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Biomarkers of angiogenesis in hepatocellular carcinoma: a novel sunshine road

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Abstract: Background: Hepatocellular carcinoma (HCC) is a global health problem associated with chronic liver disease. The pathogenesis of chronic liver disease varies according to the underlying etiological factor, although in most cases it develops from a liver cirrhosis. The worsening progression of liver disease is accompanied by pathological angiogenesis, which is a prerequisite that favors the development of HCC. The aim of this study is to evaluate the clinical utility of circulating angiogenic markers VEGF, Ang-1, Ang-2, the Angiopoietin receptor (Tie1/2), HGF and PECAM-1 to screen early onset patients and to follow the evolution of HCC.

Materials and Methods: We enrolled 62 patients; 33 out of 62 subjects were diagnosed for HCC and 29/62 for liver cirrhosis of different etiology without signs of neoplasia. Patients underwent venous blood sampling before and after treatments for VEGF, Ang-1, Ang-2, Tie1, Tie2, HGF and PECAM-1 measurement.

Results: Ang-1 and Ang-2 are detectable not only in patients already suffering from HCC but also in cirrhotic patients without signs of cancer. Patients with HCC show higher HGF concentrations than patients with cirrhosis. A significant reduction in serum levels of Ang-2, Ang-2/Ang-1 and Ca 19-9 after DAAs therapy was observed. Moreover, VEGF levels were increased after treatment of HCC.

Conclusion: The preliminary study here presented confirms that the mechanism of tumor angiogenesis is very complex and involves a very large number of factors. The integration of different methodologies and multi-marker algorithms is likely to emerge for the early diagnosis of HCC and the monitoring of the risk of relapse.

Keywords: Hepatocellular carcinoma; cirrhosis; neoangiogenesis factors

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a global health problem associated with chronic liver disease; it represents the second leading cause of cancer death in males and the sixth in females (males/females~2:1), with an annual incidence of 13,000 new cases (in 2020) in Italy, 3% of all new cases of cancer[1]. The pre-existence of risk factors takes in account over 70% of primary liver tumors, primarily related to the prevalence of hepatitis C virus (HCV) infection. Also hepatitis B virus (HBV) infection is related to the onset of the disease, with a prevalence in Asia and Africa, while in the other Countries its role is predictably destined to decrease because of vaccination campaigns from 1978 onwards[2]. Among non-infective risk factors the aflatoxins taken with food[3,4] and alcohol consumption play an important role.

The pathogenesis of chronic liver disease varies according to the underlying etiological factor, although in most cases it develops from a liver cirrhosis.

Crucial steps towards worsening progression and carcinogenesis include, chronic inflammation, tumor micro and macroenvironment, angiogenesis. Both intrinsic individual genetic predisposition and extrinsic risk factors can lead to the development of HCC[5].

The worsening progression of liver disease is accompanied by pathological angiogenesis, which is a prerequisite that favors the development of HCC. Angiogenesis takes place through different progressive steps and represents the limiting factor for the the speed of tumor growth[6]. The growth of avascular tumors is limited by the distance from near vessels for the uptake of oxygen, nutrients, and the discharge of catabolic products through the interstitium. An angiogenic "switch", through the production of angiogenetic factors, is therefore a needful feature of a tumor that can grow[7]. In normal situations, there is a balance between endogenous angiogenic inducers and endogenous angiogenic inhibitors that keeps the angiogenic process under control and prevents inappropriate tissue vascularization. Angiogenesis inhibitors are often derived from circulating extracellular matrix proteins (as results of injury to the matrix), eg. fibronectin, prolactin, collagen XVIII (endostatin), NK1 fragment of the Hepatocyte Growth Factor (HGF) and angiostatin[8]. Virtually all endogenous angiogenesis inhibitors suppress tumor growth in animal models.

Vascular endothelial growth factor (VEGF) is best known as the most potent stimulator of normal and pathological angiogenesis[9]. VEGF release increases under hypoxic conditions. Its expression is regulated by the inducible factor of hypoxia (HIF-1a), which triggers the VEGF transcription[10]. This indicates that VEGF participates in the initial phase of angiogenesis. As a matter of fact, the transition of endothelial cells from an inactive to an active state can occur along with their proliferation, migration and formation of new vessels.

The tyrosine kinase receptors (Tie1 and Tie2) and their angiopoietin 1-4 ligands (Ang1, -2, -3 and -4) play a key role during the late phase of angiogenesis and are responsible for the maturation of newly established vascular structures. Ang1 and Ang2 are the best described and characterized angiopoietins[11]. The activity of the angiopoietin/Tie system determines the stabilization of new vessels. Both Ang1 and Ang2 interact with the same Tie2 receptor site having a similar affinity towards it, but only Ang1 induces its phosphorylation and the subsequent activation[12].

There is growing evidence that the angiopoietin/Tie signal can influence the outcome of inflammation[13]. Ang1 appears to be a powerful activator of Tie2, as well as a regulator of blood vessel formation and maturation. Experimental studies have shown that Ang1 acts as an anti-inflammatory molecule[14] but it can induce significant complications such as pulmonary hypertension[15]. Ang1 neutralizes Tissue factor (TF) activity that is relevant for the negative control of coagulation, thrombosis, and inflammatory response. Furthermore, Ang1

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reduces the adhesion of VEGF-related leukocytes to the endothelium[16,17]. On the contrary, Ang2 acts as a competitive antagonist of Ang1, deregulates the signal pathway of Tie2[12] and exerts pro-inflammatory effects[18,19]. Additionally, significantly elevated serum Ang2 levels have been observed during carcinogenesis in HCC patients[20].

It has been shown that HGF is over expressed in HCC compared to normal liver[21,22]. Stellate cells and myofibroblasts are induced to secrete HGF from tumor cell products and HGF, in turn, stimulates the invasiveness of tumor cells[23]. Recently published data show that higher HGF serum levels negatively correlate with patient survival time[24] and positively with tumor size[25,26].

PECAM-1 (platelet endothelial cell adhesion molecule-1) also known as CD31 is normally expressed on the surface of endothelial cells, platelets, leukocyte subpopulations and Kupffer cells[27]: these intercellular interactions are crucial for the angiogenesis process. In this context, PECAM-1 mediate both homophilic and heterophilic adhesion[28]. Its identification can help in assessing the degree of tumor angiogenesis, which may indicate a rapidly growing tumor[29].

Stemming from this background, it could be possible to keep subjects at risk of HCC under control. However, it is not always possible to carry out an accurate screening of the population at risk of HCC, especially for the costs of the procedure would have. Here, we aim to evaluate the clinical utility of circulating angiogenic markers VEGF, Ang-1, Ang-2, the Angiopoietin receptor (Tie1/2), HGF and PECAM-1 to screen early onset patients and to follow the evolution of HCC.

MATIERIAL AND METHODS

Patients

This is a non-profit interventional study, that involved the recruitment of patients belonging to the Liver Diseases Outpatient Clinic, suffering from liver cirrhosis of different etiology, and/or HCC. The subjects under examination underwent, after signing the informed consent, peripheral blood sampling, to measure circulating levels of the main neoangiogenesis factors as biomarkers of carcinogenesis.

Patients underwent a first blood draw (an 8 mL tube of serum) at the first visit. Then, they undertook normal outpatient clinical monitoring, which varied according to the stage of the disease. Patients treated with antiviral therapy underwent a second sampling at the end of therapy. The duration of therapy was variable, based on the ongoing patient response.

HCC patients underwent venous blood sampling before and after the different tumor treatments: percutaneous alcoholization (PEI), radiofrequency ablation (RFA), intra-arterial chemoembolization (TACE), intra-arterial radioembolization (TARE), surgical resection, transplantation, systemic antiangiogenic treatment. After the baseline assessment, the neoangiogenesis factors were re-evaluated at scheduled controls, according to international guidelines, after treatment, both in the presence and absence of disease recurrence.

Patients with chronic liver disease of viral etiology treated with the new Direct Acting Antiviral Agents (DAAs), without HCC, underwent the measurement of the neoangiogenetic factors with the same timing.

Inclusion criteria: patients aged 18 years or older; patients with liver cirrhosis of different etiology; patients with liver cancer at diagnosis; patients who have given written informed consent.

Exclusion criteria: presence of infections other than HCV and HBV; severe comorbidities at the time of enrollment; participation in other clinical trials involving the use of drugs; pregnant women.

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Among the 82 subjects evaluated for this study, we enrolled 62 patients (35 men and 27 women aged between 26 and 85 years) that met all the inclusion criteria: 33 out of 62 subjects were diagnosed for HCC and 29/62 for liver cirrhosis of different etiology without signs of neoplasia. Patient demographic and clinical characteristics are summarized in Table 1.

Laboratory procedures

For the measurement of Ang-1, Ang-2, VEGF, Tie1/2, HGF and PECAM-1 levels, serum samples were centrifuged at 2000g/min for 15 minutes and stored at -80 °C until their use. The assessment of these biomarkers was carried out at the Laboratory of Clinical Immunology and Molecular Hepatology of the Department of Medical Sciences of the Fondazione Policlinico Universitario "A. Gemelli"- I.R.C.C.S (Rome), and at the Institute of General Pathology of the Università Cattolica del Sacro Cuore (Rome), using ELISA assays (FineTest®, Wuhan Fine Biotech Co., Ltd) for Tie1 and the use of the Bio-Plex Multiplex (BIO-RAD) system for Ang-1, Ang-2, VEGF, Tie2, HGF and PECAM-1.

All the tests were performed in a single analytical session, following the instructions provided by the manufacturers, and the determinations were performed by an operator without knowledge of the clinical information of the handled sample. Each sample was tested twice to minimize eventual discrepancies, and all tests were performed in the same laboratory with the same instruments.

Ethical Consideration

The ethic committee of our institution (Fondazione Policlinico Universitario "A. Gemelli" I.R.C.C.S., Università Cattolica del Sacro Cuore) approved the study (Protocol ID: 2078). All patients gave written informed consent to the use of their clinical and serological data in this study. The whole study was conducted according to the Declaration of Helsinki, as revised in 2013.

Statistical analysis

The continuous variables were tested to verify their normality by analyzing the QQplot and the Shapiro-Wilk test. Some of the analyzed biomarkers -as reported in the appendix - showed significant deviations from the normality. Therefore, unless explicitly stated the database was analyzed using static non-parametric methods.

First, the presence of correlations among the various plasma biomarkers considered and some basic characteristics of the patients, such as age, the MELD score (Model for End stage Liver Disease) used to estimate the severity of the disease and the fibrosis index (FI = 8.0 − 0.01 × Plt (10^3/μl) − Alb (g/dl))[30] was evaluated. For this purpose, the Spearman correlation index (ϱ) was evaluated for each pair of variables, which can also be used for ranges of values and does not assume the normality of the data. The values of ϱ have been calculated using the programming language R, with particular reference to the algorithms implemented in the corrPlot function of the Hmisch package. The statistical significance of the correlation coefficients was evaluated by means of a power analysis.

The obtained values were summarized in matrix form and displayed by means of a false color map. The correlation matrices thus obtained were normally symmetrical with respect to the main diagonal.

In order to make the matrix easy to read, a double color code and size of the points of the map were used, in order to allow immediate assessment of the strength of the correlation and its direction. As indicated by the color scale on the right of the graph, negative correlations are displayed by means of an orange

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color that increases in intensity as the strength of the measured correlation increases, while for positive correlations a color scale based on the light blue.

RESULTS

Analysis of angiogenetic biomarkers in cirrhotic patients and correlation with clinical parameters

The analysis was conducted on a total of 29 cirrhotic patients of which 18 women and 11 men, aged between 26 and 78 years; 22/29 were diagnosed with metabolic cirrhosis and 7/29 with viral cirrhosis. The following comorbidities were highlighted: hypertension (n°8); esophageal varices (n°16); diabetes (metabolic n°17; insipidus n°1); portal hypertension (n°7); presence of ascites (n°6) and encephalopathy (n°7).

In Figure 1 B-G, the data relating to the presence of some significant correlations associated with the plasma markers Ang-1 and Ang-2 are analyzed. Figure 1B shows the trend of Ang-2 as a function of AFP values (average negative correlation ϱ= -0.43, p= 0.02). The results of the linear regression are shown in the graph, together with the 95% confidence interval of the best fit line (gray area) and the prediction bands (dashed orange lines). An examination of Figure 1B and of the coefficient shows that, although the correlation between the two variables is statistically significant, the measured AFP concentrations explain only a negligible portion of the variability found in Ang-2 measurements.

Figure 1C shows the trend of Ang-1 values as a function of the MELD (ϱ= -0.73, p <0.0001). It should be noted that - in principle - the application of a standard linear regression model would not be allowed in this case, since the MELD coefficient is not a normally distributed continuous variable, being also of an ordinal nature. However, we decided to show the result of this analysis because of its potential clinical applications and as it clearly shows that the determination of serum Ang-1 has potential applications in the development of new quantitative and objective methods for the staging of the disease. To confirm this result, considering the sample size, we decided to divide the MELD coefficient into two categories by setting the cut-off at 10, thus grouping the patients into only two classes (MELD <10 or >10). There was a statistically significant difference in the two groups for Ang-1 concentrations (p <0.001), in the ratio Ang-2/Ang-1 (p= 0.001), INR (p <0.001) and total bilirubin (p = 0.012) (Figure 2).

Figure 1 E-G shows the data of Ang-1 (ϱ= -0.52, p= 0.007), Ang-2 (ϱ= + 0.53, p= 0.003) and of the ratio Ang-2/Ang-1 (ϱ= 0.61, p= 0.0009) respectively, as a function of the fibrosis index. It is interesting to note that in the case of Ang-1 there is a negative correlation with the fibrosis index, in the case of Ang-2 the data are positively correlated and, consequently, how the correlation is accentuated in the relationship between two indices. This result is particularly interesting and confirms the potential of these plasma markers for quantitative staging of cirrhosis.

Evaluating the fibrosis index as a function of classes A, B, C of the Child-Pugh score a significant increase in the fibrosis index is observed as the score classes increase, as expected.

The subjects were then divided into two groups according to whether they received a diagnosis of metabolic cirrhosis (group MC) or of viral cirrhosis (group VC). Then, the variables shown in Figure 1 were analyzed in the two groups to identify the presence of possible statistically significant differences, of potential diagnostic interest and useful in identifying specific molecular mechanisms underlying the genesis and development of the disease. Statistically significant differences were found in the measured levels of LDL (p<0.001),

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triglycerides (p=0.05), total cholesterol (p=0.0026), INR (p=0.03) and PECAM-1 (p=0.03) which exhibit higher values than subjects with viral cirrhosis.

From the evaluation of the possible impact of the presence of the found comorbidities on a selection of clinical and diagnostic parameters a statistically significant alterations in Ang-1 levels were found in patients with hypertension, which are greater than in the control group (p=0.0022) and in the Ang-2/Ang-1 ratio values which, on the other hand, are consequently reduced (p=0.04). Surprisingly, there is a reduction in the MELD index (p=0.0029) whose origin deserves a more in-depth study.

In diabetic patients there is a statistically significant reduction in LDL(p=0.04), INR (p=0.0013) and Bilirubin values (p=0.03), reductions expected as a function of the degree of severity or absence of pathology. In subjects suffering from encephalopathies, an increase in sodium values (p=0.04) and a decrease in albumin concentration (p=0.038) are observed. In this group of patients there is also a possible increase in the MELD index (p= 0.05).

Regarding the remaining comorbidities, no statistically significant differences were found in the diagnostic markers.

In Figure 2, we showed a comparative analysis carried out on a selected set of biomarkers acquired before and after antiviral treatment. Of the 29 cirrhotic patients recruited for the study, we have data regarding the follow-up only of the 7 patients with viral cirrhosis who underwent treatment with DAAs. The values of the markers are compared in Figure 2 by using the Box-plot in which the experimental measurements relating to each patient, before and after treatment, are connected by a continuous line. The result of a Wilcoxon test for dependent samples is shown in each graph. The comparative analysis on selected patients shows a significant reduction after treatment of the following biomarkers: Ang-2 and, consequently, of the Ang-2/Ang-1 ratio, INR and Ca19-9. An increase in the platelet count is also observed.

Analysis of angiogenetic biomarkers in patients with hepatocellular carcinoma and correlation with clinical parameters

Analysis was conducted on a total of 33 patients diagnosed with HCC (24 men and 9 women) aged between 49 and 85 years: 24 patients were affected by cirrhosis, 4 by alcoholic steatosis and the remaining 5 showed no further liver disease. Twenty-seven patients have at least one other comorbidity (Table 1): 17 subjects show signs of portal hypertension and 4 of deep vein thrombosis.Six patients underwent hepatic resection, 3 underwent a transplant, 4 underwent pharmacological treatment with sorafenib, 9 undergo local-regional treatment and 4 received best supportive care. At the end of the study there were 5 deaths.

Firstly - as was done for the group of cirrhotic patients without HCC - the presence of statistically significant correlations between the various plasma biomarkers considered and some basic characteristics of the patients, such as age and MELD score, was assessed. Figure 3A shows a correlation matrix of the Spearman correlation coefficients, ϱ. As in the previous case, all the correlation coefficients calculated for each pair of variables above the main diagonal of the matrix and only the statistically significant correlations ($p < 0.05$) below the main diagonal are reported.

Figures 3 B-M show the scatter plots for some pairs of variables selected for their relevance for the present study among the pairs of variables for which a statistically significant coefficient ϱ is obtained. In particular, the data show a strong positive correlation in the case of Ang-2 and HGF (ϱ= 0.62) and a moderate positive correlation for the remaining variables: ϱ= 0.56 between Ang-1 and platelets, ϱ= 0.42 in the case of Tie2/ALT, ϱ= 0.49 for Ang-1/Ca 19-9, ϱ= 0.27 Ang-1/VEGF, ϱ= 0.36 Ang-2/PECAM-1, ϱ= 0.44 PECAM-1/MELD, ϱ= 0.51 HGF/VEGF and ϱ= 0.16 INR/PECAM-1.

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The variables were analyzed by stratifying the patients according to the etiology of the HCC, which can be of the metabolic, viral, cryptogenic type, none because neoplasm arising on a healthy liver.

We revealed the presence of higher levels of platelets (p=0.012), Ang-1 (p=0.027) and INR (p=0.012) in patients with cryptogenic HCC than in the remaining groups. It should be noted that, given the low sample size, this data must be confirmed on a larger dataset. It is also possible to observe a statistically significant increase in the number of platelets in patients with HCC with healthy liver compared to patients with metabolic or viral HCC, related to the presence or absence of portal hypertension. Conversely, low INR values are found in this category compared to the remaining and higher Ang-1 levels compared to subjects diagnosed with viral HCC.

A comparative analysis of the levels of some markers in three different groups of HCC patients is made, stratified according to the underlying liver disease. We report the presence of a reduced platelet count in cirrhotic patients when compared with subjects without further liver disease (p=0.012). The presence of significantly higher values of the INR index is shown in patients with additional liver diseases, such as cirrhosis (p=0.009) or fatty liver disease(p=0.012), compared to subjects for whom HCC is not associated with other liver diseases. Finally reduced Ang-1 levels in cirrhotic subjects compared to the remaining groups were observed (p=0.018).

After that we considered a set of biomarkers according to the Child-Pugh classification. In order not to limit too much the sample size in each group, we have chosen to group classes B, C and D into a single class, B+. The data show significantly higher levels of Ang-1 (p=0.012) and Ang-2 (p=0.018) in patients with non-cirrhotic liver, compared to the remaining groups. As the class increases, there is also a monotonous increase in the levels of ALP (p=0.0071), AST (p=0.05), INR(p=0.03) and γ-GT (p=0.04).

Analyzing the data obtained by comparing all the variables under study as a function of MELD (cut-off <10), a statistically significant difference emerged between the two groups for the concentrations of total bilirubin (p= 0.011), INR (p <0.001), total cholesterol (p= 0.009) and PECAM-1 (p <0.001).

From the comparative analysis of biomarkers as a function of the size of the nodules, only the trend of γ-GT resulted statistically significant. Although the division of the size of the nodules is typically <2cm, in the range 2-5 cm and > 5 cm, since the sample size is low, in order not to penalize the statistical power of the test, it was decided to include the group <2 and 2-5 cm in one group, <5 cm. Thus, two groups were compared, patients with nodules <5 cm and patients with nodules > 5 cm. A statistically significant difference (p= 0.021, Wilcoxon test for independent samples) was observed between the groups, which confirms an increase in γ-GT levels as the size of the nodules increases.

Figure 4 shows the comparative analysis of AFP, VEGF levels and the number of lymphocytes before and after the treatment of the disease. There is a significant reduction in AFP and a significant increase in the number of lymphocytes and levels of VEGF. Given the sample size, it is not possible to stratify by type of treatment.

Comparing the MELD coefficient (cut-off <10) with all serological markers, after treatment of HCC, a statistically significant difference emerged between the two classes compared for the concentrations of total bilirubin (p= 0.015), INR (p= 0.016), Tie-2 (p= 0.014) and a trend for the Ang-2/Ang-1 ratio (p= 0.056) and HGF (p= 0.053) with higher values in patients with MELD >10.

Finally, a comparison between the values measured on cirrhotic patients and those who received a diagnosis of HCC highlights higher levels of HGF in patients with HCC than in cirrhotic patients, suggesting that the levels of HGF

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can be used as an indicator of the progression of the disease by cirrhosis to HCC. This hypothesis could be validated by a longitudinal cohort study.

DISCUSSION

Hepatocellular carcinoma is the most common cancer affecting the liver and its incidence almost entirely reflects mortality. The high prevalence results from the high frequency in populations of developing chronic liver damage, following hepatitis and/or cirrhosis. The initial lack of symptoms does not allow an early diagnosis and therefore a timely intervention to fight the neoplasm. In fact, HCC is a potentially curable form of cancer, but unfortunately most patients present the disease at an advanced stage.

When a case of HCC is diagnosed at a relatively early stage, in which liver function is preserved, the most suitable approach and which offers a higher rate of post-operative survival is surgical resection. Despite the continuous progress of surgical techniques, of early diagnosis, the morbidity rate of patients undergoing liver resection remains very high. Therefore, compared to other types of solid tumors, the long-term prognosis (5 years after surgery) remains unsatisfactory, due to the high incidence of intrahepatic relapses[31].

In this scenario, it is essential for scientists to provide major benefits for the treatment of HCC. Targeting the hallmarks of cancer is usually one of the approaches to anchor this problem. For HCC, hallmarks include maintenance of proliferative signaling, avoidance of growth suppressors, escape immune destruction, replicative immortality, promotion of inflammation, activation of invasions and metastases, inducing angiogenesis, mediating the instability and mutation of the genome, resisting cell death, and deregulating cellular energy[32]. This means that more hallmarks, more pathways, and cytokines are involved. It is therefore necessary to search for HCC markers that allow the identification and control of those at greater risk; that they can also stratify patients according to the risk of cancer recurrence.

The progression of liver disease is accompanied by pathological angiogenesis, which is a prerequisite that favors the development of HCC. In HCC, hypoxia increases the expression of VEGF[33].

As we showed in results, the mean serum levels of VEGF were increased in patients with HCC compared to patients without signs of neoplasia, albeit not in a statistically significant way, moreover, these levels significantly correlate with the levels of Ang-1 and HGF. In addition, following the treatment of HCC, the levels of VEGF were increased compared to the values reported at the time of diagnosis, as well as the levels of lymphocytes. This can be partly explained by the rebound effect of VEGF, induced by hypoxia following locoregional treatments, often associated with treatment failure and low survival rates in patients[34].

Elevated Ang-2 levels have been reported in patients with HCC and cirrhosis. Increased Ang-2 levels have also been associated with advanced pathological features and worsening overall survival[35].

Patients with HCC enrolled for this study do not show circulating levels of Ang-1, Ang-2 and Tie1 and Tie2 receptors significantly different from patients with cirrhosis without HCC, but these molecules correlate with VEGF, Ca 19-9 and platelets count in the case of Ang-1, with PECAM-1 and HGF in the case of Ang-2. This data confirms the close relationship and interplay of these molecules in pathological angiogenesis and in the development of hepatocellular carcinoma.

Interestingly, we found a correlation between the levels of the Tie2 receptor and the levels of ALT, index of cyto-necrosis and surrogate marker of liver damage. The Tie2 receptor plays a key role during the late phase of angiogenesis and is responsible for the maturation of newly established vascular structures.

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This correlation highlights the parallel trend of hepatocellular damage and tumor angiogenesis in the progression of the disease.

Any differences within the group of patients with HCC were then evaluated between the different etiologies. Patients with HCC and cirrhosis of cryptogenic etiology had higher Ang-1, platelets count and INR levels than patients with HCC and cirrhosis of viral etiology or HCC with healthy liver.

The latter group of patients reported a higher platelet concentration and a lower INR than the other comparison groups.

Several reports provided evidence to support the role of platelets in HCC, e.g. reduction of HBV-associated experimental HCC by platelet inhibitors, antagonism of the action of sorafenib by platelet factors in HCC cell lines and complete remission of advanced HCC with sorafenib in combination with clopidogrel (antiplatelet agent)[36–38]. While the Ang-1 values were higher in the group of patients with HCC with healthy liver than in the group of patients with HCC with viral and metabolic or cryptogenic etiology.

In addition, patients with HCC show higher HGF concentrations than patients with cirrhosis. This data confirms what has already been reported in the literature, namely that the expression of HGF and its receptor supports the existence of both autocrine and paracrine mechanisms of HGF action in HCC compared to the only paracrine mechanism in the liver without neoplasia, suggesting that it also plays a role in tumor development and/or progression[21,22,39]. We also found a strong correlation between HGF levels, Ang-2 and VEGF levels, further supporting the fundamental value of these markers in tumor angiogenesis.

The PECAM-1 has been found to correlate positively with MELD, its identification can help in assessing the degree of tumor angiogenesis, which can indicate a rapidly growing tumor but also in estimating the severity of the disease and the probable survival of patients waiting for liver transplant. It has been shown that PECAM-1 promotes the formation of metastases by inducing the epithelium-mesenchymal transition in HCC by increasing the regulation of β1 integrin through the FAK/Akt[40] signaling pathway.

Since the Child-Pugh classification is the most widely used system and included in almost all staging systems, all the parameters considered in this study were evaluated according to the Child-Pugh classification. The concentrations of alkaline phosphatase, AST, γ-GT and INR increase as the class increases and therefore in proportion to the severity of the disease. Surprisingly, the levels of Ang-1, Ang-2 and albumin were higher in the group of patients with non-cirrhotic HCC than in the other patients. Furthermore, a comparison between the blood parameters and the size of the HCC nodules was determined. Therefore, the γ-GTs are the only ones to show a statistically significant difference between the grouping classes of size, and an increasing trend as the size of the nodule increases. In recent years, several studies have focused on the possible relationship between γ-GT and the incidence, development, relapse, and poor prognosis of HCC. Elevated γ-GT concentration has been suggested as a promising predictor of poor survival rates in HCC patients after hepatectomy, TACE or RFA[41,42].

Recently, the serum level of γ-GT has also been defined as a biomarker of oxidative stress and has been shown to correlate with inflammation in the microenvironment of extracellular tissue. When γ-GT is over-expressed, its pro-oxidant effect disturbs the oxidant/antioxidant balance, which carries out a continuous oxidative resistance action in the tumor; this in turn is involved in the regulation of tumor progression. Therefore, as a reflection of the inflamed liver microenvironment in patients with chronic hepatitis[43], γ-GT may be an attractive predictor for the prognosis of HCC patients. In a recent study, the

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prognostic value of γ-GT ≥ 75 U/L was shown to be higher than that of Child-Pugh staging, MELD and serum AFP[44].

Serum levels of angiogenetic biomarkers were also evaluated in patients with cirrhosis, divided in three groups according to the etiology: metabolic, cryptogenic, and viral, to identify the presence of possible statistically significant differences, of potential diagnostic interest, or useful for identifying specific molecular mechanisms underlying the genesis and development of the disease as well as the progression to HCC.

Concentrations of PECAM-1, total cholesterol, LDL, triglycerides, and INR were higher in patients with viral cirrhosis than in patients with metabolic or cryptogenic cirrhosis. Furthermore, Ang-1 levels in hypertensive patients were higher than in patients without arterial hypertension. Consequently, the Ang-2/Ang-1 ratio was decreased. Surprisingly, the MELD in hypertensive patients was lower than in non-hypertensive patients. However, the cirrhotic patient often presents a picture of circulatory dysfunction.

It is interesting to note that in the case of Ang-1 there is a negative correlation with the fibrosis index, in the case of Ang-2 the data are instead positively correlated and, consequently, how the correlation is accentuated in the relationship between the two indices. This result confirms the potential of these plasma markers for the staging of cirrhosis. In addition, Ang-1 correlates negatively with MELD, and as a direct mathematical consequence the Ang-2 / Ang-1 ratio correlates positively with MELD.

Considering the changes in markers before and after treatment, which in the case of patients with viral cirrhosis consist in the administration of DAAs, a significant reduction in serum Ang-2 levels, Ang-2/Ang-1 ratio, Ca 19-9 and INR emerged and an increase in platelets, the latter remaining however within the normal reference range. Increased serum levels of Ca 19-9 are common in chronic viral hepatitis (approximately 50% of patients); this does not necessarily indicate the concomitant presence of neoplastic pathology, but correlates significantly with the severity of the disease[45]. Therefore, the reduction in levels after antiviral treatment can be considered an indication of the success of therapy. As for the evaluation of the parameters according to the treatment of HCC, as expected the AFP concentrations following the treatment were significantly decreased. However, the combination of ultrasonography with AFP, the most used serum markers, recommended by all guidelines, remain unsatisfactory[46]. New imaging modalities and non-invasive biomarkers have been evaluated which show favorable specificity and sensitivity. Although many biomarkers have already shown diagnostic and prognostic potential, a greater number of multicenter studies including larger cohorts and long-term evaluations are needed to confirm their clinical usefulness.

In conclusion, angiogenesis is an indispensable process for tumor growth and the formation of metastases. The preliminary study presented here confirms that the mechanism of tumor angiogenesis is very complex and involves a very large number of factors. The integration of different methodologies and multi-marker models/algorithms is likely to emerge as a trend for the early diagnosis of HCC and the monitoring of the risk of relapse. It would be interesting to further characterize the significance of angiopoietin levels and other angiogenic markers also as predictors of response or resistance to therapies, particularly in the era of immunotherapy and HCC-targeted antiangiogenic drugs. The limit of this study is the sample size, but it is a pilot study that involves the recruitment of a greater number of patients and a longer-term follow-up.

The coronavirus pandemic has changed the priorities of the entire medical society. During the clinical course of COVID-19, liver damage has been observed in a significant proportion of patients, particularly those with severe or critical illness. In general, patients with pre-existing chronic liver disease may be more

susceptible to liver injury from SARS-CoV-2[47]. Stress and sepsis are particularly problematic in patients with decompensated liver cirrhosis, as both can trigger acute over chronic liver failure. COVID-19 is characterized by a significant activation of cytokines, which induces apoptosis and necrosis of hepatocytes, which in the context of reduced hepatic reserve, can lead to hepatic decompensation[48].

Patients with a history of cancer and SARS-CoV-2 infection are more vulnerable to severe disease and have a higher chance of death, as is ICU admission. Chemotherapy performed within 1 month of SARS-CoV-2 infection also increases the risk of worsening the disease. The indications for liver transplantation and locoregional therapy in HCC patients have not changed, but transplantation is reserved for highly progressive cases due to the shortage of ICU beds and the decline in the number of donors. The use of locoregional treatment as a salvage procedure is recommended to reduce the risk of HCC progression during the waiting period. For patients with resectable BCLC (Barcelona Clinic Liver Cancer) stage HCC whose surgical procedures have been canceled, transarterial therapies are recommended as a bridge to definitive treatment. Finally, it is recommended that sorafenib therapy be continued without changing the planned dose[49–51]. In the light of the above, it might be interesting to evaluate any changes in the serum distribution of neoangiogenesis markers in patients with COVID-19 and HCC compared to patients not exposed to SARS-CoV-2 infection, to see if there is a correlation with liver damage and better understand all the pathways involved.

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