502	1,928051	0,0756
miR28-5p	0,005412	0,006419	1,186068	0,3198
miR324-3p	0,01054	0,01801	1,708729	0,7762
miR382-5p	0,003769	0,006031	1,600159	0,2540
miR584-5p	0,001229	0,002152	1,751017	0,2555

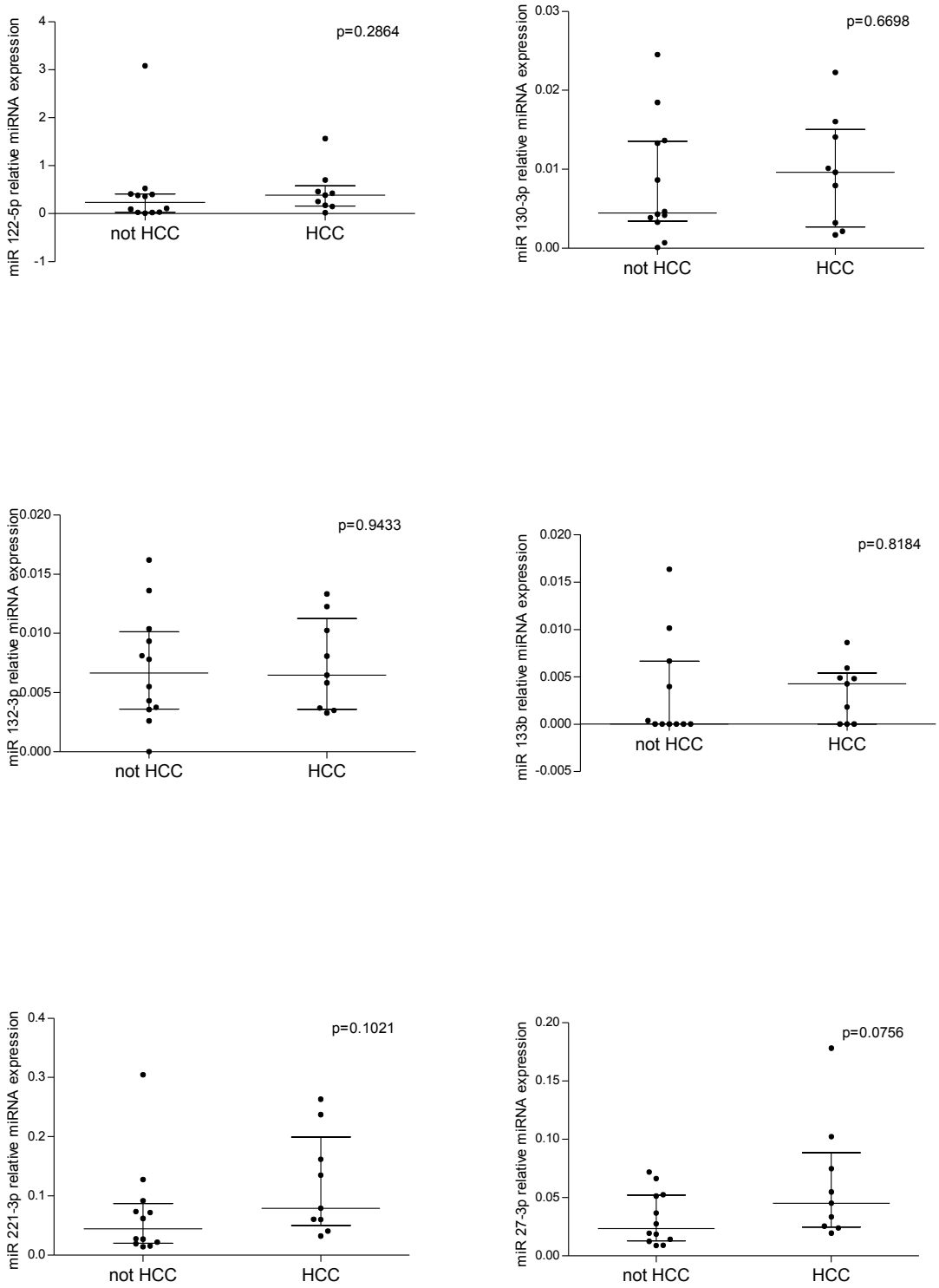


Figure 9. Baseline mi-RNAs expression in not HCC group compared to HCC group, patients treated for 24 weeks. mi-RNAs: miR122-5p, miR130b-3p, miR132-3p, miR133b, miR221-3p, miR27a3p.

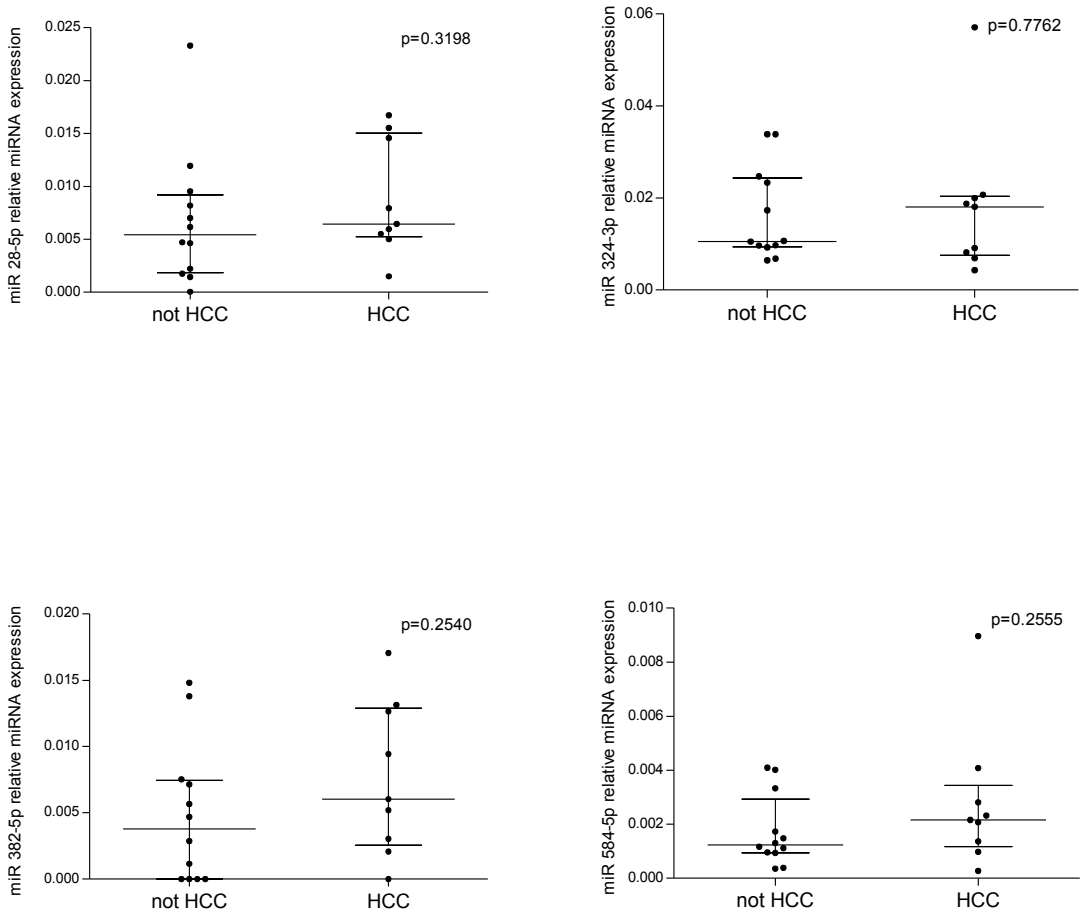


Figure 10. Baseline mi-RNAs expression in not HCC group compared to HCC group, patients treated for 24 weeks). mi-RNAs: miR28-5p, miR324-3p, miR382-5p, miR584-5p.

End of therapy validation phase in patients treated for 24 weeks with DAAs

The same mi-RNAs evaluated at baseline in the HCC and not HCC group have been evaluated at the end of therapy. In not HCC group there are not statistical differences in the mi-RNAs expression between the start and the end of therapy (Table 10). In the HCC group, at the end of therapy compared with the baseline, there was a reduction of the levels of miR-28-5p and miR-122-5p (Table11) (figure 11 and 12).

Table 10. mi-RNAs expression baseline vs end therapy in not HCC group (patients treated for 24 weeks) .

not HCC group	Baseline (Median)	End of therapy (Median)	**Fold change**	**p-value**
miR122-5p	0,234	0,0120	0,051282	0,2190
miR130b-3p	0,00447	0,0139	3,10962	0,1787
miR132-3p	0,00666	0,00296	0,444444	0,1511
miR133b	0,00000238	0,0000887	37,26891	0,0894
miR221-3p	0,0446	0,0458	1,026906	0,5140
miR27a-3p	0,0233	0,0207	0,888412	0,2926
miR28-5p	0,00541	0,00516	0,953789	0,5220
miR324-3p	0,0105	0,00895	0,852381	0,1820
miR382-5p	0,00377	0,00131	0,34748	0,0891
miR584-5p	0,00123	0,000166	0,134959	0,2456

Table 11. mi-RNAs expression baseline vs end therapy in HCC group (patients treated for 24 weeks) .

HCC group	Baseline (Median)	End of therapy (Median)	**Fold change**	**p-value**
miR122-5p	0,386	0,0168	0,043523	**0,0349**
miR130b-3p	0,00962	0,0198	2,058212	0,3260
miR132-3p	0,00648	0,00490	0,756173	0,5183
miR133b	0,00426	0,000	0,000000	0,0631
miR221-3p	0,0791	0,0460	0,581542	0,1292
miR27a-3p	0,0450	0,0389	0,864444	0,8173
miR28-5p	0,00642	0,000132	0,020561	**0,0159**
miR324-3p	0,0180	0,00694	0,385556	0,1029
miR382-5p	0,00603	0,00388	0,643449	0,6943
miR584-5p	0,00215	0,000311	0,144651	0,4564

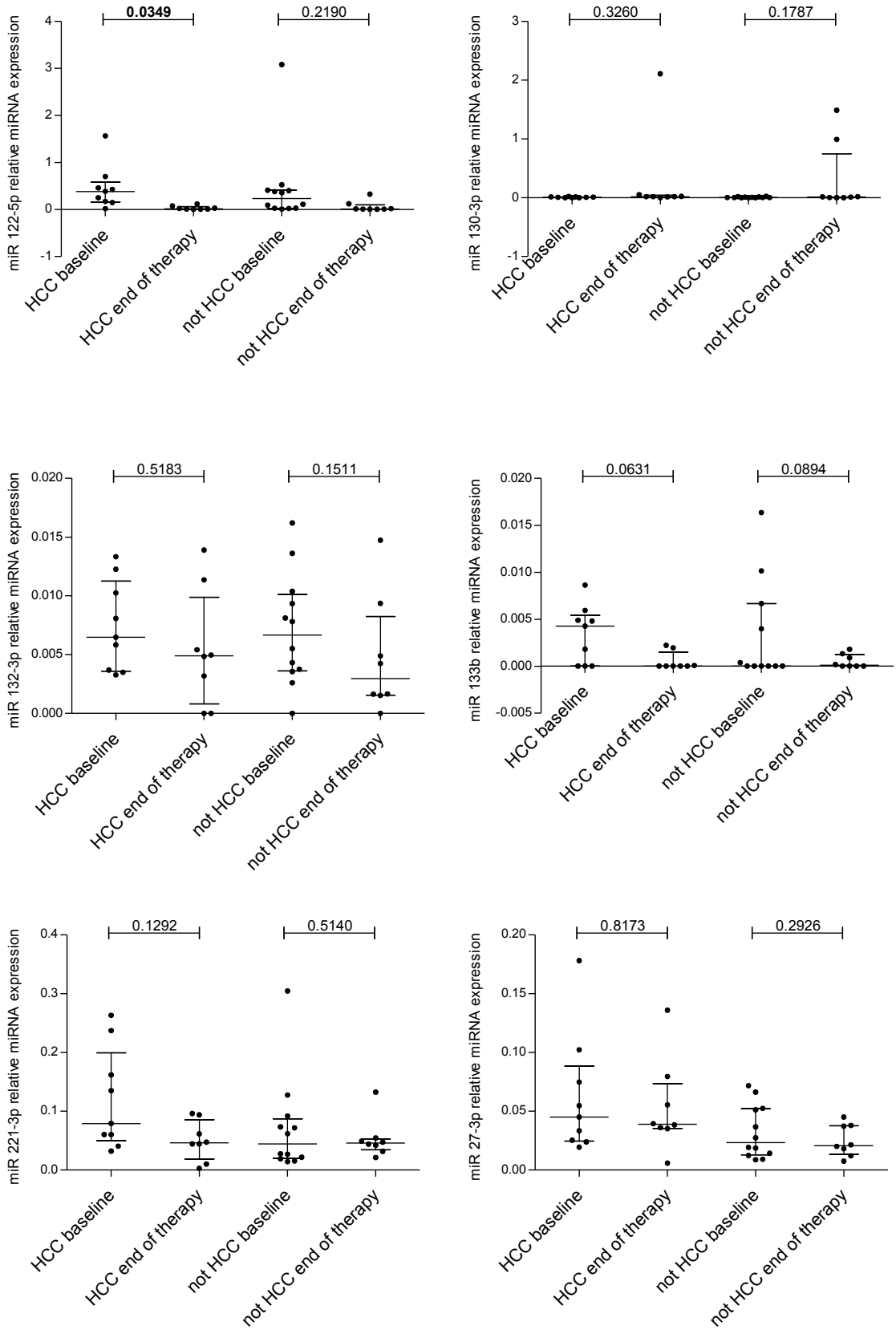


Figure 11. mi-RNAs expression in HCC and not HCC group: end of therapy and baseline (patients treated with DAAs for 24 weeks). mi-RNAs: miR122-5p, miR130b-3p, miR132-3p, miR133b, miR221-3p, miR27a3p.

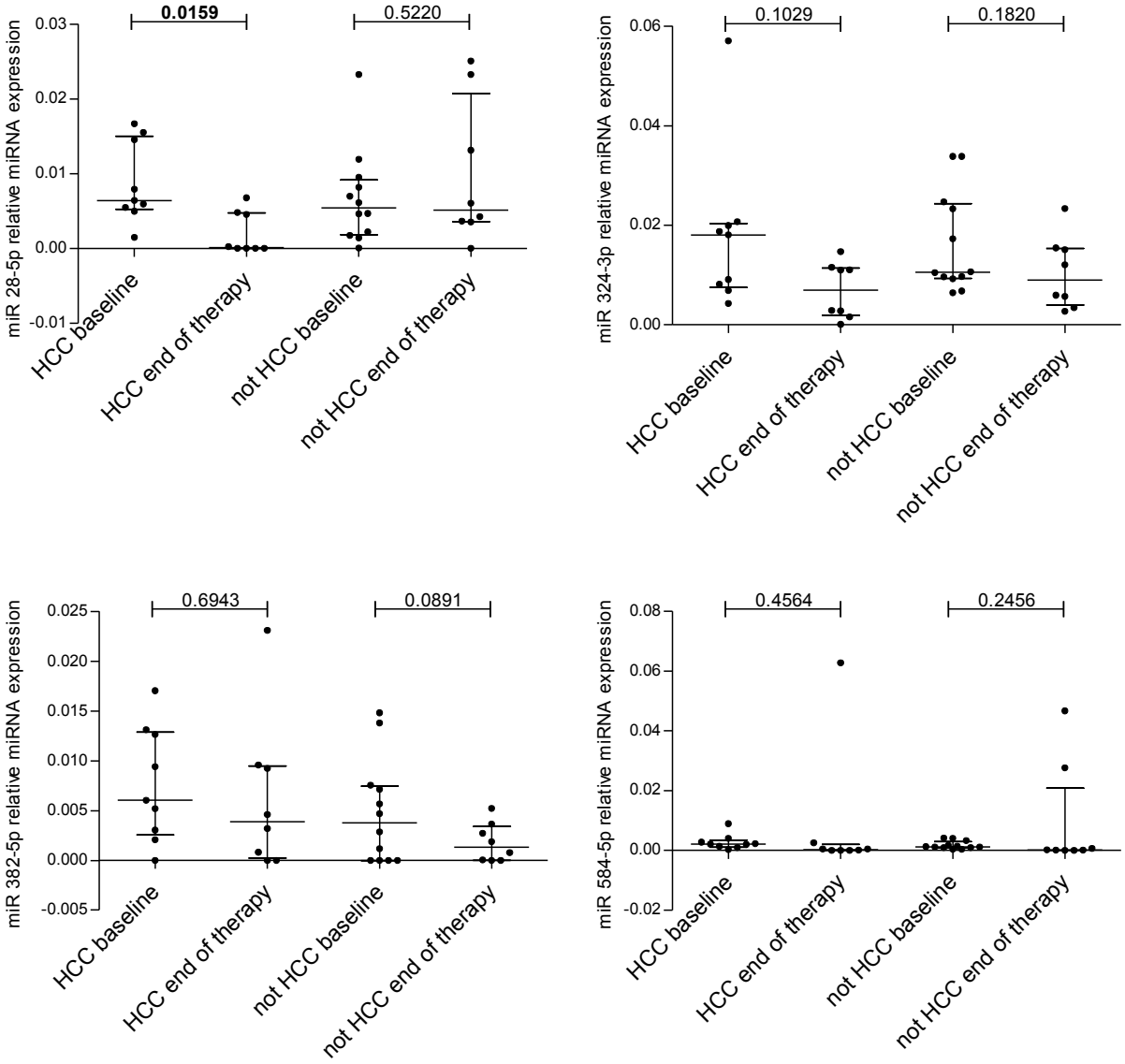


Figure 12. mi-RNAs expression in HCC and not HCC group: end of therapy and baseline (patients treated with DAAs for 24 weeks). mi-RNAs: miR28-5p, miR324-3p, miR382-5p, miR584-5p.

Discussion and Conclusions

This descriptive study shows that baseline levels of miR-28-5p in cirrhotic patients treated for 12 weeks with DAAs who develop HCC are higher compared with the not HCC group, confirming the results of the screening phase were miR28-5p is resulted significantly higher in HCC group with a fold change of *1.74*. In HCC group (n=11) treated with DAAs for 12 weeks, HCC occurrence is diagnosed between the start of therapy (baseline) and a maximum of 9 months from the beginning of the therapy, with a 91% of patients who develop HCC after the end of therapy (3-9 months after the start of DAAs) and 9% during therapy. In this setting the increasing of miR28-5p should be a predictive marker of HCC development. In the HCC group treated for 12 weeks, we have evaluated the predictive value of miR-28-5p, using a ROC curve. The area under the curve (AUC) is resulted of 72.9 % with a p value of 0,018, corresponding to a moderate diagnostic accuracy of miR-28-5p.

In a second smaller group (n=9) of patients with HCC, treated with DAAs for 24 weeks, the expression of miR28-5p is not higher compared with the HCC group. In these patients, HCC occurrence is diagnosed between the start of therapy (baseline) and a maximum of 12 months from the beginning of the therapy, with a 67% of patients who develop HCC after the end of therapy (6-12 months after the start of DAAs) and 33% during therapy. In this group, the lenght of therapy and the timing of HCC occurrence may have probably led to a different expression of miR28-5p.

In patients treated with DAAs for 12 weeks, when we have evaluated the miRNAs expression at the end of therapy compared to baseline, six mi-RNAs are resulted significantly lower than the baseline, in both groups either in not HCC group. In HCC group, in addiction to the reduction of the six mi-RNAs, has also been underlined a significant reduction of miR 28-5p. Considering that the change of mi-RNAs expression is the same in both group, we could

hypothesize that the reduction of the six mi-RNAs is related to the effect of HCV therapy. Conversely miR28-5p has resulted differently express at the baseline in HCC group compared with not HCC group and after DAAs therapy is resulted reduced only in the HCC group. Therefore, also in patients treated for 24 weeks who develop HCC, miR28-5p is reduced at the end of therapy, and associated to the reduction of miR 122-5p. This change of expression of miR28-5p should be explained by its involvement in the pathophysiology of HCC. miR28-5p has been associated with the development of several tumors, including gastrointestinal tumors and ovarian tumor. The expression of miR28-5p has resulted lower in patients with colorectal cancer and prostate cancer, suggesting its possible role as tumor suppressor. While, miR28-5p expression is resulted increasing in the brain cancer and ovarian cancer suggesting an oncogenic role[144]. Recently miR28-5p has shown to be correlated with the development of HCC. The expression of miR 28-5p has been tested on HCC biopsy of patients underwent to liver resection and with different ethiologies of liver disease. In these patients a reduction of expression of miR 28-5p (in HCC tissue) is resulted associated with a poor survival, tumour metastasis and tumour recurrence. In the same study has been demonstrated that the effect of miR 28-5p deficiency on HCC progression was dependent on IL-34 mediated tumour-associated macrophage (TAM) infiltration. mir28-5p is inversely correlated with IL-34 expression and TAM infiltration. Moreover TAM induced by miR28 5p inhibit its expression on HCC cells by transforming growth factor beta[145]. Further in human hepatoma cells, down-regulated miR-28-5p was associated with tumor growth through PI3K/ AKT pathway by targeting IGF.[146]

Considering that miR28-5p is known to be involved in the development and progression of HCC, its expression in patients treated with DAAs who develop HCC should be directly correlated with the appeareance and progression of HCC. The expression of miR 28-5p at baseline is increased in patients treated with DAAs for 12 weeks who develop HCC,

suggesting its potential prognostic role in HCC related to HCV. Further investigations are requested to clarify the phisyopatolgical role of miR 28-5p in this contest.

Study 3

Preliminary results

"The role of pro-carcinogenic host protein Osteopontin in modulating tumour-specific immune responses in Hepatocellular Carcinoma."

Host Institution

Dr Shilpa Chokshi, Chief Scientific Officer

Institute of Hepatology, Foundation for Liver Research

London, UK

AWARDED 2018

EASL Andrew K.Burroughs Short-term Fellowship.

Introduction

Osteopontin (OPN) is a multi-functional extracellular matrix protein that is produced by a different number of cell types (epithelial tissue, smooth muscle cells, osteoblasts) including immune and tumour cells. OPN plays a key role in the cellular signalling pathways that mediate inflammation, tumor progression and metastasis[147]. Several data have demonstrated the key role played by OPN in mediating hepatic inflammation, hepatic fibrogenesis and in several different aspects of the response of the liver to injury[148]. OPN is also highly expressed in Hepatocellular Carcinoma (HCC) and multiple retrospective cohort studies have investigated the diagnostic potential of OPN for HCC, which is strongly correlated with worse outcomes[149, 150]. Indeed OPN overexpression appears to be correlated with HCC grade, HCC stage and early recurrence[151]. We also know that OPN can enhance hepatitis B (HBV) replication directly, which we know is a direct causal factor in HCC development[152-156]. More

recently observations have provided evidence that OPN induced by hepatitis C (HCV) is critical for HCV replication and assembly[157].

Therefore has been showed that Osteopontin acts as modulator of immune system. OPN has a dominant role in balancing lymphoid and myeloid cells during emergency granulopoiesis (for example during an infection), exercising a pro-apoptotic role on myeloid progenitor cells and inhibiting apoptosis in T and B-cells[158]. Other studies have established that OPN acts as a bridge between innate and adaptive immune system, up-regulating interleukin-(IL-) 12 in macrophages and stimulating Thelper (Th)1 development[159]. Other studies have confirmed that OPN is intimately involved in modulating functions of neutrophils monocytes/macrophages and lymphocytes during liver inflammation, injury and disease[148].

As well, the high expression of OPN in colon carcinoma is inversely correlated with patient survival. OPN expression in colon cancer is repressed by interferon regulatory factor 8 (IRF 8) that is a ligand for CD44 on T cells. OPN overexpression acts as a potent T cell suppressor conferring host tumor immune tolerance and acting as an immune check-point to regulate T cells activation[160].

The immune cells are pivotal in anti-HCC immunity and are the targets of the new immunotherapeutic treatments, such as anti-programmed death-1, aimed at harnessing their anti-cancer potential[161]. However, the role of OPN in anti-HCC immunity is not understood or defined.

Aim of the study

The main aims of this study are to better define the role of OPN in HCC and elucidate the immunoregulatory functions of OPN in Hepatocellular Carcinoma.

Methods

Experimental plan

Using techniques that are well established at host institution[162-166], the following experimental analysis has been performed:

1. Measurement of OPN in the plasma of patients with HCC with chronic hepatitis B (CHB) or without HBV (non-viral) compared to patients with chronic hepatitis B (CHB) and with healthy control patients (healthy controls). The commercially available ELISA (R&D systems) has been used[167].
2. Measurement of OPN in supernatant of Peripheral blood mononuclear cells (PBMCs) of HCC patients analyzed with enzyme linked immunospot procedure (ELISPOT) for IFN-γ (BD Bioscences) and Granzyme B (BD Bioscences). ELISPOT has been done on PBMCs of patients with HCC and CHB (HCC-CHB) or HCC without CHB (HCC-non viral) and of healthy controls, incubated with HCC-derived tumor antigen libraries (protein-53 antigen (p-53), Human cancer- testis antigen (NYESO-1) and glypican 3 antigen, available from JPT solutions) and with or without antibody neutralizing (anti) programmed cell death protein 1(PD-1). ELISPOT has also been done on PBMCs from patients with HCC-CHB incubated with HBV-antigens (Capsid protein (HBV-CP) and Large Envelope Protein (HBV-LEP), available from JPT solutions) and with or without anti-PD-1. IFN-γ and Granzyme B have been quantified with ELISPOT. For OPN analysis has been used ELISA (R&D systems)[167].
3. Assessment of the impact of recombinant OPN on modulating the phenotype and function of innate and HCC-specific adaptive immune cells in PBMCs from patients with HCC-CHB or HCC-non viral. This has been done using the following assays: PBMCs of patients with HCC have been cultured with: HCC-derived tumor antigen libraries (protein-53 antigen (p-53), Human cancer-testis antigen (NYESO-1) and glypican 3 antigen, available from JPT

solutions), for T cells analysis or with interleukin 12 and 18 (IL-12 and IL-18 respectively from R&D systems and MBL) and human myeloid leukemia cell lines (K562) for NK cells, plus recombinant OPN (r-OPN, available from R&D systems) to assess the impact of OPN on immune function, OPN-aptamer (OPN-APT, available from Pharmacon) to neutralises OPN function and SHAM-Aptamer (SHAM-APT, available from Pharmacon) as negative control. After NK cells and T cells phenotype has been characterized using flow cytometry (Fluorescence-activated cell sorting (FACS)) analysis.

The enzyme linked immunospot procedure (ELISPOT)

Elispot assay using BD Biosciences reagents for either_IFN-γ and Granzyme is designed to detect the frequencies of cells that produce and secrete cytokines. Day one of the method involves an overnight incubation of PBMCs with antigen and coating of ELISPOT plates with cytokine capture antibody. On day 2 peripheral blood mononuclear cells (PBMC) are transferred to coated ELISPOT plates and cytokines secreted from antigen stimulated cells are captured during an overnight incubation. On day 3 cells are washed away and captured cytokines are detected by the addition of a biotinylated specific cytokine detection antibody followed by streptavidin HRP. Addition of AEC substrate enables the formation of spots which correspond to the number of cytokine secreting cells which are enumerated using an ELISPOT plate reader.

Fluorescence-activated cell sorting (FACS) analysis

Analysis of NK cells and T cells phenotype has been analyzed by Fluorescence-activated cell sorting (FACS) analysis, using a 18-colour FACS analyser (FACS Fortessa).

Assessment of the following parameters have been performed: a) *NK cells characterization using flow cytometry:* PBMCs have been stained for NK cells phenotype markers using anti-CD56 (AF700), anti-CD3 (BV510) and anti-CD16 (APCCy7) antibodies (available from Biolegend). Further staining has been performed for inhibitory and activating receptors with anti-NKG2A (PEVio 770, available from Miltenyi), anti-programmed death -1(PD-1) (PE, available from eBioscience) and anti-NKG2D (BV 605, available from Biolegend). PBMCs have also been stained for natural cytotoxic receptors with anti-NKp46 (BV 786, available from BD) and anti-NKp30 (BV 395,available from BD) and with anti-TNF-related apoptosis inducing ligand (TRAIL) (BV 421, available from Biolegend) . NK cells functions have been assessed with anti-IFN-γ (BUV 737) and anti-CD107a (BV711) antibodies (available from BD and Biolegend respectively). PBMCs have been been further stained with anti-TIM-3 (FITC, available from eBiosciences), anti-CD134 (BV 650, available from BD Biosciences), anti-CD44 (PerCP-Vio700, available from Miltenyi), anti CD38 (PE-Cy5.5, available from Novus).

b) T cells characterization using flow cytometry: PBMCs have been stained for T cells phenotypic markers using the following antibodies: anti-CD3 (BV510, available from Biolegend), anti-CD4 (APC-R700, available from BD Biosciences), and anti-CD8 (BUV 395, available from BD Biosciences). T cells function has been assessed with antibodies anti-IFN-γ (BUV 737, available from BD), IL-10 (BV711, available from BD Biosciences), and anti-CD107a (PECy7, available from Biolegend),). PBMCs have been been further stained with anti-PD1 (PE, available from eBiosciences), anti-TIM-3 (FITC, available from eBiosciences), anti-FoxP3 (BV 421, available from Biolegend), anti-CD134 (BV 650, available from BD Biosciences), anti-CD25 (BV 785, available from Biolegend), anti-CD44 (PerCP-Vio700, available from Miltenyi), and anti CD38 (PE-Cy5.5, available from Novus).

NK cells characterization method: a) day 1 requires the preparation of PBMCs and HCC antigens, and OPN aptamers and their addition to design wells of the plates. Day 1 NK functional assay: CD107a antibodies are added to all wells except the designed K562 wells. Then IL12/IL-18 or PMA/Inomycin or any other stimulation are added to the designed wells and left for 2 hours at 37°C, after protein inhibitor cocktail is added to only IL-12/IL-18 and PMA/Inomycin stimulated wells and the plate is incubated at 37 °C overnight**.** *b)* day 2 provides NK degranulation assay: CD107a antibodies are added only to wells where K562 will be added, after K562 cells are added to the designed wells. Aptamers are replenished in the wells where K562 cells are added, then plates are left for 2 hours at 37°, after protein inhibitor cocktail is added and then plates are left 4 hours at 37°**.** Subsequently PBMCS are stained with intracellular or surface antibodies and then analyzed with FACS .

T cell characterization method: a) day 1: requires preparation of PBMCs, aptamers and antigens. Addiction of each antigen, aptamer and PBMCs to plates following the established T cells plate layout. After IL-2 is added to each well and the plates are incubated at 37 °C. for 48 hours. b) Day 3: requires addiction of CD4 and CD107a to wells, then addiction of PMA/Inomycin to designed wells and incubation for 2 hours at 37°C. After protein inhibitorcocktail is added to each well and plates are incubated overnight at 37 °C. c) day 4 is the T cells staining day: PBMCs are stained with cells surface and intracellular antibodies and then analyzed with FACS.

Statistic

A comparison between more than two groups is performed with Kruskal-Wallis test. If test results positive ($p<0.05$), it is made a comparison analysis between couple of groups using Mann-Whitney test (non parametric samples). Mann-Whitney test is used also for comparison between two indipendent groups. For paired samples comparison, it is used Wilcoxon signed

rank test. Statistical significance accepted is a p value<0.05. Statistical analyses are performed with the MedCalc™ software (vers. 15.6 MedCalc Software, Mariakerke, Belgium) and GraphPad Prism 5.01.

Results

OPN plasma measurement

OPN has been analyzed in the plasma of patients with CHB, with HCC-CHB, and of healthy controls. 5 groups of patients have been analyzed: 1) healthy controls (n=15), 2) patients with CHB and different stages of fibrosis not in treatment for HBV (CHB not treated, n=14), 3) patients with CHB and different stages of fibrosis and in treatment for HBV (CHB treated, n=14), 4) patients with CHB in treatment for HBV who develops HCC (HCC-CHB, n=11), 5) patients with chronic hepatitis not viral related (alcholic or metabolic) who develop HCC (HCC-non viral (n=15).

OPN levels are significantly higher in CHB group compared to the healthy controls (p=0.0004). In patients with CHB treated, levels of OPN are reduced significantly (p=0.0026).

In the group of patients with HCC-CHB, the levels of OPN are higher than patients with CHB–treated (p=0.0016). The group of patients with HCC non-viral has a higher levels of OPN compared with patients with CHB and with patients with HCC-CHB (HCC-non viral vs. HCC- CHB, p=0.0063).

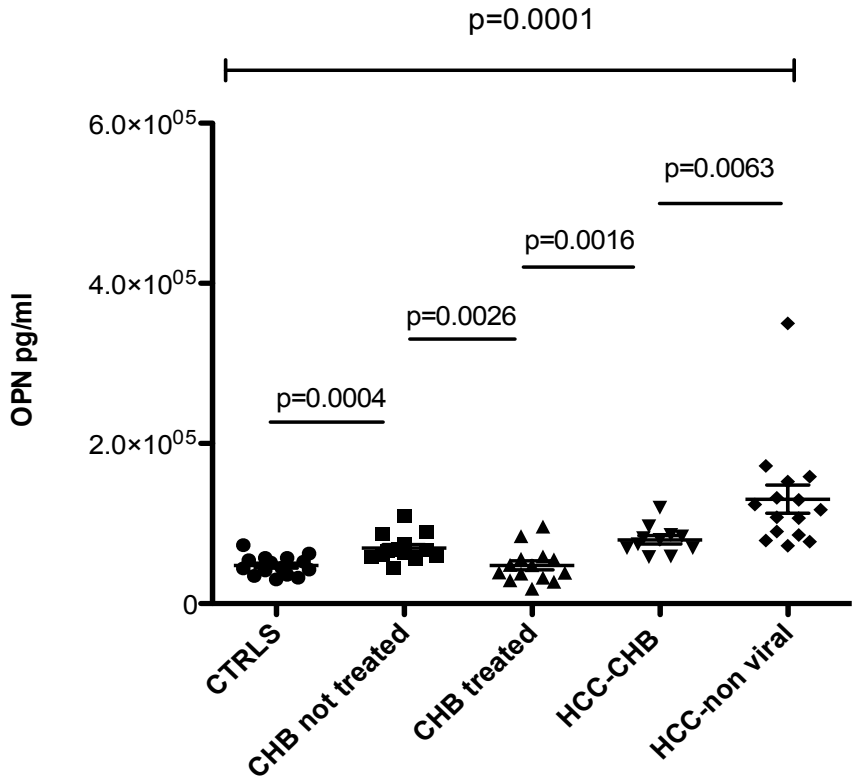


Figure 1. OPN levels (measured with ELISA) in healthy controls, patients with CHB and patients with HCC. *CTRLs: healthy controls.*

IFN-γ and Granzyme B analysis with ELISPOT

IFN-γ and Granzyme B have been analyzed with ELISPOT assay on PBMCs of patients with HCC incubated with HBV antigens or HCC-antigens and with or without anti-PD-1. ELISPOT data of patients with HCC-CHB (n=11) and patients with HCC-non viral (n=8) have been analyzed. Data from ELISPOT analysis in HCC-CHB incubated with HBV antigens have not showed significant different levels of IFN-γ and Granzyme B when PBMCs have been incubated with anti-PD-1 (Figure 2).

ELISPOT analysis of IFN-γ and Granzyme B on PBMCs of patients with HCC and incubated with HCC-antigens with or without anti-PD1 is reported in figure 3 and figure 4.

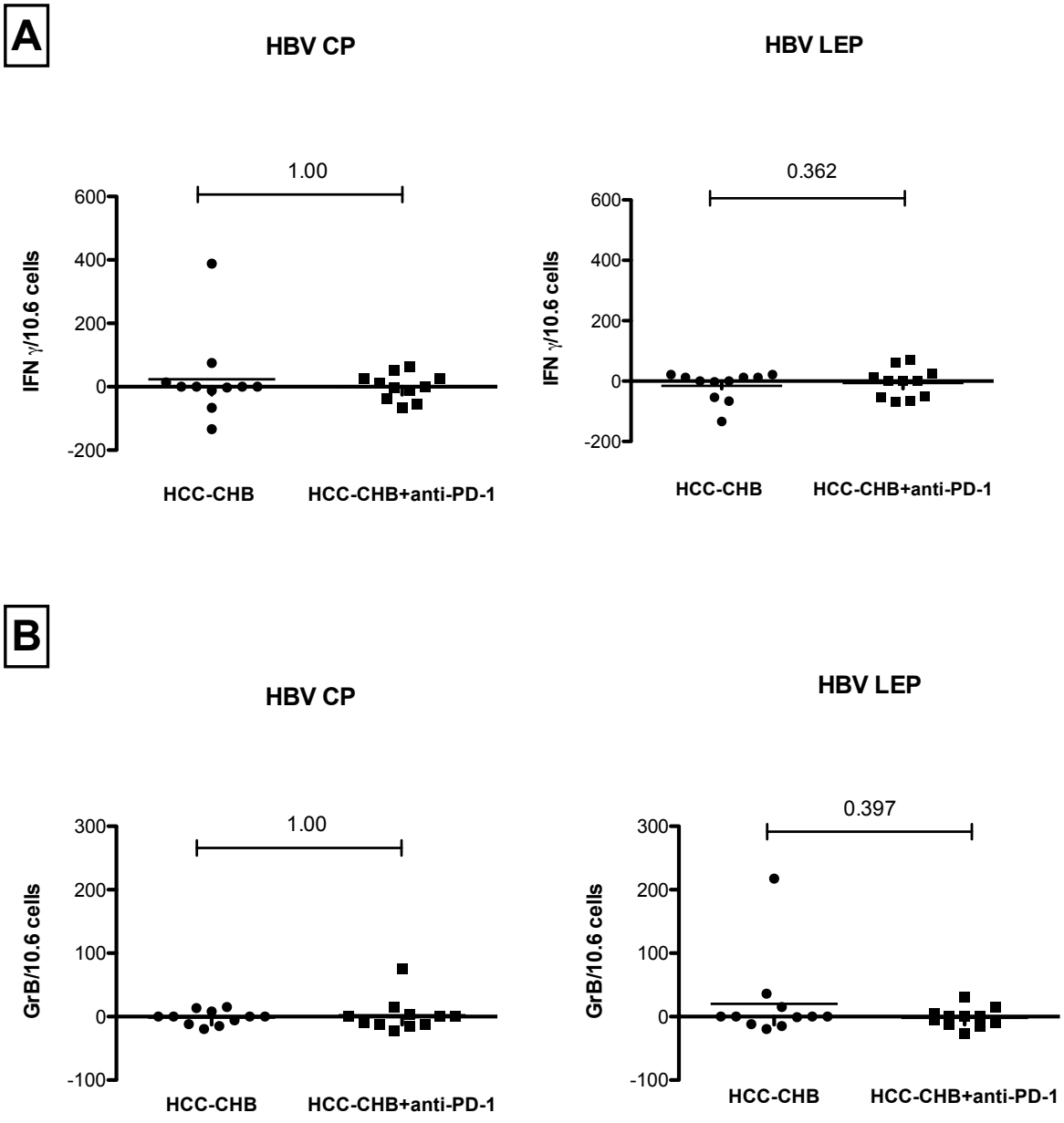


Figure 2: ***IFN-γ and Granzyme B analysis with ELISPOT on PBMCs of HCC-CHB patients incubated with HBV antigens:*** ***A)*** *IFN-γ levels on PBMcs of patients with HCC-CHB incubated with HBV antigens (HBV CP or HBV LEP) with or without anti-PD-1.* ***B)*** *Granzyme B -γ levels on PBMcs of patients with HCC-CHB incubated with HBV antigens (HBV CP or HBV LEP) with or without anti-PD-1.*

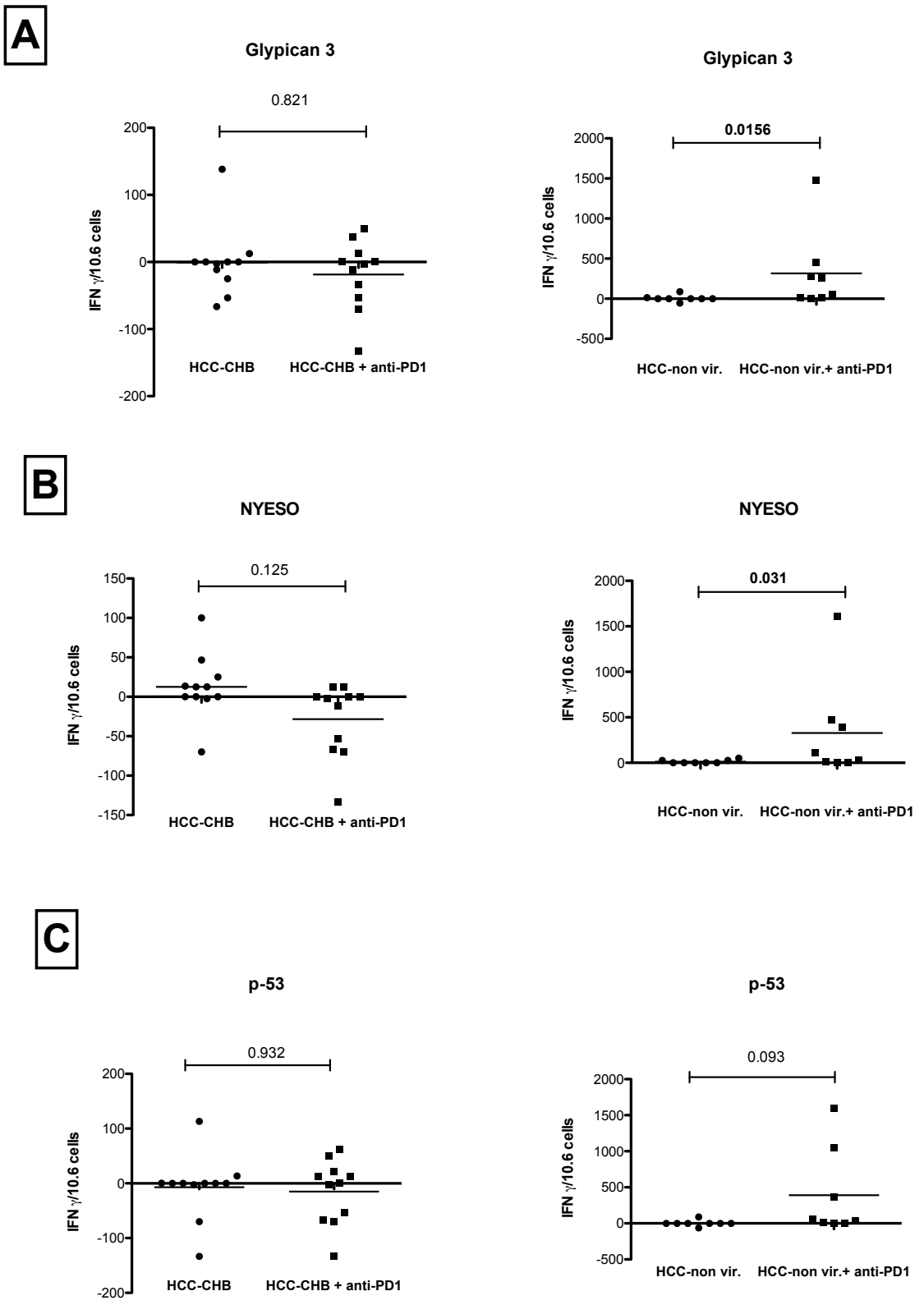


Figure 3: ***IFN-γ analysis with ELISPOT on PBMCs of patients with HCC-CHB and HCC-non viral (HCC-non vir.) incubated with HCC antigens (glypican 3, NYESO1, p53):*** ***A)*** *IFN-γ levels on PBMcs (HCC-CHB or HCC-non viral) incubated with glypican 3 with or without anti-PD1* ***B)*** *IFN-γ*

levels on PBMcs (HCC-CHB or HCC-non viral) incubated with NYESO1 with or without anti-PD1. ***C)*** *IFN-γ levels on PBMcs (HCC-CHB or HCC-non viral) incubated with p-53 with or without anti-PD1.*

In HCC-non viral group incubated with Glypican 3 and NYESO1, IFN-γ is significant increased following PD1 blockade (p=0.0156 and p=0.031 respectively) (figure 3 B and 3C).

Also Granzyme B is significant increased following PD1 blockade in HCC-non viral group incubated with p53 (p=0.0078) (figure 4 C).

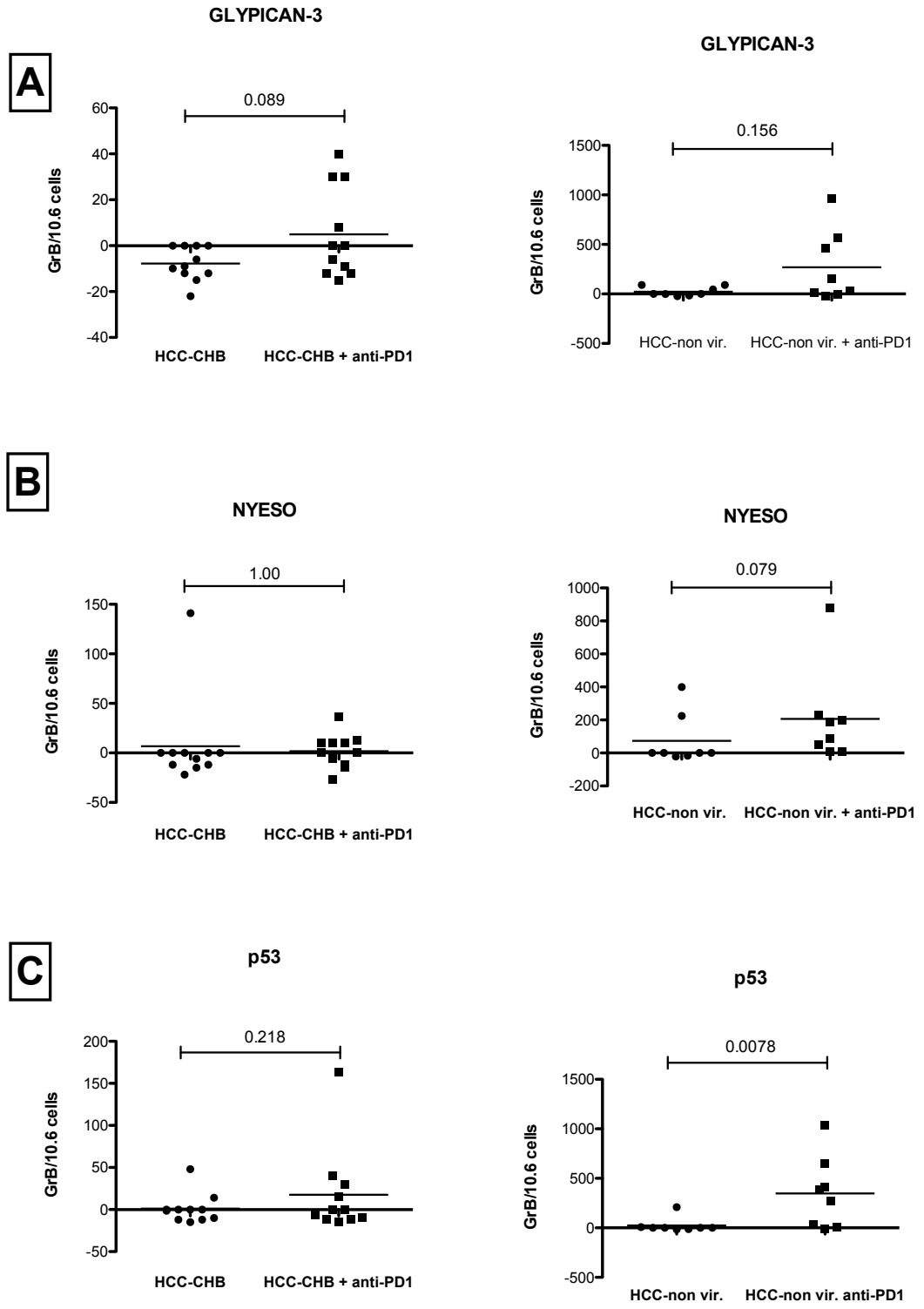


Figure 4: ***Granzyme B analysis with ELISPOT on PBMCs of patients with HCC-CHB and HCC-non viral (HCC-non vir.) incubated with HCC antigens (glypican 3, NYESO1, p53):*** *A) Granzyme B levels on PBMcs (HCC-CHB or HCC-non viral) incubated with glypican 3 with or without anti-*

PD1 ***B)*** *Granzyme B levels on PBMcs (HCC-CHB or HCC-non viral) incubated with NYESO1 with or without anti-PD1.* ***C)*** *Granzyme B levels on PBMcs (HCC-CHB or HCC- non viral) incubated with p-53 with or without anti-PD1.*

OPN analysis on supernatant of ELISPOT assay

Supernatant of ELISPOT for IFN-γ and Granzyme B has been used for the analysis of OPN with ELISA. Supernatant from PBMCs incubated with HBV antigens or with HCC-antigens (p-53, glypican 3 and NY-ESO1) and with or without anti-PD-1 has been analyzed in three group of patients: healthy controls (CTRLs, n=6), patients with HCC-CHB (n=11) and patients with HCC-non viral (n=8). In PBMCs of patients with HCC-CHB incubated with HBV-CP, anti-PD-1 has significantly modified the expression of OPN (p=0.0029) (figure 5).

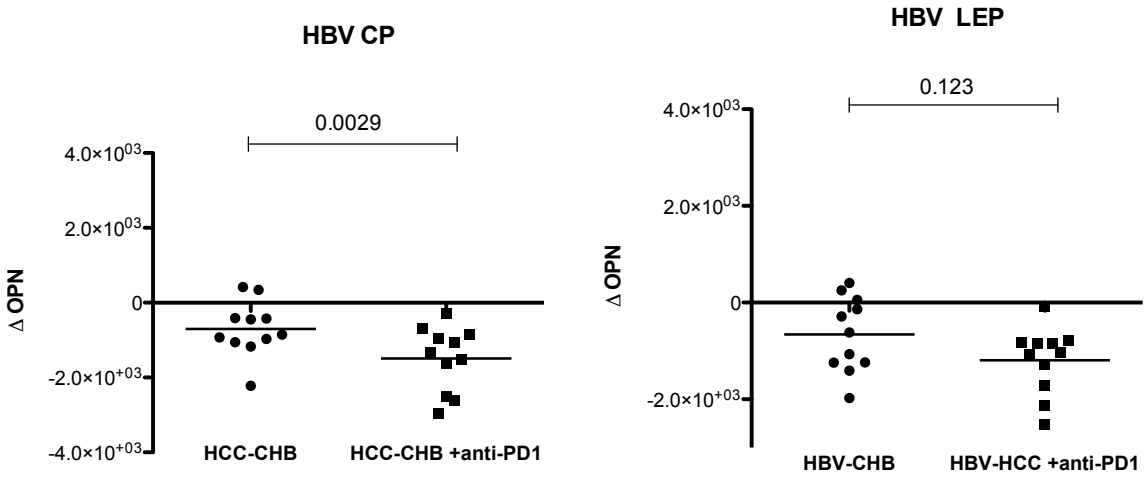


Figure 5: OPN analysis on supernatant of ELISPOT on PBMCs of HCC-CHB incubated with HBV antigens (HBV CP pr HBV LEP) with or without anti-PD1. *OPN is expressed as Delta OPN: to each value of OPN is subtracted the value of OPN of the healthy controls with only medium (controls not incubated with HCC-antigens or anti-PD-1).*

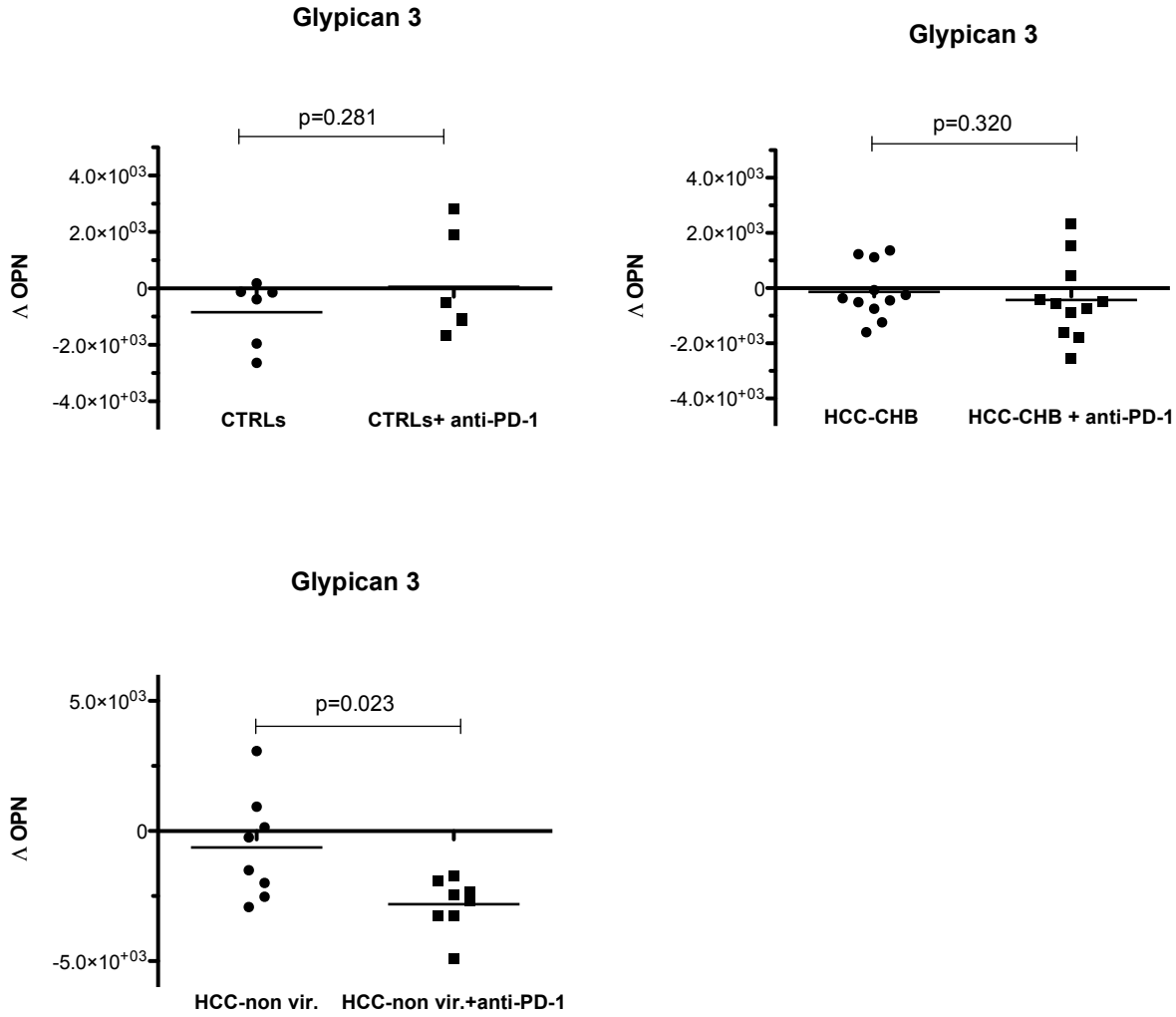


Figure 6: OPN analysis on supernatant of ELISPOT on PBMCs of HCC and healthy controls incubated with glypican 3 with or without anti-PD1. *OPN is expressed as Delta OPN: to each value of OPN is subtracted the value of OPN of the healthy controls with only medium (controls not incubated with HCC-antigens or anti-PD-1). CTRLS: healthy controls. HCC-non vir.: HCC non viral.*

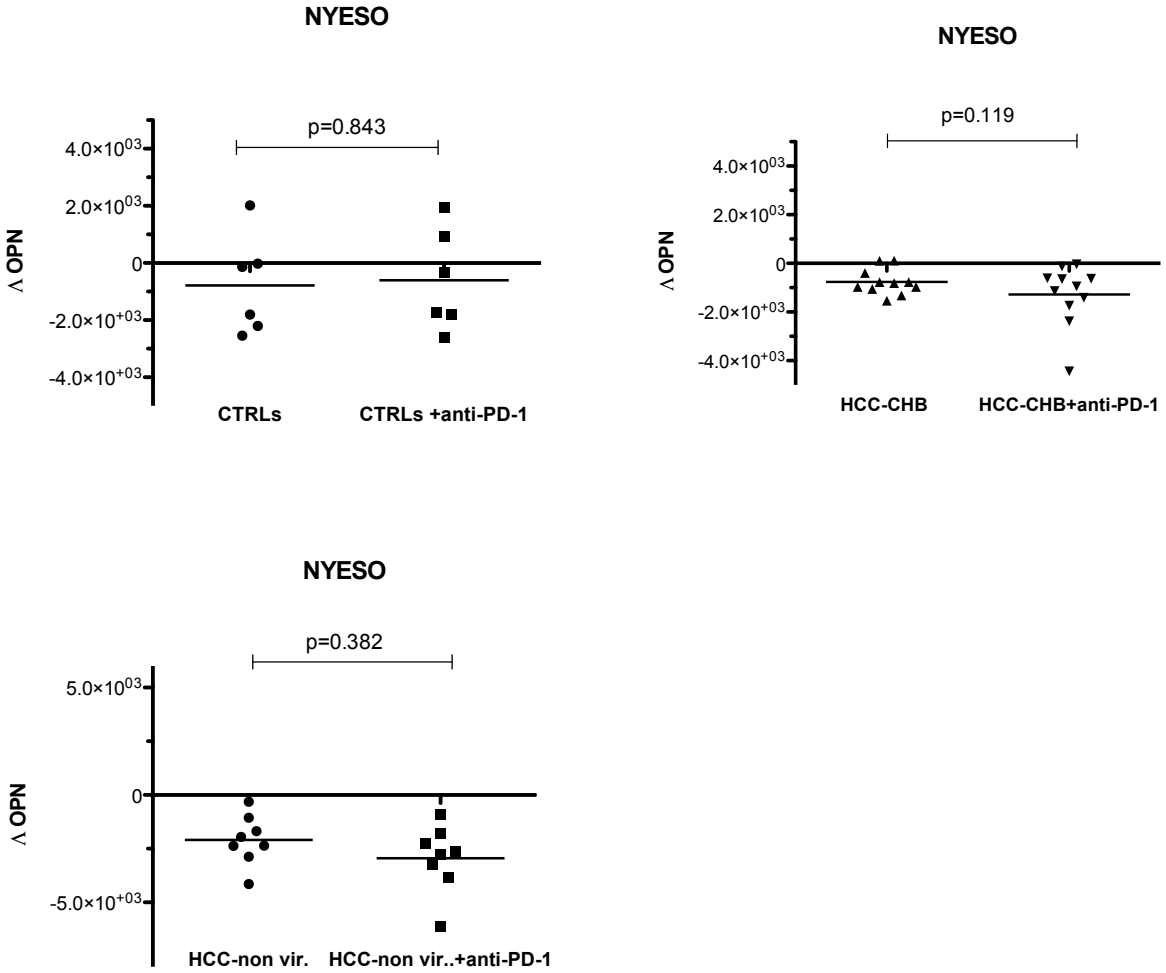


Figure 7: OPN analysis on supernatant of ELISPOT on PBMCs of HCC and healthy controls incubated with NYESO1 with or without anti-PD1. *OPN is expressed as Delta OPN: to each value of OPN is subtracted the value of OPN of the healthy controls with only medium (controls not incubated with HCC-antigens or anti-PD-1). CTRLS:healthy controls. HCC-non vir.: HCC non viral.*

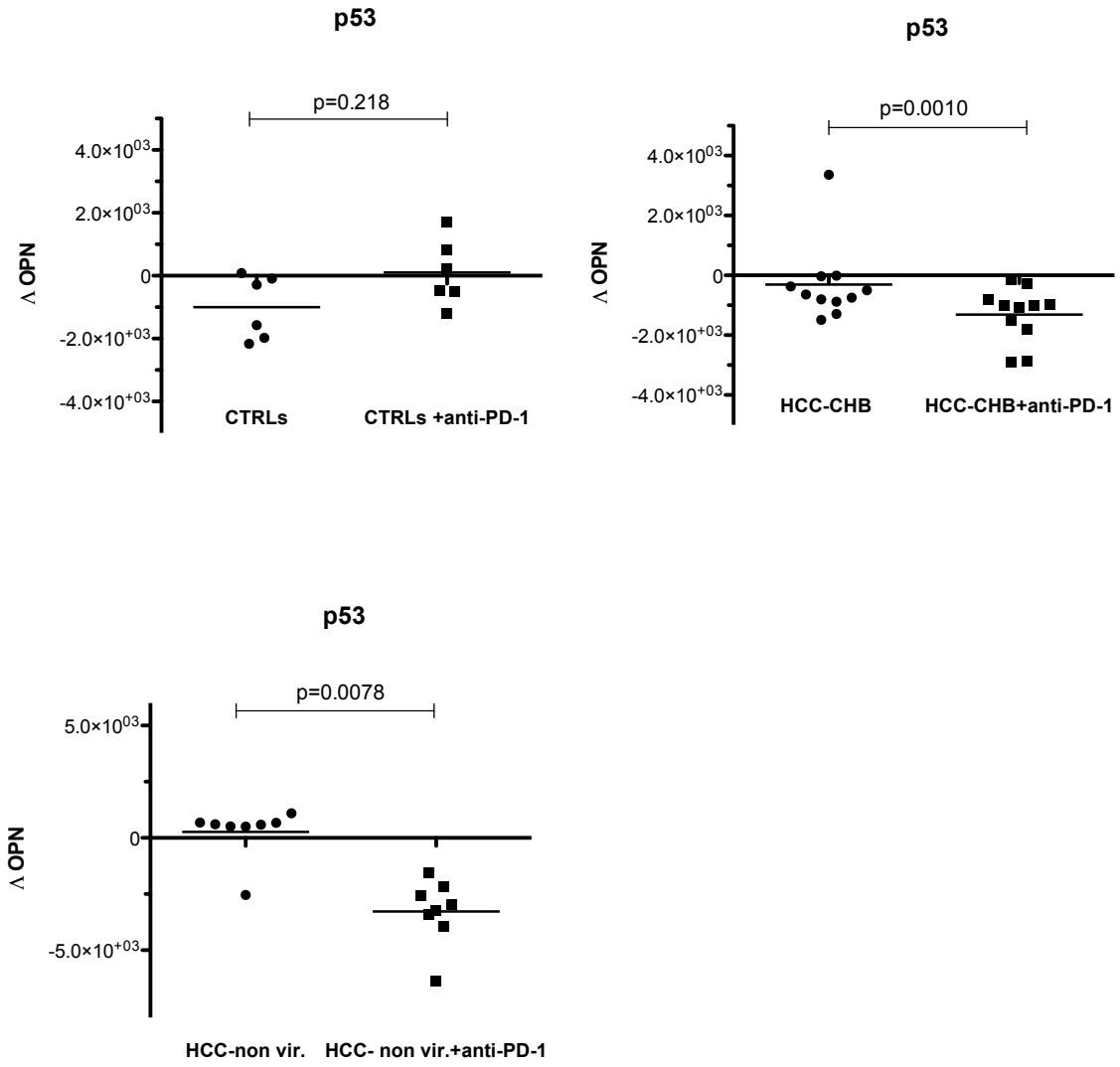


Figure 8: OPN analysis on supernatant of ELISPOT on PBMCs of HCC and healthy controls incubated with p53 with or without anti-PD1. *OPN is expressed as Delta OPN: to each value of OPN is subtracted the value of OPN of the healthy controls with only medium (controls not incubated with HCC-antigens or anti-PD-1). CTRLS: healthy controls. HCC-non vir.:HCC non viral*

The expression of OPN is significantly lower in PBMCs of HCC-non viral incubated with anti-PD1 and glypican 3 (figure 6). The expression of OPN is not modified in PBMCs of HCC-CHB and HCC-nonviral incubated with anti-PD1 and NYESO (figure 7). OPN is significantly lower in PBMCs of HCC-CHB and HCC-non viral incubated with anti-PD1 and p53. (figure 8).

Preliminary NK cells characterization using flow cytometry

A preliminary analysis of NK cells phenotype has been done for the surface activating receptor NKG2D and for NK function through evaluation of intracellular IFN-γ expression .

PBMCs from HCC-CHB (n=4) and HCC-non viral (n=4) have been cultured with IL-12 and 18 or medium and plus rOPN or OPN-APT and then stained using anti-NKG2D or anti-IFN-γ and analyzed with flow cytometry (Figure 9 and Figure 10).

In HCC-CHB patients, percentage (% frequence of parent) of NKG2D positive NK cells (NKG2D+NK cells) was not significant different in PBMCs cultered with r-OPN or OPN-APT. In HCC-CHB patients, percentage of NKG2D+NK cells was not different when PBMCs have been cultered with r-OPN or OPN APT (p=0.87 and p=1.0 respectively). While in HCC-non viral there was a trend to reduction of NKG2D+ NK cells in PBMCs cultered with r-OPN, without a statistical significance (p=0.25) (Figure 9).

The evaluation of IFN-γ expression in NK cells in patients with HCC-CHB has showed an increasing trend of NK cells positive for IFNγ (IFNγ+NK cells) in PBMCs cultered with r-OPN and a tendency to reduction of IFNγ+NK cells in PBMCs cultered with OPN-APT, but without statistical significance. In HCC-non viral there was not difference in IFNγ+NK cells

in PBMCs cultured with r-OPN. While there was a trend to reduction of IFNγ+NK cells in PBMCs cultured with OPN-APT, without statistical significance (Figure 10).

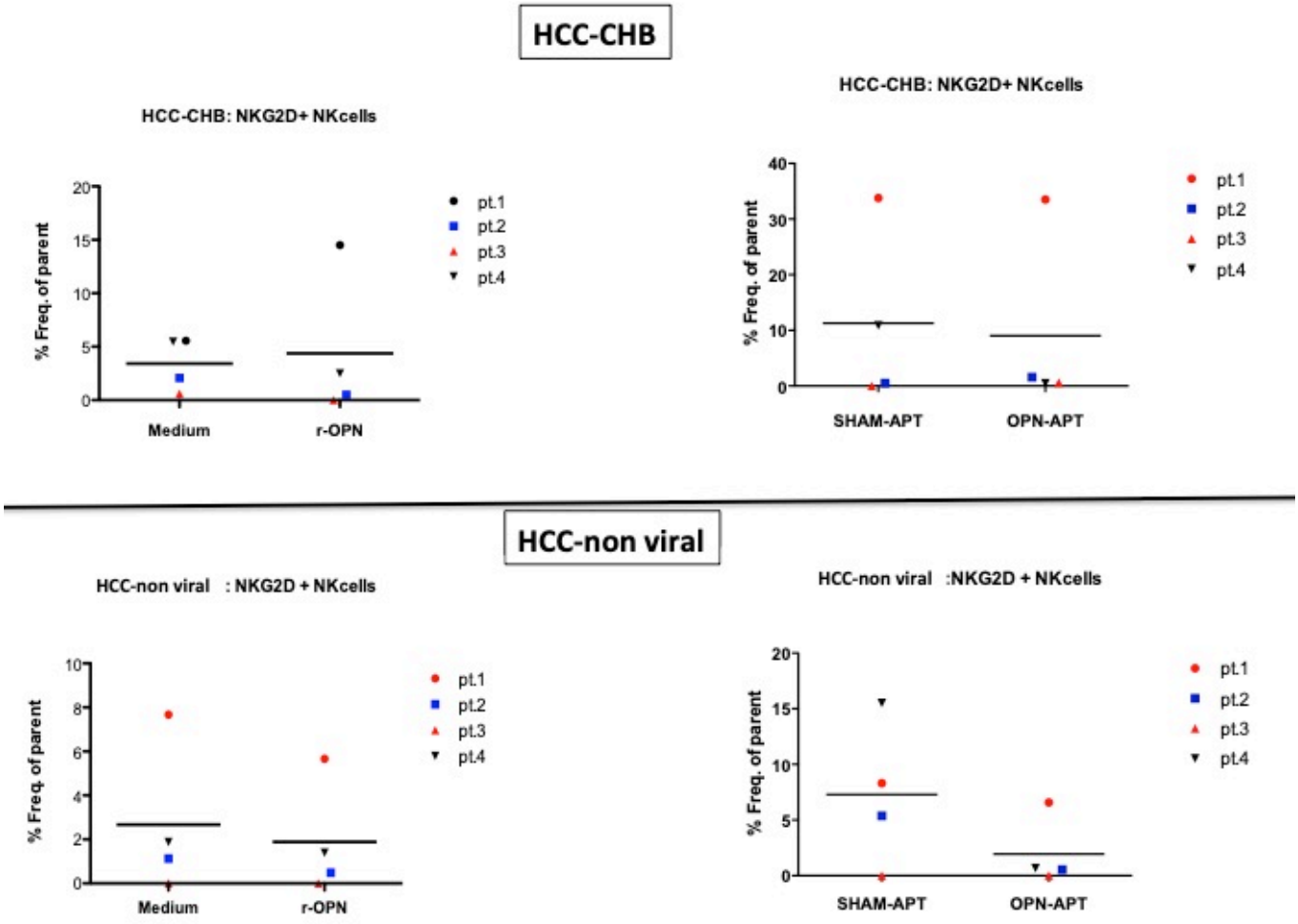


Figure 9. ***NK cells characterization for NKG2D using flow cytometry:*** *PBMCs from HCC-CHB and HCC-non viral have been cultured with medium and with rOPN or OPN-APT and then stained using anti- NKG2D and analyzed with flow cytometry. SHAM APT: negative control. NKG2D+NK cells (NK cells positive for NKG2D).*

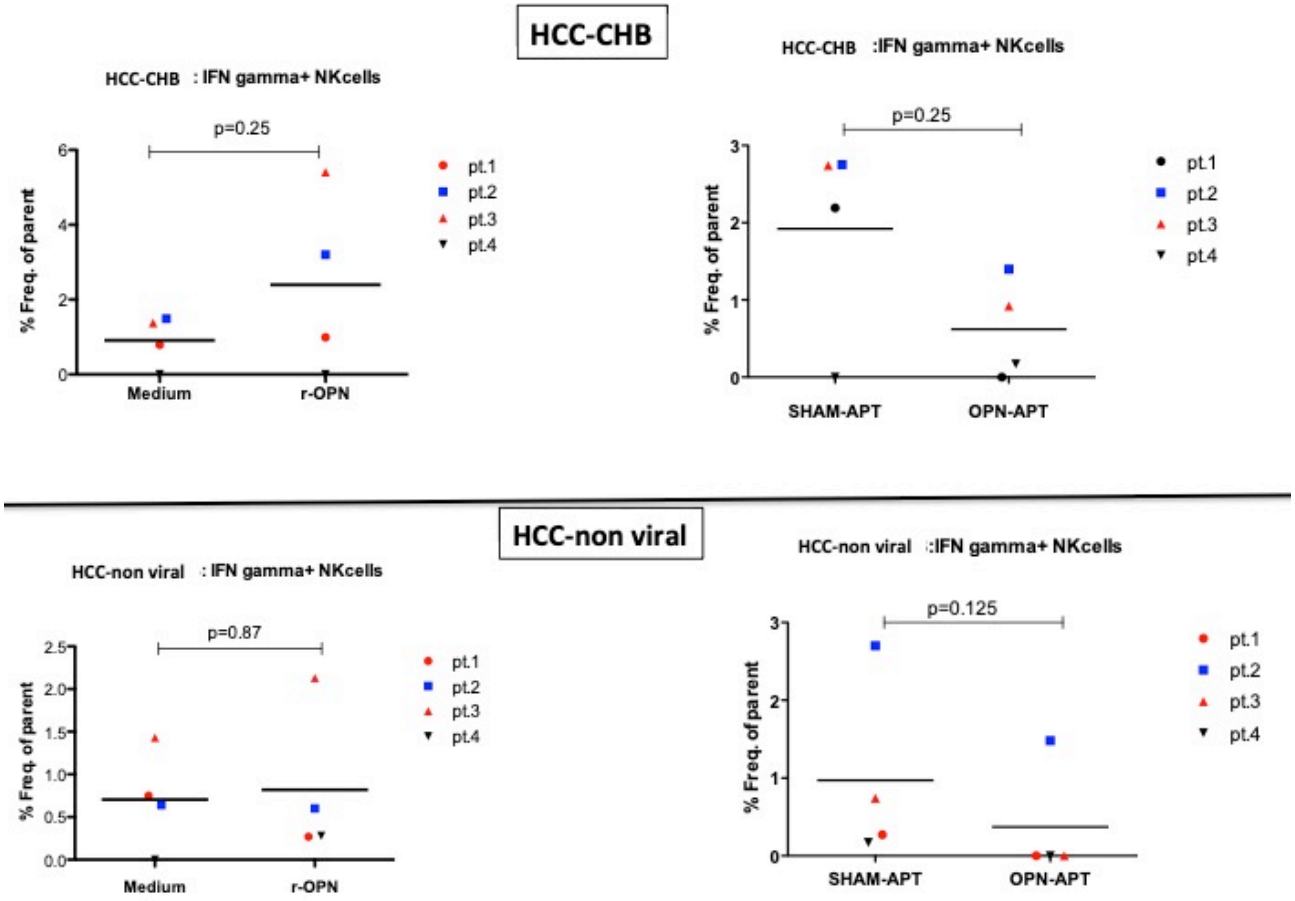


Figure 10. ***NK cells characterization for IFNγ using flow cytometry:*** *PBMCs from HCC-CHB and HCC-non viral have been cultured with IL-12 and IL-18 and with rOPN or OPN-APT and then stained for with anti-IFN**γ** and analyzed with flow cytometry . SHAM APT:negative control. IFNγ +NK cells (NK cells positive for IFNγ).*

Discussion and Conclusions

OPN data on plasma have confirmed the role of OPN in HBV replication[152-156], indeed levels of OPN in CHB patients are significant higher then healthy controls. Furthermore, the levels of OPN go down when CHB patients are in treatment for HBV, confirming the close correlation between OPN expression and HBV replication.

Moreover OPN expression in HCC-CHB patients in treatment for HBV is significantly over-expressed compared to CHB treated patients. Confirming the role of OPN in the development of HCC-HBV and as early biomarkers of HCC.

Data on OPN, in patients with HCC not related to chronic hepatitis B suggest the role of OPN in HCC development and progression also in patients with a different ethiology of HCC. In these patients the expression of OPN is higher then controls and CHB patients, and is significantly higher then HCC non viral. This should be related to the different phisiopatolgy of HCC in HCC-non viral patients and to the absence of the interfering role of HBV that is known to be closely related to OPN espression[152-156].

In the ELISPOT analysis, following PD1 blockade, the number of IFN-γ producing Glypican 3+T cells was augmented (p=0.015) while OPN production was reduced (p=0.023) in HCC-non viral but not HCC-CHB or Healthy controls. We also observed an induction in the frequency of Graenzyme 3 producing p53+T cells and a reduction in OPN in both HCC- non viral (p=0.0078) and HCC-CHB (p=0.0010).

Limited and preliminary data regarding the analysis of effect of r-OPN and OPN-APT on NK cells phenotype and don't allow to achieve conclusions.

In conclusion, this preliminary data confirm the strong role of OPN in the development of HCC and its potential role as early biomarker of HCC both related to CHB that related to other ethiologies.

Therefore this study reports the immunoregulatory properties of OPN showing its novel role in restoration of anti-HCC T-cell responses during anti-PD1 blockade. These results suggest an immunomodulatory role for Osteopontin in HCC and require its further investigation as an immunotherapeutic target for HCC.

Conclusions

The aim of the studies presented in this thesis was to define HCC incidence in HCV patients treated with DAAs, and investigate the role of novel targets and circulating biomarker in HCC related to chronic viral hepatitis. The study 1 obtained in a large, prospective, population study, shows that in patients with advanced hepatitis C receiving DAAs, the risk of "de novo" hepatocarcinoma during the first year is not higher (HCC incidence was 0.46%) , and might be lower, than that of untreated patients, and further declines thereafter. These data need to be confirmed in the same population with a longer follow-up.

In the study 2 the expression of miR 28-5p is altered in patients treated with DAAs who develop HCC compared with patients without HCC, suggesting its role in the pathophysiology of HCC and its potential prognostic role in HCC related to HCV. Further investigations are requested to clarify the phisyopatolgical role of miR 28-5p in this contest. The main aim of study 3 is to better define the role of OPN as novel target and circulating biomarker in patients with HCC. In the group of patients with HCC HCC-CHB and with HCC non-viral related, the levels of OPN are higher than in patients with chronic hepatitis without HCC ($p=0.0016$ and $p=0.0063$ respectively). Moreover this study reports the immunoregulatory properties of OPN showing its novel role in restoration of anti-HCC T-cell responses during anti-PD1 blockade. These results confirm the role of OPN as biomarker and suggest an immunomodulatory role for Osteopontin in HCC that require further investigation.

Bibliography:

1. Laura Kulak, Hashem B. El-Serag. Epidemiology and Management of Hepatocellular Carcinoma. Gastroenterology 2019;156:477–491.
2. White DL, Thrift AP, Kanwal F, et al. Incidence of hepatocellular carcinoma in all 50 United States, from 2000 through 2012. Gastroenterology 2017;152:812–820.
3. IARC. Fact sheets by Population-Globocan-IARC.http://globocan.iarc.fr/Pages/fact _sheets_population.aspx (accessed Dec 18, 2016).
4. El-Serag HB. Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology 2012; 142: 1264–73.e1.
5. Morgan TR, Mandayam S, Jamal MM. Alcohol and hepatocellular carcinoma. Gastroenterology 2004; 127: S87–96.
6. Asia, S., Asia, S., & Hdi, H. (2018). Liver Cancer Global WHO Report. 876, 2018–2019. Retrieved from http://gco.iarc.fr/today.
7. Armstrong GL, Wasley A, Simard EP, et al. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. Ann Intern Med 2006; 144:705–714.
8. Kallwitz ER, Daviglus ML, Allison MA, et al. Prevalence of suspected nonalcoholic fatty liver disease in Hispanic/ Latino individuals differs by heritage. Clin Gastroenterol Hepatol 2015;13:569–576.
9. Mittal S, Sada YH, El-Serag HB, et al. Temporal trends of nonalcoholic fatty liver disease–related hepatocellular carcinoma in the veteran affairs population. Clin Gas- troenterol Hepatol 2015;13:594–601.
10. Forner A, Reig M, Bruix J. Hepatocellular carcinoma. Lancet 2018; 391: 1301–14
11. Schafer DF, Sorrell MF. Hepatocellular carcinoma. Lancet 1999; 353: 1253–57.
12. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: a cancer journal for clinicians. 2018. CA Cancer J Clin. 2018; 68:394-424.

13. Prashanth Rawla, Tagore Sunkara, Pradhyumna Muralidharan, Jeffrey Pradeep Raj. Update in global trends and aetiology of hepatocellular carcinoma. Review. Contemp Oncol (Pozn) 2018; 22: 141–150.
14. Giannini EG, Marenco S, Bruzzone L, Savarino V, Fabio Farinati F, Del Poggio P, et al. Hepatocellular carcinoma in patients without cirrhosis in Italy. Digestive and Liver Disease 2013; 45:164–169.
15. Kim H, El-Serag HB. The Epidemiology of Hepatocellular Carcinoma in the USA. Current Gastroenterology Reports 2019; 21: 17.
16. Kanwal F, Kramer J, Asch SM, Chayanupatkul M, Cao Y, El-Serag HB. Risk of Hepatocellular Cancer in HCV Patients Treated With Direct-Acting Antiviral Agents. Gastroenterology 2017; **153**:996–1005 .
17. Ioannou GN, Green PK, Berry K. HCV eradication induced by direct-acting antiviral agents reduces the risk of hepatocellular carcinoma Journal of Hepatology 2017. [Epub ahead of print]
18. Sui-Weng Wong, Yi-Wen Ting and Wah-Kheong Chan . Epidemiology of non-alcoholic fatty liver disease-related hepatocellular carcinoma and its implications. journal of gastroenterology and hepatology 2018; 2: 235–241.
19. Sayiner M, Golabi P, Younossi ZM. Disease Burden of Hepatocellular Carcinoma: A Global Perspective. Digestive Diseases and Sciences 2019; 64:910–917 .
20. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004; 127:35-50.
21. Li JT, Liao ZX, Ping J, Xu D, Wang H. Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies. J Gastroenterol 2008; 43: 419-428.
22. Kirmaz C, Terzioglu E, Topalak O, Bayrak P, Yilmaz O, Ersoz G, Sebik F. Serum transforming growth factor-beta1(TGF-beta1) in patients with cirrhosis, chronic hepatitis B

and chronic hepatitis C. Eur Cytokine Netw 2004; 15: 112-116 .

23. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, Gores G. Hepatocellular carcinoma. Nat Rev Dis Primers 2016; 2: 16018.
24. Plentz RR, Caselitz M, Bleck JS, Gebel M, Flemming P, Kubicka S, Manns MP, Rudolph KL. Hepatocellular telomere shortening correlates with chromosomal instability and the development of human hepatoma. Hepatology 2004; 40: 80-86.
25. Girón-González JA, Martínez-Sierra C, Rodriguez-Ramos C, Macías MA, Rendón P, Díaz F, Fernández-Gutiérrez C, Martín- Herrera L. Implication of inflammation-related cytokines in the natural history of liver cirrhosis. Liver Int 2004; 24: 437-445 .
26. Byul A Jee, Ji-Hye Choi, Hyungjin Rhee, Sarah Yoon, So Mee Kwon, Ji Hae Nahm, Jeong Eun Yoo, Youngsic Jeon, Gi Hong Choi, Hyun Goo Woo, Young Nyun Park. Dynamics of genomic, epigenomic, and transcriptomic aberrations during stepwise hepatocarcinogenesis. Cancer research 2019. [Epub ahead of print].
27. Schulze K, Nault J-C, Villanueva A. Genetic profiling of hepatocellular carcinoma using next-generation sequencing. J Hepatol 2016; 65: 1031–42.
28. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860–7.
29. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39:1–10.
30. Atanasov et al. Immunologic cellular characteristics of the tumour microenvironment of hepatocellular carcinoma drive patient outcomes . World Journal of Surgical Oncology 2019; 17:97.
31. Wang JC, Livingstone AM. Cutting edge: CD4+ T cell help can be essential for primary CD8+ T cell responses in vivo. J Immunol 2003; **171**: 6339-6343
32. Makarova-Rusher OV, Medina-Echeverz J, Duffy AG, et al. The yin and yang of evasion and immune activation in HCC. J Hepatol 2015; 62: 1420–1429.

33. Hato T, Goyal L, Greten TF, et al. Immune checkpoint blockade in hepatocellular carcinoma: current progress and future directions. Hepatology 2014; 60: 1776–1782.
34. Cai L, Zhang Z, Zhou L, Wang H, Fu J, Zhang S, Shi M, Zhang H, Yang Y, Wu H, Tien P, Wang FS. Functional impairment in circulating and intrahepatic NK cells and relative mechanism in hepatocellular carcinoma patients. Clin Immunol 2008; 129: 428-437.
35. Sherman M, Furlan A, Marin D, et al. Surveillance for hepatocellular carcinoma. Best Pract Res Clin Gastroenterol 2014; 28: 783–793.
36. Singal A, Volk ML, Waljee A, et al. Meta-analysis: surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with cirrhosis. Aliment Pharmacol Ther 2009; 30: 37–47.
37. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology 2011; 53: 1020–1022.
38. Lok AS, Sterling RK, Everhart JE, et al. Des-γ-carboxy prothrombin and α-fetoprotein as biomarkers for the early detection of hepatocellular carcinoma. Gastroenterology 2010; 138: 493–502.
39. Zhang B, Yang B. Combined alpha fetoprotein testing and ultrasonography as a screening test for primary liver cancer. J Med Screen 1999; 6: 108–110.
40. Forner A, Vilana R, Ayuso C, et al. Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma. Hepatology 2008; 47: 97–104.
41. Sangiovanni A, Manini MA, Iavarone M, et al. The diagnostic and economic impact of contrast imaging technique in the diagnosis of small hepatocellular carcinoma in cirrhosis. Gut 2010; 59: 638–644.
42. Leoni S, Piscaglia F, Golfieri R, et al. The impact of vascular and nonvascular findings on the noninvasive diagnosis of small hepatocellular carcinoma based on the EASL and AASLD

criteria. Am J Gastroenterol 2010; 105: 599–609.

43. Khalili KT, Kim TK, Jang HJ, et al. Optimization of imaging diagnosis of 1-2 cm hepatocellular carcinoma: an analysis of diagnostic performance and resource utilization. J Hepatol 2011; 54: 723–728.
44. American College of Radiology. Liver Imaging Reporting and Data System version 2017. https://www.acr.org/~/media/ACR/Documents/PDF/QualitySafety/Resources/LIRADS/2017/LIRADS_2017 (accessed Nov 27, 2017).
45. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2018; 69 :182–236.
46. Forner A, Díaz-González A, Liccioni A, Vilana R. Prognosis prediction and staging. Best Pract Res Clin Gastroenterol 2014; 28**:** 855–865.
47. Bruix J, Reig M, Sherman M. Evidence-based diagnosis, staging, and treatment of patients with hepatocellular carcinoma. Gastroenterology 2016; 150: 835–853.
48. He X, Wang Y, Zhang W, Li H, Luo R, Zhou Y, Liao CL, Huang H, Lv X, Xie Z, He M. Screening differential expression of serum proteins in AFP-negative HBV-related hepatocellular carcinoma using iTRAQ -MALDI-MS/MS. Neoplasma 2014; 61: 17-26.
49. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, Taketa K, Endo Y, Nagataki S. Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med 1993; 328: 1802-1806.
50. Huang TS, Shyu YC, Turner R, Chen HY, Chen PJ. Diagnostic performance of alpha-fetoprotein, lens culinaris agglutinin-reactive alpha-fetoprotein, des-gamma carboxyprothrombin, and glypican-3 for the detection of hepatocellular carcinoma: a systematic review and meta-analysis protocol. Syst Rev 2013; 2: 37.
51. Witjes CD, van Aalten SM, Steyerberg EW, Borsboom GJ, de Man RA, Verhoef C, Ijzermans JN. Recently introduced biomarkers for screening of hepatocellular carcinoma: a

systematic review and meta-analysis. Hepatol Int 2013; 7: 59-64.

52. Zhang J, Shao C, Zhou Q, Zhu Y, Zhu J, Tu C. Diagnostic accuracy of serum squamous cell carcinoma antigen and squamous cell carcinoma antigen-immunoglobulin M for hepatocellular carcinoma: A meta-analysis. Mol Clin Oncol 2015; 3: 1165-1171.
53. Rachner TD, Thiele S, Göbel A, Browne A, Fuessel S, Erdmann K, Wirth MP, Fröhner M, Todenhöfer T, Muders MH, Kieslinger M, Rauner M, Hofbauer LC. High serum levels of Dickkopf-1 are associated with a poor prognosis in prostate cancer patients. BMC Cancer 2014; 14: 649.
54. Sodek, J., Ganss, B., and McKee. Osteopontin. Crit. Rev. Oral Biol. Med. 200; 11: 279–303.
55. Koh, A., daSilva, A. P. B., Bansal, A. K., Bansal, M., Sun, C., Lee, H., Glogauer, M., Sodek, and Zohar, R. Role of osteopontin in neutrophil function. Immunology 2007 [Epub ahead of print].
56. Denhardt,D.T.,Noda,M.,O'Regan,A.W.,andPavlin,D. Osteopontin as a means to cope with environmental insults: Regulation of inflammation, tissue remodeling, and cell survival. J. Clin. Invest 2001: 107: 1055–1061
57. Luo, J. H., Ren, B., Keryanov, S., Tseng, G. C., Rao, U. N., Monga, S. P., Strom, S., Demetris, A. J., Nalesnik, M., Yu, Y. P., et al.. Transcriptomic and genomic analysis of human hepatocellular carcinomas and hepatoblastomas. Hepatology 2006: 44: 1012–1024.
58. Kim J, Ki SS, Lee SD, et al. Elevated plasma osteopontin levels in patients with hepatocellular carcinoma. Am J Gastroenterol 2006;101: 2051-2069.
59. Abu El Makarem MA, Abdel-Aleem A, Ali A, et al. Diagnostic significance of plasma osteopontin in hepatitis C virus-related hepatocellular carcinoma. Ann Hepatol 2011;10:296-305.
60. Shang S, Plymoth A, Ge S, et al. Identification of osteopontin as a novel marker for early hepatocellular carcinoma. Hepatology 2012;55:483-90.

61. Shevde LA, Das S, Clark DW, Samant RS. Osteopontin: an effector and an effect of tumor metastasis. Curr Mol Med 2010;10:71-81.

62. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009;10:704–714.

63. Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. Nat Rev Mol Cell Biol 2010;11:252–263.

64. ArreseM, EguchiA, FeldsteinAE. CirculatingmicroRNAs:emergingbiomarkers of liver disease. Semin Liver Dis 2015;35:43–54.

65. Giordano S, Columbano A. MicroRNAs: new tools for diagnosis, prognosis, and therapy in hepatocellular carcinoma? . Hepatology 2013;57:840–857.

66. Chen X, Ba Y, Ma L, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008;18:997–1006.

67. Cheng Peng, Yanshuo Ye, Zhanpeng Wang, Lianyue Guan, Suriguga Bao, Bo Li, Wei Li Circulating microRNAs for the diagnosis of hepatocellular carcinoma. C. Peng et al. / Digestive and Liver Disease 2019;59: 621–631.

68. Roayaie S, Jibara G, Tabrizian P, et al. The role of hepatic resection in the treatment of hepatocellular cancer. Hepatology 2015; 62**:** 440–451.

69. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. Hepatology 2003; 37: 429–42.

70. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008; 359: 378–90

71. Kudo M, Finn RS, Qin S, et al. Lenvatinib versus sorafenib in first- line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet 2018 (Epub ahead of print).

72. Bruix J, Qin S, Merle P, et al. Regorafenib for patients with hepatocellular carcinoma who

progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet 2017; 389: 56–66.

73. Prieto J, Melero I, Sangro B. Immunological landscape and immunotherapy of hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol 2015; 12: 681–700.
74. El-Khoueiry AB, Sangro B, Yau T, et al. Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase ½ dose escalation and expansion trial. Lancet 2017; 389: 2492–502.
75. Lu L-C, Cheng A-L, Poon RTP. Recent advances in the prevention of hepatocellular carcinoma recurrence. Semin Liver Dis 2014; 34: 427–434.
76. Mazzaferro V, Regalia E, Doci R, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 1996; 334: 693–99.
77. Sapisochin G, Bruix J. Liver transplantation for hepatocellular carcinoma: outcomes and novel surgical approaches. Nat Rev Gastroenterol Hepatol 2017; 14: 203–221.
78. Cucchetti A, Piscaglia F, Cescon M, et al. Cost-effectiveness of hepatic resection versus percutaneous radiofrequency ablation for early hepatocellular carcinoma. J Hepatol 2013; 59: 300–307.
79. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: chemoembolization improves survival. Hepatology 2003; 37: 429–442.
80. Burrel M, Reig M, Forner A, et al. Survival of patients with hepatocellular carcinoma treated by transarterial chemoembolisation (TACE) using Drug Eluting Beads. Implications for clinical practice and trial design. J Hepatol 2012; 56: 1330–1335.
81. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008; 359: 378–390.
82. Bruix J, Qin S, Merle P, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-

controlled, phase 3 trial. Lancet 2017; 389**:** 56–66.

83. Manns MP, Buti M, Gane E, Pawlotsky JM, Razavi H, Terrault N, et al. Hepatitis C virus infection. Nat Rev Dis Primers. 2017; 3:17006. Review.
84. Pawlotsky JM. New Hepatitis C Therapies: The Toolbox, Strategies, and Challenges. Gastroenterology 2014; 146: 1176–1192.
85. Feld JJ, Jacobson IM, Hézode C, Asselah T, Ruane PJ, Gruener N, et al. For the ASTRAL-1 Investigators Sofosbuvir and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. New England Journal of Medicine 2015; 373: 2599-2607.
86. Jacobson IM, Lawitz E, Kwo PY, Hézode C, Peng CY, Howe AYM, et al. Safety and efficacy of Elbasvir/Grazoprevir in patients with Hepatitis C, Virus infection and compensated cirrhosis: an Integrated Analysis. Gastroenterology 2017; 152:1372-138.
87. Welzel TM, Petersen J, Herzer K, Ferenci P, Gschwantler M, Wedemeyer H, et al. Daclatasvir plus sofosbuvir, with or without ribavirin, achieved high sustained virological response rates in patients with HCV infection and advanced liver disease in a real-world cohort. Gut 2016; 65:1861–1870.
88. Foster GR, Irving WL, Cheung MCM, Walker AJ, Hudson BE, Verma S, et al. On behalf of HCV Research, UK. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. Journal of Hepatology 2016; 64: 1224–1231.
89. Curry MP, O'Leary JG, Bzowej N, Muir AJ, Korenblat KM, Fenkel JM et al. for the ASTRAL-4 Investigators. Sofosbuvir and Velpatasvir for HCV in Patients with Decompensated Cirrhosis**.** N Engl J Med 2015; *373***:**2618-2628.
90. Charlton M, Everson G T, Flamm S L, Kumar P, Landis C, Brown RS Jr, et al. For the SOLAR-1 Investigators. Ledipasvir and sofosbuvir plus ribavirin for treatment of HCV infection in patients with advanced liver disease. Gastroenterology 2015; 149: 649–659 .
91. Belli LS, Berenguer M, Cortesi PA, Strazzabosco M, Rockenschaub SR, Martini S, et al.

Delisting of liver transplant candidates with chronic hepatitis C after viral eradication: A European study. Journal of Hepatology 2016; 65: 524–531.

92. Van der Meer AJ, Berenguer M. Reversion of disease manifestations after HCV eradication. Journal of Hepatology 2016; 65: 95–108.
93. Cheung MCM, Walker AJ, Hudson BE, Verma S, McLauchlan J, Mutimer DJ, et al. HCV Research UK. Outcomes after successful direct-acting antiviral therapy for patients with chronic hepatitis C and decompensated cirrhosis. Journal of Hepatology 2016; 65: 741–747.
94. Backus LI, Belperio PS, Shahoumian TA, Mole LA. Impact of Sustained Virologic Response with Direct-Acting Antiviral Treatment on Mortality in Patients with Advanced Liver Disease. Hepatology 2017 [Epub ahead of print].
95. Delmonte RJ, Andrews B, Richards LM, Soto R, Collier S, Kuo A et al. Treatment of HCV with direct acting antivirals significantly reduces liver related hospitalizations in patients with cirrhosis. AASLD. The Liver Meeting 2017 Washington DC. October 20-24.
96. Reig M, Mariño Z, Perelló C, Iñarrairaegui M, RibeiroA, Lens S, et al. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. Journal of Hepatology 2016; 65: 719-726.
97. Kozbial K, Moser S, Schwarzer R, Laferl H, Al-ZoairyR, Stauber R, et al. Unexpected high incidence of hepatocellular carcinoma in cirrhotic patients with SVR following IFN-free DAA treatment. Journal of Hepatology 2016; 65: 856–858.
98. Conti F, Buonfiglioli F, Scuteri A, Crespi C, Bolondi L, Paolo Caraceni P, et al. Early occurrence and recurrence of hepatocellular carcinoma in HCV-related cirrhosis treated with direct-acting antivirals. Journal of Hepatology 2016; 65 : 727-733.
99. Nault JC, Colombo M. Hepatocellular carcinoma and direct acting antiviral treatments: Controversy after the revolution. Journal of Hepatology 2016; 65: 663–665.
100. Kobayashi M, Suzuki F, Fujiyama S, Kawamura Y, Sezaki H, Hosaka T, et al.

Sustained Virologic Response by Direct Antiviral Agents Reduces the Incidence of Hepatocellular Carcinoma in Patients With HCV Infection. Journal of Medical virology 2016; 89: 476-483.

101. Kozbial K, Moser S, Al-Zoairy R, Schwarzer R , Datz C, Stauber R, et al. Follow-up of sustained virologic responders with hepatitis C and advanced liver disease after interferon/ribavirin-free treatment. Liver International 2017 [Epub ahead of print].

102. Bruno S, Stroffolini T, Colombo M, Bollani S, Benvegnù L, Mazzella G, et al. On behalf of the Italian Association of the Study of the Liver Disease (AISF). Sustained virological response to interferon is associated with improved outcome in HCV-related cirrhosis: A Retrospective Study. Hepatology 2007; 48: 579-587.

103. Cardoso AC, Moucari R, Figueiredo-Mendes C, Ripault MP, Giuily N, Castelnau C, et al. Impact of peginterferon and ribavirin therapy on hepatocellular carcinoma: Incidence and survival in hepatitis C patients with advanced fibrosis. Journal of Hepatology 2010; 52: 652–657.

104. Benvegnu` L, Gios M, Boccato S, Alberti A. Natural history of compensated viral cirrhosis: a prospective study on the incidence and hierarchy of major complications. Gut 2004; 53: 744–749.

105. Sangiovanni A, Prati GM, Fasani P, Ronchi G, Romeo R, Manini M, et al. The natural history of compensated cirrhosis due to hepatitis C virus: a 17-Year Cohort Study of 214 Patients. Hepatology 2006;43: 1303-1310.

106. Van der Meer AJ, Feld JJ, Hofer H, Hofer H, Almasio PL, Calvaruso V, et al. Risk of cirrhosis-related complications in patients with advanced fibrosis following hepatitis C virus eradication. Journal of Hepatology 2016; 66: 485.

107. Waziry R, Hajarizadeh B, Grebely J, Amin J, Law M, Danta M, et al. Hepatocellular carcinoma risk following direct-acting antiviral HCV therapy: A systematic review, meta-

analyses, and meta-regression. Journal of Hepatology 2017. [Epub ahead of print].

108. Kanwal F, Kramer J, Asch SM, Chayanupatkul M, Cao Y, El-Serag HB. Risk of Hepatocellular Cancer in HCV Patients Treated With Direct-Acting Antiviral Agents. Gastroenterology 2017; 153:996–1005.

109. Ioannou GN, Green PK, Berry K. HCV eradication induced by direct-acting antiviral agents reduces the risk of hepatocellular carcinoma Journal of Hepatology 2017. [Epub ahead of print].

110. European Association for the Study of the Liver, European Organisation for Research and Treatment of Cancer. EASL–EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. Journal of Hepatology 2012; 56:908-943.

111. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. Hepatology 1994; 20: 15–20.

112. http://www.webaisf.org/media/39700/documento_hcv_241017.pdf. Aghemo A, Bruno R, Ciancio A, Coco B, Petta S. AISF 2014.

113. Decreto_222_4.8.15_LineeIndirizzoDAA_EpC.pdf. Alberti A, Vincenzi V, De Boni M, Carlotto A, Pellizzer G, Fabris P, et al. Veneto Region 2015.

114. Fujiwara N, Friedman SL, Goossens N, Hoshida Y. Risk factors and prevention of hepatocellular carcinoma in the era of precision medicine. Journal of Hepatology 2017. [Epub ahead of print].

115. Prenner S, VanWagner LB, Flamm SL, Salem R, Lewandowski RJ, Kulik .Hepatocellular carcinoma decreases the chance of successful Hepatitis C virus therapy with direct-acting antivirals. Journal of Hepatology 2017; 66:1173-1181.

116. Vescovo T, Refolo G, Vitagliano G, Fimia GM, Piacentini M. Molecular mechanisms of hepatitis C virus-induced hepatocellular carcinoma. Clinical Microbiology and Infection

2016; 22; 853-861.

117. Cariani E, Pilli M, Barili V, Porro E, Biasini E, Olivani A et al. Natural killer cells phenotypic characterization as an outcome predictor of HCV-linked HCC after curative treatments. Oncoimmunology 2016; 5: e1154249.

118. Spaan M, Van Oord G, Kreefft K, Hou J, Hansen BE, Janssen HL, et al. Immunological analysis during interferon-Free therapy for Chronic Hepatitis C Virus infection reveals modulation of the natural killer cell compartment. The Journal of Infectious Diseases 2016; 213: 216–223.

119. Serti E, Chepa-Lotrea X, Kim YJ, Keane M, Fryzek N, Liang TJ, et al. Successful interferon-free therapy of chronic hepatitis C virus infection normalizes natural killer cell function. Gastroenterology 2015; 149:190–200.Hengst J, Falk CS, Schlaphoff V, Deterding K, Manns MP, Cornberg M, et al. Direct-acting antiviral induced hepatitis C virus clearance does not completely restore the altered cytokine and chemokine milieu in patients with chronic hepatitis C. The Journal of Infectious Diseases 2016; 214: 1965–1974.

120. Meissner EG, Decalf J, Casrouge A, Casrouge A, Masur H, Kottilil S, et al. Dynamic changes of post-translationally modified forms of CXCL10 and Soluble DPP4 in HCV subjects receiving Interferon-Free Therapy. PLoS ONE 2017: 10; 1-9.

121. Benvegnu`L, Alberti A. Patterns of hepatocellular carcinoma development in hepatitis B virus and hepatitis C virus related cirrhosis. Antiviral Research 2001; 52: 199–207.

122. Wedemeyer, H.; Dore, G.J.; Ward, J.W. Estimates on HCV disease burden worldwide-filling the gaps. J. Viral Hepat 2015, 22, 1–5.

123. Foster, G.R.; Irving, W.L.; Cheung, M.C.; Walker, A.J.; Hudson, B.E.; Verma, S.; McLauchlan, J.; Mutimer, D.J.; Brown, A.; Gelson, W.T.; et al. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. J. Hepatol. 2016; 64: 1224–1231.

124. European Association for the Study of the Liver: EASL Recommendations on Treatment of Hepatitis C 2018. Journal of Hepatology; 2018; 69: 461–511.

125. Benvegnu` L, Gios M, Boccato S, Alberti A. Natural history of compensated viral cirrhosis: a prospective study on the incidence and hierarchy of major complications. Gut; 2004; 53: 744–749.

126. Van der Meer AJ, Feld JJ, Hofer H, Hofer H, Almasio PL, Calvaruso V, et al. Risk of cirrhosis-related complications in patients with advanced fibrosis following epatitis C virus eradication. Journal of Hepatology; 2016; 66: 485.

127. Reig M, Mariño Z, Perelló C, Iñarrairaegui M, Ribeiro A, Lens S, et al. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. Journal of Hepatology; 2016; 65: 719-726.

128. Ioannou GN, Green PK, Berry K. HCV eradication induced by direct-acting antiviral agents reduces the risk of hepatocellular carcinoma. Journal of Hepatology; 2018; 68: 25-32.

129. Romano A, Angeli P et al. Newly diagnosed Hepatocellular Carcinoma in patients with advanced hepatitis C treated with DAAs: a prospective population study. Journal of Hepatology; 2018; 69: 345-352.

130. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell; 1993; 75: 843-854.

131. Valery, P.C.; Laversanne, M.; Clark, P.J.; Petrick, J.L.; McGlynn, K.A.; Bray, F. Projections of primary liver cancer to 2030 in 30 countries worldwide. Hepatology 2018, 67, 600–611.

132. Bolondi L. Screening for hepatocellular carcinoma in cirrhosis. J Hepatol 2003;39:1076–1084.

133. Singal A, Volk ML, Waljee A, Salgia R, Higgins P, Rogers MA, et al. Meta- analysis: surveillance with ultrasound for early-stage hepatocellular carcinoma in patients with

cirrhosis. Aliment Pharmacol Ther 2009;30:37–47.

134. Hu Li, Jian-Dong Jiang, Zong-Gen Peng. MicroRNA-mediated interactions between host and hepatitis C virus. World J Gastroenterol; 2016; 22(4): 1487-1496.

135. Jonel Trebicka, Anadol E, Elfimova N, et. al. Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis. Journal of Hepatology; 2013; 58: 234–239.

136. Van der Meer, A.J., Farid, W.R., Sonneveld, M.J., et al. Sensitive detection of hepatocellular injury in chronic hepatitis C patients with circulating hepatocyte-derived microRNA-122. J. Viral Hepat; 2013; 20 (3): 158–166.

137. Yoshiki Murakami and Norifumi Kawada. MicroRNAs in hepatic pathophysiology. Hepatology Research; 2017; 47: 60–69.

138. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell; 2009; 136: 215-233.

139. Ambros V. The functions of animal microRNAs. Nature; 2004; 431: 350-355.

140. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet; 2011; 12: 99-110.

141. Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat Struct Mol Biol; 2012; 19: 86-593.

142. European Association for the Study of the Liver, European Organisation for Research and Treatment of Cancer. EASL–EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma. Journal of Hepatology 2012; 56:908-943.

143. miRCURYTM RNA Isolation Kit - Biofluids Instruction manual v1.7 #300112 and #300113 November 2015.

144. Almeida MI, Nicoloso MS, Zeng L, Ivan C, Spizzo R, Gafa R et al (2012) Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. Gastroenterology 2012 142: 886–896.

145. Zhou SL, et al. miR-28-5p-IL-34-macrophage feedback loop modulates hepatocellular carcinoma metastasis. Hepatology 2016;63:1560–1575.

146. Xiaomin Shi,Fei Teng. Down-regulated miR-28-5p in human hepatocellular carcinoma correlated with tumor proliferation and migration by targeting insulin-like growth factor-1 (IGF-1). Mol Cell Biochem 2015; 408:283–293.

147. Castello LM, Raineri D, Salmi L, Clemente N, Vaschetto R, Quaglia M, Garzaro M, Gentilli S, Navalesi P, Cantaluppi V, Dianzani U, Aspesi A, Chiocchetti A. Osteopontin at the Crossroads of Inflammation and Tumor Progression. Mediators Inflamm. 2017.

148. Ramaiah S K, and Rittling S. Pathophysiological Role of Osteopontin in Hepatic Inflammation, Toxicity, and Cancer. TOXICOLOGICAL SCIENCES 2008;103: 4–13.

149. Van Hees S, Michielsen P, Vanwolleghem T. Circulating predictive and diagnostic biomarkers for hepatitis B virus-associated hepatocellular carcinoma. World J Gastroenterol. 2016;22:8271-8282.

150. Tsuchiya N, Sawada Y, Endo I, Saito K, Uemura Y, Nakatsura T. Biomarkers for the early diagnosis of hepatocellular carcinoma. World J Gastroenterol. 2015; 2:10573-83.

151. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 2003;9:416-23.

152. Chokshi S, Cooksley H, Riva A, Phillips S, Williams R, Gaggar A, Naoumov NV. Identification of serum cytokine profiles associated with HBeAg seroconversion following antiviral treatment interruption. Viral Immunol. 2014; 27:235.

153. Wen Y, Jeong S, Xia Q, Kong X. Role of Osteopontin in Liver Diseases. Int J Biol Sci. 2016 ; 12:1121-8.

154. Phillips S, Mistry S, Riva A, Cooksley H, Hadzhiolova-Lebeau T, Plavova S, Katzarov K, Simonova M, Zeuzem S, Woffendin C, Chen PJ, Peng CY, Chang TT, Lueth S,

De Knegt R, Choi MS, Wedemeyer H, Dao M, Kim CW, Chu HC, Wind-Rotolo M, Williams R, Cooney E, Chokshi S. Peg-Interferon Lambda Treatment Induces Robust Innate and Adaptive Immunity in Chronic Hepatitis B Patients. Front Immunol. 201; 8:621.

155. Phillips S, Coombes JD, Mistry S, Williams R, Syn WK, Chokshi S. Osteopontin is Involved in Chronic HBV Infection and Enhances HBV Replication and HBsAg secretion. Hepatology. 2015; 62, 292-295.

156. Shashi K. Ramaiah and Susan Rittling. Pathophysiological Role of Osteopontin in Hepatic Inflammation, Toxicity, and Cancer. Review. Toxicological Sciences 2008; 103; 4–12.

157. Iqbal J, Sarkar-Dutta M, McRae S, Ramachandran A, Kumar B, Waris G. Osteopontin Regulates Hepatitis C Virus (HCV) Replication and Assembly by Interacting with HCV Proteins and Lipid Droplets and by Binding to Receptors αVβ3 and CD44. Journal of Virology 2018; 92:1-19.

158. N. Clemente, D. Raineri, G. Cappellano, et al. Osteopontin Bridging Innate and Adaptive Immunity in Autoimmune Diseases. Review Article. Journal of Immunology Research 2016. http://dx.doi.org/10.1155/2016/7675437.

159. Anthony W. O'Regan, Jason M. Hayden, and Jeffrey S. Berman. Osteopontin augments CD3-mediated interferon-γ and CD40 ligand expression by T cells, which results in IL-12 production from peripheral blood mononuclear cells. Journal of Leukocyte Biology 2000; 68; 495-502.

160. Klement J D, Ozato K, Liu K. An osteopontin/CD44 immune checkpoint controls $CD8^+$ T cell activation and tumor immune evasion. *J Clin Invest.* 2018; 128:5549-5560.

161. Kim HY, Park JW. Current immunotherapeutic strategies in hepatocellular carcinoma: recent advances and future directions. Therap Adv Gastroenterol. 2017;10: 805-815.

162. Markwick LJ, Riva A, Ryan JM, Cooksley H, Palma E, Tranah TH, Manakkat Vijay GK, Vergis N, Thursz M, Evans A, Wright G, Tarff S, O'Grady J, Williams R, Shawcross DL, Chokshi S. Blockade of PD1 and TIM3 restores innate and adaptive immunity in patients with acute alcoholic hepatitis. Gastroenterology. 2015;148: 590-602.

163. Markwick LJ, Riva A, Ryan JM, Cooksley H, Palma E, Tranah TH, Manakkat Vijay GK, Vergis N, Thursz M, Evans A, Wright G, Tarff S, O'Grady J, Williams R, Shawcross DL, Chokshi S. Blockade of PD1 and TIM3 restores innate and adaptive immunity in patients with acute alcoholic hepatitis. Gastroenterology. 2015;148:590-602.

164. Phillips S* and Chokshi S*, Chatterji U, Riva A, Bobardt M, Williams R, Gallay P, Naoumov NV. Alisporivir inhibition of hepatocyte cyclophilins reduces HBV replication and hepatitis B surface antigen production. Gastroenterology. 2015;148:403-14. *joint 1st author

165. Coombes JD, Swiderska-Syn M, Dollé L, Reid D, Eksteen B, Claridge L, Briones-Orta MA, Shetty S, Oo YH, Riva A, Chokshi S, Papa S, Mi Z, Kuo PC, Williams R, Canbay A, Adams DH, Diehl AM, van Grunsven LA, Choi SS, Syn WK. Osteopontin neutralisation abrogates the liver progenitor cell response and fibrogenesis in mice. Gut. 2015 ;64:1120-31

166. Phillips S, Coombes JD, Mistry S, Williams R, Syn WK, Chokshi S. Osteopontin is Involved in Chronic HBV Infection and Enhances HBV Replication and HBsAg secretion. Hepatology. 2015; 62, 292-295.

167. Quantikine ELISAHumanOsteopontin (OPN) Immunoassay. (R&D systems) Catalog Number DOST00 SOST00 PDOST00.