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TITOLO TESI

IMPACT OF PROTEIN INDUCED BY VITAMIN K ABSENCE (PIVKA-II) IN DIAGNOSIS AND MONITORING OF PATIENTS WITH HEPATOCELLULAR CARCINOMA

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LIST OF ABBREVIATIONS

HCC	Hepatocellular carcinoma
HBV	Hepatitisvirus B
HCV	Hepatiticvirus C
SERCA1	Sarco-EndoplasmicReticulumCalciumATPase
AP-1	Activating Protein-1
Nf-kb	Nuclearfactor κ B
TP53	Tumor Protein p53
NS3-NS5A	Non-structural 3-5A proteins
TNF-α	Tumor necrosis factor α
c-myc	Avian myelocytomatosis virus oncogene cellular homolog
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
G-T	Guanine-Tymine
K-ras	Kirsten rat Sarcoma
Rb	Retinoblastomaprotein
PI3K	Phosphoinositide 3 kinase
MAPK	Mitogen-activatedproteinkinase
JAK	Janus-family tyrosinekinase
STAT	Signal transducer and activator of transcription
miRNAs	microRNAs
GSK3β	Glycogen Synthase Kinase-3 Beta
RAF	RapidlyAcceleratedFibrosarcoma
AKT=PKB	Protein kinase B
mTOR	mammalian target of rapamycin
PIP2	phosphatidylinositol 4,5-biphosphate
PTEN	Phosphatase and tensin homolog
SOCS	Suppressor of cytokinesignalling
SS1	Sucrose synthase 1
RFA	Radiofrequency Ablation
US	Ultrasound
BCLC	Barcelona Clinic LiverCancer
ECOG	Eastern CooperativeOncology Group
LR	Liver resection
LT	Liver Transplantation
TACE	Transarterialchemoembolization
FDA	Food and Drug Admistration

AFP	Alpha-fetoprotein
AFP-L3	Lectin-bound AFP
GPC3	Glypican-3
GS	Glutamine synthase
CK19	Cytokeratin 19
GP73	Golgi protein 73
OPN	Osteopontin
SCCA	Squamous cell carcinoma antigen
FGF 3/4	Fibroblast growth factor 3/4
GGT	Gamma glutamyltransferase
lncRNAs	Long non-coding RNAs
CTCs	Circulating Tumor Cells
cfDNA	Cell-free DNA
HSP70	Heat shock protein 70
PIVKA-II	Protein induced by vitamin K absence or antagonist II
DCP	Des-gamma-carboxyprothrombin
CPG	Clinical practice guidelines
MVI	Microvascular invasion
5-FU	5-fluorouracil
Gla	Glutamic acid
KH2	Vitamin K hydroquinone
KO	Vitamin K 2,3-epoxide
PLC/PRF/5	Primary Liver Carcinoma/Poliomyelitis Research Foundation/5
EGFR	Epidermal growth factor receptor
HGF	Hepatocyte growth factor
VEGF	Vascular-EndothelialGrowthFactor
MMP-2	Matrix metalloproteinase-2
GCP	Good Clinical Practice

1. HEPATOCELLULAR CARCINOMA

1.1. Epidemiology and risk factors

Hepatocellular carcinoma (HCC) is an important cause of death worldwide, representing the fifth most common cancer worldwide and the second cause of cancer death and it is the most malignant tumor diagnosed in males, with a male-female ratio of about 2-2.5:1 (1). The incidence of liver cancer and mortality shows a stable increase worldwide with854,000 new cases and 810,000 deaths per year, accounting for 7% of all cancers (2). There are geographical variations in incidence relating to the epidemiological distribution of infections with viral hepatitis B (HBV) and C infections (HCV), which represent the more prevalent causes of liver disease leading to HCC development.In Southeast Asia and Sub-Saharan Africa, between 40% and 90% of HCCs result from chronic HBV infection (3).

❖ Hepatitis virus B

HBV represents the main cause of HCC onset, because it holds direct oncogenic power.
HBV is a DNA virusthat presents a peculiar life cycle that includes a phase of integration of the viral genome within the host cellular genome inducing modifications in the expression of endogenous genes, such as cyclin A andSarco-Endoplasmic Reticulum Calcium ATPase (SERCA1) that stimulate cellular proliferation and, causing uncontrolled hepatic regeneration (4).
Furthermore, the integration of the HBV- DNA in the host cellular genome may deregulate the expression of oncogenes and tumor suppressors that, physiologically, are able to check and balance the signals of cell death and survival.
The HBV genome, codes for a regulatory element, the protein X, one of the non-structural components of the HBV genome, whose functional role has not yet been fully elucidated which acts as an activatorof transcription of many genessuch as AP-1 (activating protein-1) and NF-kB (nuclear factor kappa B); it is supposed that in the virus-infected liver cell,this protein may alter the normal mechanisms of cell growth control activating proto-oncogenes of the host cell and suppressing the control of the cyclecellular (5). Through its ability to act at the level of specific promoters,HBx is able to modify the expression of some genes that are involved in the control of the cell cycle, such as, for example, the gene that encodes the proteinp53, an important tumor suppressor and inducer of the

apoptotic process whose activity is suppressed in HCC. These effects of protein X on the p53 gene could promote malignant transformation of infected hepatocytes from HBV.
Summarizing these findings, it can be said that HCC on HBV represents a somewhat more complex model of HCC: with other models of *noxae* of chronic inflammation the mechanisms of necrosis and hepatocellular regeneration ultimately leading to fibrotic process, and in turn, to neoplastic development. In addition, it has direct pro-carcinogenic mechanisms, including the integration into the host genome, and the production of antigens once meaning (6).

❖ Hepatitis virus C

HCV, presumablyactsindirectly, inducing chronic inflammation with consequent death cyclescell and regeneration that would predispose to the onset and accumulation ofpotentially malignant mutations. HCV has numerous viral proteins associated with carcinogenesis such as core non-structural 3-5A proteins(NS3 and NS5A), which inhibit the post-transcriptional expression of the cyclin-dependent p21 inhibitor, resulting in cell-cycle dysregulation.
The HCV core proteins therefore, play a key role in HCV-related hepatocarcinogenesis through the modulation of cell proliferation, apoptosis and the immune response.
Contrary to HBV, HCV is an RNA virus that does not integrate into the cellular genome of the host but is able to induce hepatocarcinogenesis through protein interactions. In the process of infection, after an initial phase of adsorption, the virus releases its genomewithin the cell cytoplasm. It is known that once the genome has penetrated the cytoplasmof HCV, being constituted by an RNA filament of positive polarity, it comes directlytranslated into a polyprotein that undergoes a series of proteolytic processes which they will then carryto the production of mature viral proteins.
In particular, the core protein and NS3 and NS5A are the most involved factors in the HCV related hepatocarcinogenesis process.
The HCV core protein, in fact, acts as a polyfunctional protein with regulatory capabilities of cell proliferation and transcriptional regulation of some involved genes el control of cell growth, such as protooncogene avian myelocytomatosis virus oncogene cellular homolog (c-myc). Furthermore, this protein could induce hepatocarcinogenesis through other mechanisms, such as the modification of the release of tumor necrosis factor α (TNFα) which, as is known, influences equilibrium physiological that occurs between processes of death and cell survival (7).

❖ Non-alcoholic Fatty Liver Disease&Non-alcoholicSteatohepatitis

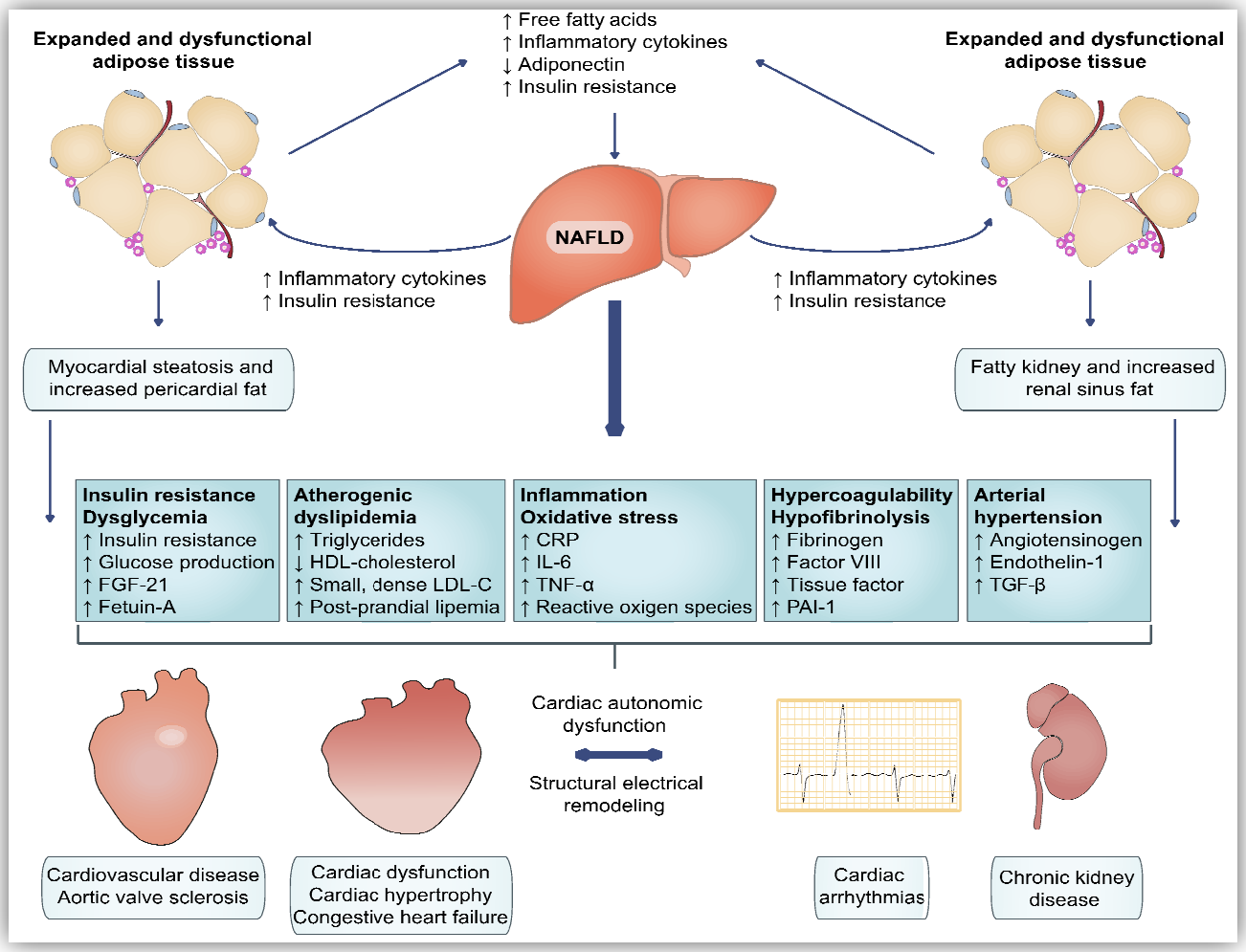


Figure 1: Byrne CD.et al.JHepatol 2014 [8].

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the western world. By definition, itoccurs in subjects who have a slight or no alcohol consumption. It is one complex and multifactorial pathologyas it is closely associated with

metabolic alterations such as obesity, insulin resistance, dyslipidemia, type 2 diabetes mellitus, arterial hypertension.

It is characterized by the presence of liver fatintraepatocitary (steatosis, consisting of an accumulationthe triglyceride cell> 5%) that can promote inflammation and necrosis. According to etiopathogenetic profile, the NAFLD includes a wide spectrum of diseases ranging from simple hepatic or non-alcoholic steatosis to NASH (non-alcoholic steatohepatitis) which presents some peculiar abnormalities such as damage to hepatocytes, inflammation and activation of fibrogenesis. NASH in at least one third of cases may progress to advanced fibrosis and to cirrhosis with the onset of HCC (9).

This pathology is an emerging problem in developed countries, with a prevalence that is increasing significantly, especially in terms of surveillance as HCC can also occur on non-cirrhotic liver.

In the last 10 years NASH has become the second indication for liver transplantation in the United States and its importance is growing in Europe also.

Although the application of the programs ofscreening for HCC in non-cirrhotic patients is not currently recommended by the guidelines, it is desirable that in the future selection tools of the individuals at higher risk for HCC are identified in order to stratify the general population and allow the development of surveillance strategies more targeted.

Indeed, the long-lasting liver disease and liver cirrhosis are the main risk factors for HCC, and only 10-25% of cases develop in absence of cirrhosis.

In addition to hepatitis viruses, NAFLD&NASH, which account for the overwhelming majority of cases of HCC, the onset of cancer may be associated with numerous other conditions, such as hemochromatosis, α1-deficiency antitrypsin, Wilson's disease, autoimmune hepatitis, tyrosinemia, primary biliary cirrhosis.Above all the incidence of HCC, has been progressively increasing in developed countries, probably because of modifications in lifestyle, which in turn are causing a progressive increase in liver disease due to alcohol abuse, environmental contaminants of industrial origin (azo dyes, aromatic amines, nitrosamines, vinyl chloride, organic solvents, pesticides, arsenic), derivatives of tobacco combustion (benzopyrene), metabolic syndrome.

Finally, another important etiological association was identified with somefood contaminants, in particular aflatoxin B1, found in grains andin moldy peanuts, especially in some regions of Africa and in ChinaSouthern; this mycotoxin would be able to induce a specific mutation Guanine-Tymine(G-T) in codon 249 of the tumor suppressor gene p53 (10).

1.2. Molecular mechanisms

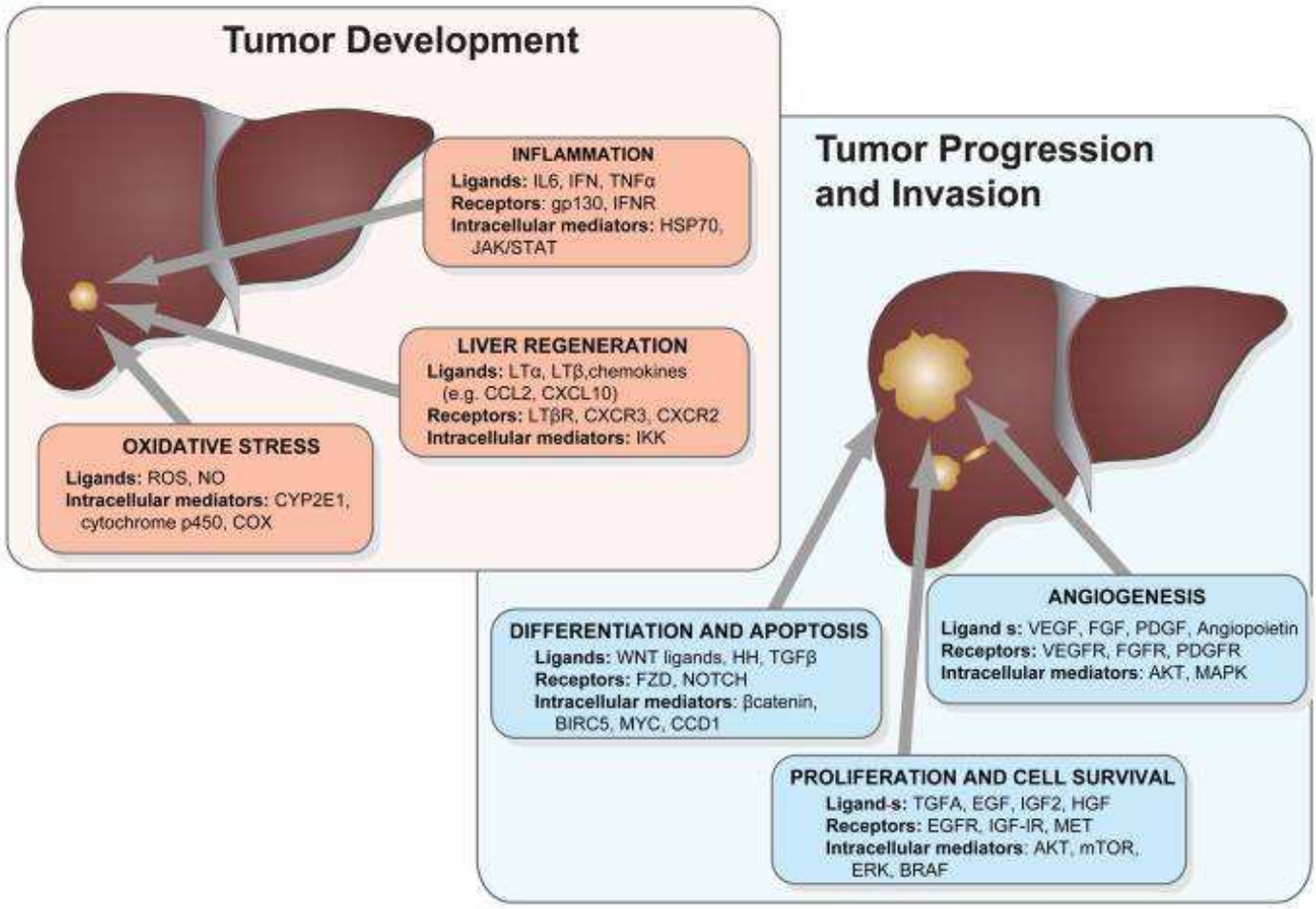


Figure 2: Signaling pathways frequently dysregulated in HCC [11].

At the base of the development of HCC there is a complex, multistep process,that derived from a combination of genetic and environmental factors that determine an abnormal activation or inactivation of single or multiple cellular pathways including those of cell proliferation, cell survival, differentiation and angiogenesis.

According to recent studies, the mechanisms underlying the onset of HCC concern the inactivation of multiple tumor suppressor genes (such as p53), abnormal activation of oncogenes Kirsten rat sarcoma (K-ras), c-myc, Retinoblastoma protein (pRb) and multiple signaling pathways: Phosphoinositide-3-kinase(PI3K), Mitogen-activated protein kinase (MAPK), Janus-family tyrosine kinase/Signal transducer and activator of transcription(JAK/STAT), NF-κB, Wnt/β-catenin), abnormal regulation of epigenetic events as the dysregulation of the expression of microRNAs(miRNAs), and even exosomes that deliver a large number of protumorigenic molecules are all involved in HCC development and progression and which together determine a genomic instability, in the majority of cancers affecting the human race. However, different studies often yield diverse results and the global view on the landscape of genomic changes is still not very clear.

Currently, some signal transduction pathways involved in the onset of HCC are at the center of experimental studies as they could represent the target of new antiproliferative therapies:

-Wnt/*β*-catenin signaling pathway is involved in maintaining cellular homeostasis via cell proliferation, differentiation, motility and apoptosis. The pathway comprises ligand Wnt protein, frizzled receptor, and regulator proteins, such as Glycogen Synthase Kinase-3 Beta (GSK-3β) and β-catenin. Approximately 20%-40% of HCC shows mutations in this pathway. The upregulation of frizzled-7 gene and the dephosphorylation of β-catenin are also noticed in HCC. Wnt/*β*-catenin activation is correlated with HCV infection and aflatoxin B1 exposure (11);

-Ras/Raf/MAPK Pathwayis the pivotal signal transduction pathway involved in HCC development ant its dysregulation due to aberrant upstream signals, inactivation of the Raf kinase inhibitor protein, and the presence of HBV and HCV proteins, results in anomalous cellular activity leading to cancer;

- PI3/AKT/mTORPathway ,the activation of this route occurs in 30%-50% of HCC.

The membrane lipid phosphatidylinositol 4,5-biphosphate (PIP2) is phosphorylated by PI3 kinase (PI3K), which binds to and activates serine threonine kinase (Akt).

Tumor suppressor gene, Phosphatase and tensin homolog(PTEN), which targets the lipid products of PI3K fordephosphorylation, acts as a negative regulator of this pathway in normal cells. PTEN mutation decreases PIP3 level and overactivates the PI3/AKT/mTOR pathway, thus inhibiting apoptosis and inducing tumor development(12);

- JAK/STAT Pathway, Janus Kinase (JAK) is a signal transducer and activator of a family of transcription factors STATs. The JAK/STAT pathway is activated by various cytokines and growth factors and it is involved in multiple cellular functions such as differentiation, proliferation, and apoptosis. Activated JAK triggers the transcription of SOCS genes, which belong to the negative feedback loop in the JAK/STAT pathway. The deregulation of the inhibitors of this pathway, particularly SOCS-1 and SS-1 (a JAK-binding protein), has been detected in HCC. Studies showed that STAT3 is preferentially activated in human HCC and active STAT3 is linked with aggressive tumor phenotype.

Although numerous genes are altered in associationwith HCC, only a small number of them are consideredalterations that drive clonal expansion and invasion.

Most of the somatic alterations appear to be passengers that are neutral for tumor cell selection.So far, most of the genetic events that initiate HCCremain unknown.

Therefore, the identification of keydriver genes in HCC is crucial to elucidating the geneticmechanism of hepatocarcinogenesis and providingnew molecularly targeted therapies for HCC patients. Identification of the dominant proliferative signals and key aberrations will allow for a personalized therapy.However, the introduction of new high-throughput genomic technologies (microarrays, deep sequencing) and increased sophistication of computation biology (bioinformatics, biomodeling) opens the field to new strategies in oncogene and tumor suppressor discovery (13).

In light of what has emerged, HCC is characterized by a mortality of about 90% which reflects the lack of adequate therapeutic tools even in the face of early diagnosis during screening programs. The poor prognosis of HCC has always been the source of a constant search for the most suitable procedures for achieving early diagnosis. In the past, HCCs were diagnosed when they were symptomatic and therefore at an advanced stage. However, recent acquisitions related to the natural history of this neoplasm have documented that HCC is mostly a slow-growing tumor whose natural history is often parallel to that of the underlying cirrhosis. Cirrhotic patients represent a high-risk group for HCC development and should undergo surveillance for HCC on a regular basis. Early detection of HCC through surveillance methods have increased patient survival by providing effective initial treatments such as primary curative hepatectomy and locoregional ablative therapy(14).

As for other tumors, the stage at the diagnosis strongly affects the prognosis: the majority of patients are diagnosed with late-stage disease with metastasis has occurred, being

HCC a slow-growing tumor with mild or no symptoms until advanced and this determines an overall 5-year survival rate of < 16%, while patients at early stage have a 5-year survival rate of >70%(15). On the basis of these premises, surveillance is the most important step in diagnosing liver cancer.Current guidelines recommend that abdominal ultrasound (US) should be performed every six months for surveillance of cirrhotic patients but it has now been established that US is clearly not reliable for detecting HCC at an early stage, while it is essential to improve the early diagnosis and monitoring of recurrence by sensitive markers to be used in high risk populations and also in the screening programs. Because of these limitations, the performance of US in early detection of HCC is highly dependent on the expertise of the operator and the quality of the equipment (16).

1.3. Diagnosis

The anearly diagnosis of HCC represents a fundamental step for defining the most appropriate therapeutic pathway and for defining the prognosis of patients.
A correct diagnostic approach involves the early identification of focal hepatic lesions, in particular on the cirrhotic liver, and their characterization. International guidelines suggestthe radiological diagnosis and histologyas priority, when it's not possible to get non-invasive diagnosis with imaging techniques. A lesion should be suspected for HCC when it shows a typical vascular behaviours characterized by a rapid and homogeneous absorption of the contrast medium in the arterial phase (wash-in) followed by a rapid discharge in the venous/late phase (wash-out) with respect to the surrounding parenchyma. New recommendations for the diagnosis of HCC include an initial distinction based on the diameter of the identified hepatic focal lesion; if the diameter is less than 1 cm, the US follow-up indication remains unchanged on a quarterly basis and any further diagnostic analysis is initiated in the event of an increase in size or changes in the lesion characteristics. An early and correct characterization of focal hepatic lesions is the crucial parameter in determing the chance of a succesfull treatment for HCC (17).

1.4. Clinical staging and treatment

The classification currently longer effective is that proposed by the Barcelona Clinic Liver Cancer (BCLC), capable of stratifying patients with HCCinto five stages (0, A,B, C and D)

in terms of both prognosis and ideal therapeutic choice taking into account the following parameters: tumour status (number and size of nodules, presence of vascular invasion, extraepatic spread), liver function (defined by Child- Pugh's class, bilirubin, albumin,clinically relevant portal hypertension, ascites) and health performance status (defined by the Eastern CooperativeOncology Group (ECOG) classification(Figure 3).The outcome of the treatment of HCC is affected by the correct staging of the patient, which in turn drives the correcttherapeutic choice.Clinically effective staging should be able to identify patients in prognostic terms and must indicate the most appropriate. To that end, therefore, staging must not or only measure the number and size of nodules, vascular infiltration and extrahepatic spread, but must also evaluate the residual liver function and general clinical conditions, all variables that influence prognosis and the therapeutic choice (18).

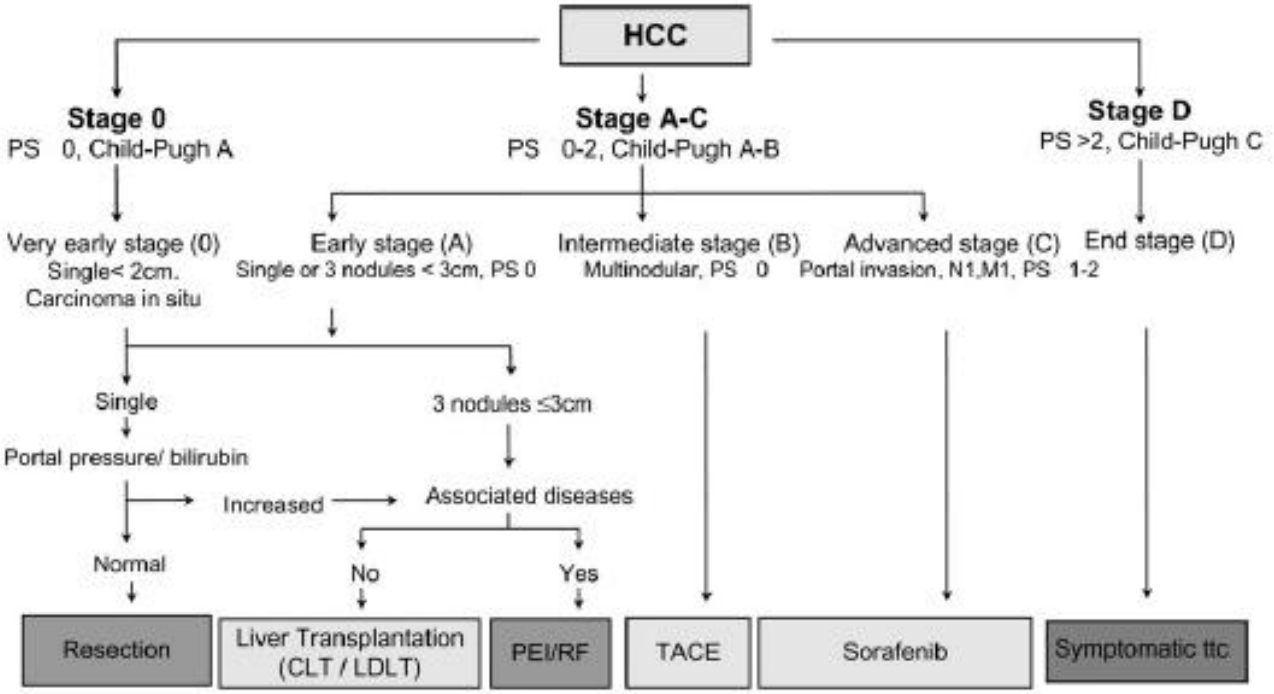


Figure 3: BCLC staging and treatment schedule [19]

Surgery is the mainstay of HCC treatment, leading to the best outcomes of any treatment available in well-selected candidates (five-year survival of 60–80%). Liver resection (LR) and liver transplantation (LT) represent the first option in patients with early tumours on an

intention-to-treat perspective. Thermal ablation with radiofrequency (RFA) is the standard of care for patients with BCLC 0 and A tumours not suitable for surgery. RFA in single tumours 2 to 3 cm in size is an alternative to LR based on technical factors (location of the tumour), hepatic and extrahepatic patient conditions. In patients with very early stage HCC (BCLC-0) RFA in favourable locations can be adoptedas first-line therapy even in surgical patients.Transarterialchemoembolization (TACE) is recommended for patients with BCLC stage B and should be carried out in a selective manner.TACE should not be used in patients with decompensated liver disease, advanced liver and/or kidney dysfunction, macroscopic vascular invasion or extrahepaticspread .Sorafenib, a multikinase inhibitor, is the standard first-line systemic therapy for HCC. It is indicated for patients with well-preserved liver function (Child-Pugh A) and with advanced tumours (BCLC–C) or earlier stage tumours progressing upon or unsuitable for loco-regional therapies (evidence high; recommendation strong). Lenvatinib has been shown to be non-inferior to sorafenib and is also recommended in first-line therapy for HCC given its approval. It is indicated for patients with well-preserved liver function (Child-Pugh A class), good performance status and with advanced tumours .There are no clinical or molecular biomarkers established to predict response to first or second-line systemic treatments. Regorafenib is recommended as second-line treatment for patients tolerating and progressing on sorafenib and with well-preserved liver function (Child-Pugh A class) and good performance status. Recently, Cabozantinib has shown survival benefits vs. placebo in this setting. Based on uncontrolled but promising data, immune therapy with nivolumab has received Food and Drug Admistration (FDA) approval in second- line treatment, pending phase III data for conventional approval (2).Capecitabine, an oral fluoropyrimidinecarbamate, that acts as a 5-fluorouracil (5-FU) pro-drug to mimic the continuous infusion of 5-FU, is a potentially important treatment option for patients with advanced HCC and may even represent a cure in certain cases especially, for patients unfitfor other treatments and its administration according to a standard schedule has been evaluated in the postoperative adjuvant setting after curative HCC resection, in advanced disease. This adjuvant therapy with capecitabine was well tolerated, postponed and reduced the risk of HCC recurrence (20).Finallypatients at BCLC D stage, who are not candidates for liver transplantation should receive palliative support, including management of pain, nutrition and psychological support. In general, they should not be considered for clinical trials (2).

2. BIOMARKERS

Surveillance and diagnostic methods for HCC can include, in association with image techniques, the use of serological biomarkers, namely molecules measured in plasma samples.

A number of non invasive diagnostic markers are under evaluation at this purpose:alpha-fetoprotein(AFP), lectin-bound AFP (AFP-L3), glypican-3 (GPC3), glutamine synthase (GS), cytokeratin 19 (CK19), Golgi protein 73 (GP73), midkine, osteopontin (OPN), squamous cell carcinoma antigen (SCCA), Annexin A2, fibroblast growth factor 3/4 (FGF3/4), gamma-glutamiltransferasi (GGT), novel cytokine, microRNAs (miRNAs), Long non-coding RNAs (lncRNAs), circulating tumor cells (CTCs), cell-free DNA (cfDNA), heat shock protein 70 (HSP70) and protein induced by vitamin K absence or antagonist II (PIVKA II) have been widely used to detect HCC and analyze treatment response (21).

2.1. Alpha-fetoprotein

AFPis the most known and most widely used marker of surveillance, in combination with images techniques.The alpha-fetoprotein was discovered in 1956 by Bergstrand and Czar, who used the paper to perform the electrophoretic separation of human AFP in serum. The first evidence of the usefulness of AFP as a diagnostic marker of HCC was described in 1964 by Tatarinov and in 1968 by Abelev(22). The human alpha-fetoprotein gene is located on chromosome 4 (4q11-q13) and is part of the superfamily of albuminoid genes which also encode other numerous proteins,including albumin and vitamin (23).This marker is a glycoprotein of 591 aminoacids with a molecular weight of about 70 Kda, its synthesis occurs at the level of the endodermal cells of the yolk sac in the early stages of fetal development and subsequently at the level of embryonic hepatocytes (24).In adults, high levels are found not only in liver regenerative processes but also in other tumoral pathologies of gastrointestinal tract, pancreatic and testicular carcinoma, and in embryonic carcinomas, as well as, obviously, during pregnancy(25). About diseases hepatic, an increase in AFP levels can be seen already in the inflammatory phase of the pathology, with possible oscillations linked to reactivations, and even more after evolution in cirrhosis: from this statement it is clear that it is extremely difficult to establish a

"threshold" level of AFP that has an adequate accuracy for HCC. The exact biological function of this molecule still remains unclear; due to its similarities with albumin, it appears to be involved in the molecular transport of different ligands such as bilirubin, fatty acids, retinoids, steroids, metals, flavonoids, phytoestrogens and a variety of drugs. Since Tatarinov discovered AFP in the serum of patients with HCC, this protein has become the diagnostic marker par excellence for hepatic neoplasia, but with important limitations. These restrictions include the low specificity primarly: AFP is not overexpressed in all HCC patients (26).

It was found that elevated AFP levels were not evident in around 80% of small HCCs. Only about 10%-20% of HCC tumors at the early stage can present with abnormal AFP serum levels.For this reason, the use of AFP as a screening and marker of HCC, in the absence of more effective biomarkers, is still in use in subjects at high risk of developing hepatocellular carcinoma (cirrhotic), but there is a pressing need for to identify molecules with specificity and sensitivity characteristics that are better for early diagnosis.

Another limitation of AFP includes the suboptimal performance in distinguishing intrahepatic cholangiocarcinoma and HCC.

As a result, AFP has been excluded in some guidelines on HCC surveillance and diagnosis. Indeed, the European Association for the Study of the Liver and the European Organization for Research and Treatment of Cancer (EASL-EORTC) clinical practice guidelines (CPG) for HCC screening and diagnosis do not include quantitative measurements of serum AFP and recommend surveillance by experienced personnel in the at-risk populations using abdominal US every 6 months. However, the American Association for the Study of Liver Diseases (AASLD) guidelines recommend surveillance by the US for cirrhotic adults every 6 months with optional use of AFP due to the poor sensitivity and specificity of this biomarker.

Shorter follow-up interval (every 3-4 months) is recommended in case of any of these conditions: (1) a nodule of less than one cm has been detected; (2) after liver resection; or (3) after loco-regional therapy. In contrary, the follow-up 3-6 months of serum AFP was included in the diagnostic algorithm of hepatic nodules by the Oriental guidelines for HCC management. Therefore, in the most common guidelines, it is well established and recommended that US should a be part of surveillance and most commonly combined with AFP.

The limitations of AFP highlight the need to identify novel biomarkers. Given the increasing incidence of HCC, it is necessary to explore whether other new or old serum biomarkers or

a combination of them can compete with or complement that of the US and constitute an optimal performance in the diagnosis, prognosis, treatment response, and surveillance of HCC (27).

2.2.Protein induced by Vitamin K Absence

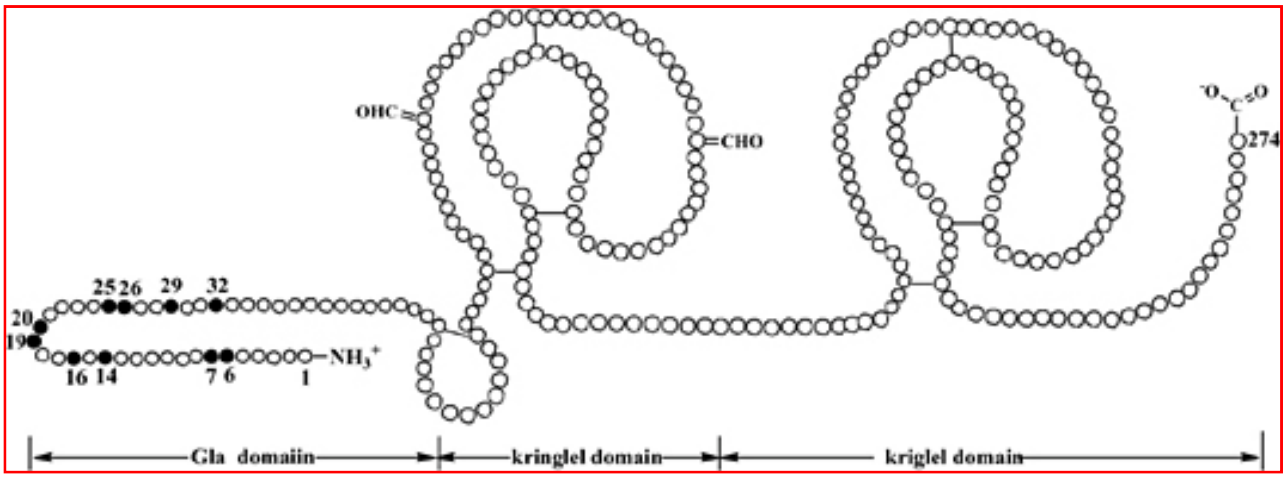


Figure 4: Diagram representing the order of carboxylation of Gla domain in prothrombin. If less than 10 Glu residues convert to Gla residues, the protein is called Des-CarboxylatedProthrombin (DCP) or PIVKA-II[31].

PIVKA-II, also called des-gammacarboxyprothrombin (DCP) or antagonist II, is an abnormal prothrombin, was first described in 1963 by Hemker.

Its chemical structure was elucidatedin 1963, Hemker et al. first indirectly observed the biosynthesis of abnormal prothrombin in plasma of dicoumarol-treated patient (28) butdirect evidence of this abnormal prothrombin was observed by Nilehn and Ganrotin (29-30). In 1972, Stenflo et al.first purified the abnormal prothrombin and compared it with normal prothrombin, that is a coagulation factor(31). It has emerged that two prothrombins had identical amino acids and carbohydrates structurally. However, the abnormal prothrombin failed to bind calcium ions and had no prothrombin activity. In 1974, Stenflo et al. reported that the normal prothrombin contained residues of modified glutamic acid, γ-carboxyglutamic acid, unlike the abnormal prothrombin (32). The γ-carboxyglutamic acid conferred the normal prothrombin with calcium ion-binding ability for its activation. Subsequent studies identified 10 γ-carboxyglutamic acids in normal prothrombin, which were absent in the abnormal prothrombin. Generation of PIVKA-II is thought to be a result

of an acquired defect in the post-translational carboxylation of the prothrombin precursor in malignant cells (33).

2.2.1. Chemical Structure

Normal prothrombin is a vitamin K-dependent zymogen composed of 579 aminoacid residues (34) assigned to three different structural domains: fragment 1 (residues 1–155), fragment 2 (residues 156–271), and a protease domain (residues 272–579) (Fig.5). Fragment 1 has a γ-carboxylated glutamic acid (Gla) domain and a kringle. Fragment 2 contains another kringle. Protease domain contains the A chain and a catalytic B chain. The Gla domain contains 10 γ-carboxylated glutamic acids in normal prothrombin, which are located at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, and 32 at the N-terminal of the protein.Normal prothrombin induces coagulation binding with calcium ions (35), which depends on its structural integrity stabilized by Gla residues. Some of the residues may directly coordinate the calcium ions to form an active complex, and other residues play a role in the bridging calcium ion-phospholipid interactions.In the absence of N-terminal Gla residues, PIVKA-IIappears to be functionally defective because it can’t bind calcium and phospholipid (36).

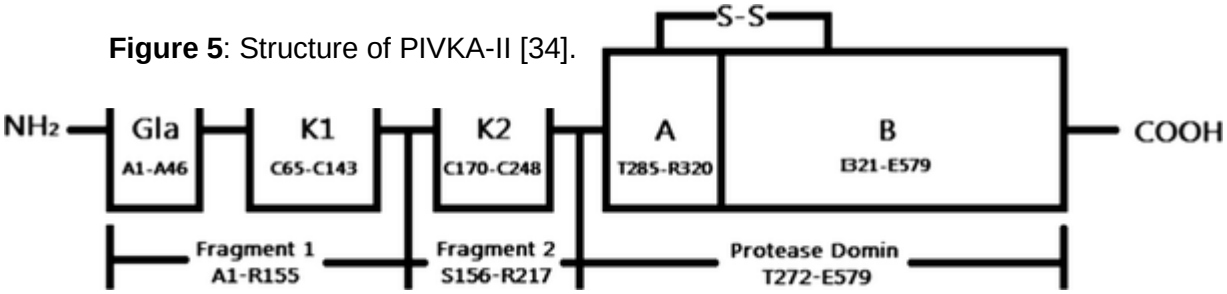


Figure 5: Structure of PIVKA-II [34].

2.2.2. Mechanism of production

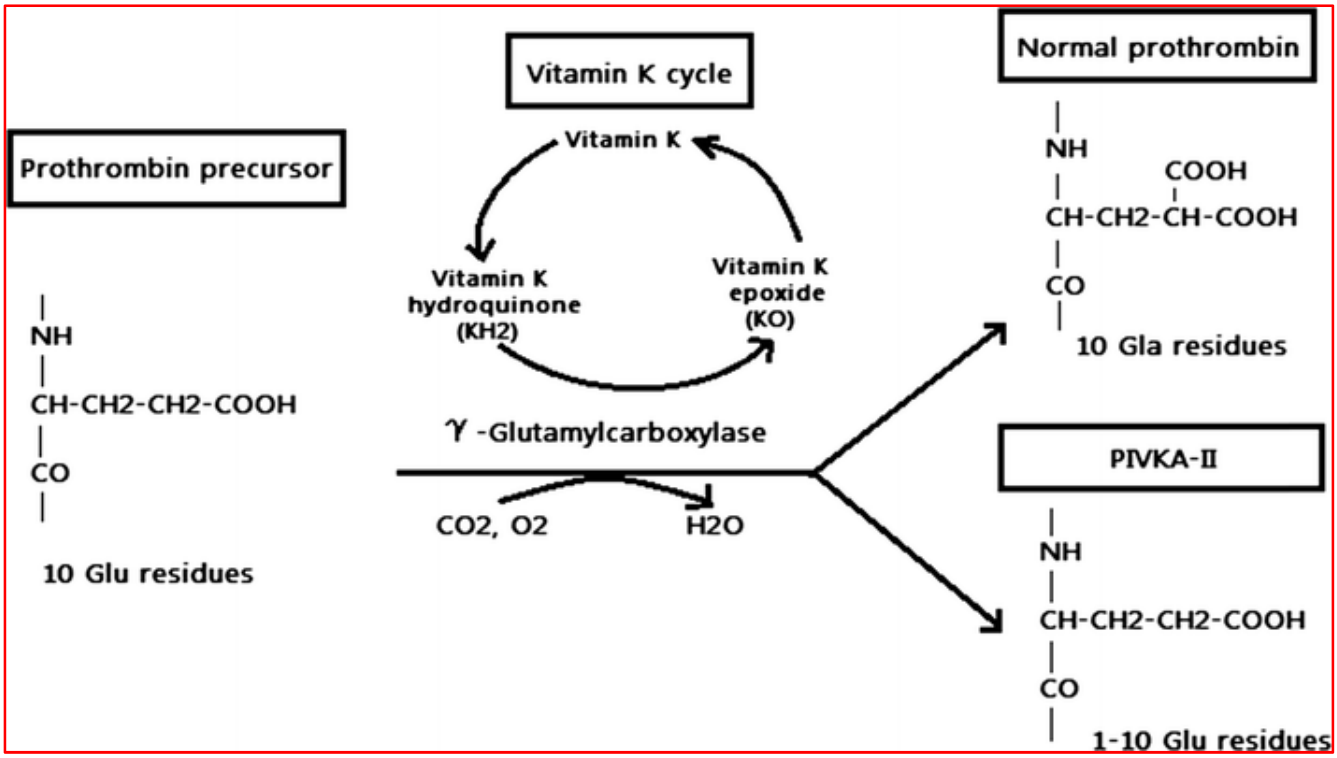


Figure 6: Mechanism of prothrombin and PIVKA-II synthesis [42].

The mechanisms of PIVKA-II production have not yet been fully understood. Some studies have revealed that PIVKA-II is not only present in HCC tissues, but also in non-cancerous tissues, for example in some benign liver diseases, such as acute hepatitis and chronic hepatitis. One of the crucial factors that implies an increase of this marker is played by the γ-glutamylcarboxylase enzyme, dependent on vitamin K, which in normal hepatocytes has the function of converting the prothrombin precursor into prothrombin, in particular catalyzes the complete conversion of 10 Glu residues in the Gla domain of the prothrombin precursor in 10 Gla residues, in the presence of vitamin K as a cofactor (Fig.6) (37).

However, in HCC cells, γ-glutamylcarboxylase is defective, consequently less than 10 Glu residues are converted into Gla residues, and all this leads to protein synthesis of PIVKA-II (38). The catalytic activity of γ-glutamylcarboxylase depends on vitamin K and there is a clear correlation between vitamin K deficiency and PIVKA-II production in HCC tissues. During the γ-carboxylation of Glu residues in lumen of endoplasmatic reticulum, vitamin K is converted from active form, vitamin K hydroquinone (KH2) to vitamin K 2,3-epoxide (KO), which must be recycled to the active form by vitamin K epoxide reductase to

maintain coagulation cycle. Clinical studies have shown that menatetrenone, an analogue of vitamin K2, inhibited HCC recurrence and PIVKA-II synthesis, after surgical resection. This marker is present in conditions where vitamin K is deficient or in patients treated with warfarin or phenprocoumon and furthermore the hypoxic situations present in the HCC tissues lead to the production of this marker (39).These results suggest that PIVKA-II is a biomarker of HCC phenotypic status (40-41).

2.2.3. Biological Function

The biochemical significance of PIVKA-II in cancer has not been completely understood. Recent studies have explored its role and mechanism in cancer survival, proliferation, invasion and metastasis. In 2005 Suzuki et al. reported that PIVKA-II purified from PLC/PRF/5 cells binds to c-Met and this binding leads to activation of the STAT3 pathway via Janus kinase-1 (figure 7) (42). C-Met inhibition by the use of antibodies, siRNA, which is a c-Met inhibitor, significantly suppressed the induction of PIVKA-II in the growth of HCC cells (43). In clinical tissues HCC, PIVKA-II and c-Met usually coexist and their absence is associated with a low risk of tumor recurrence. These results the importance of the c-Met pathway in HCC mediated by PIVKA-II. c-Met pathways, epidermal growth factor receptor (EGFR) and hepatocyte growth factor (HGF) can mediate the biological effects of PIVKA-II in HCC cells. PIVKA-II also controls HCC metastases. In a recent clinical study they found that a high level of PIVKA-II was one of the risk factors for extrahepatic metastases in HCC. Furthermore, PIVKA-II induced angiogenesis in HCC and induced angiogenesis by stimulating the expression of EGFR, VEGF and MMP-2 (44).

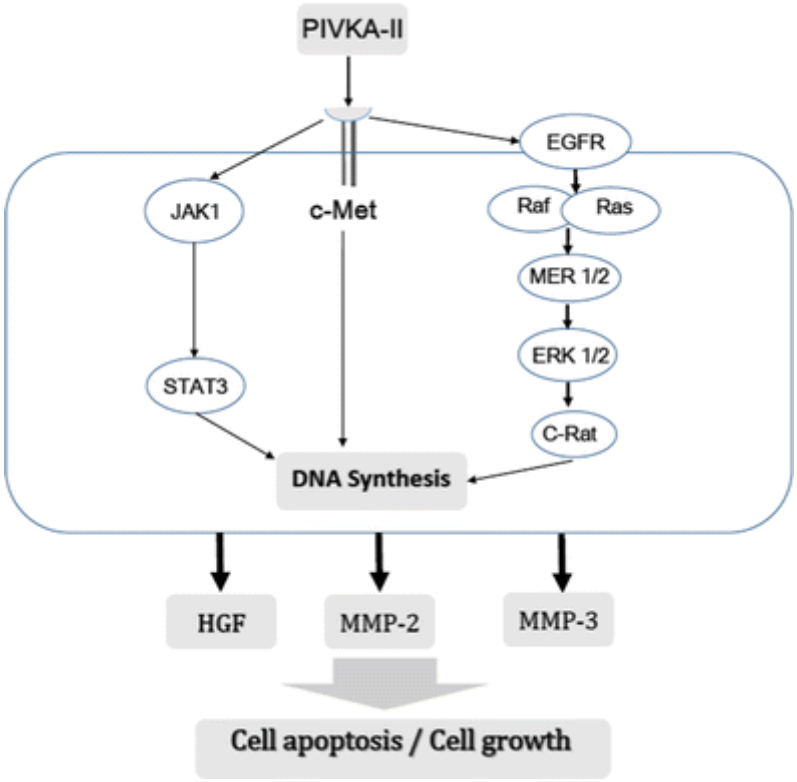


Figure 7: Signaling pathway of PIVKA-II in HCC [43-44].

2.2.4. Clinical usefulness

Preliminary studies indicate that PIVKA levels can be increased in the serum of patients diagnosed with HCC. Evidence in the scientific literature showed that this marker is more sensitive and specific than the markers commonly used in clinical practice (AFP), in particular it has the advantage of increasing directly proportional to the size of the tumor, therefore demonstrates having the property of discriminating the stage of the tumor (early or advanced). Moreover, recent data have shown that elevated serum levels of PIVKA-II and its greater tissue expression are associated with microvascular invasion (MVI), an important risk factor for recurrence and tumor mortality in HCC. In addition to these preliminary experiences, so far PIVKA-II has been used mainly in Asian countries and, despite its potential diagnostic and prognostic value, the experience with this marker in Western countries, and in particular in Europe, remains limited. This marker has shown great potential in monitoring therapeutic effects and in predicting outcomes in the early stages of HCC treatment. In addition to its role in the diagnosis of HCC, PIVKA-II alone or in combination with other biomarkers has also been used in terms of outcome monitoring

and prognosis prediction after various HCC treatments. Starting from these evidences, PIVKA-II is being evaluated as a diagnostic tool both for the surveillance of patients at risk and for the diagnosis of HCC, including the initial phases and in the monitoring of patients with liver cancer in therapy (45).Several studies have pointed out that PIVKA-II can be increased in different malignancies, such as gastric cancer, pancreatic adenocarcinoma and this observation has encouraged the search for its potentiality as cancer biomarker (46).

2.3. Other Biomarkers

❖ Glypican-3, Glutamine Synthase, Heat Shock Proteins

GPC3(Glypican-3), proteoglycan of the cell surface, belongs to the glypican family. This proteoglycan is produced in excess in HCC cells and plays a fundamental role in regulating tumor growth, in particular it is the soluble NH2-terminal fragment of GPC3 that is used as a serological biomarker. It has emerged that it has the ability to accurately distinguish between, patients with small and well-differentiated HCC tumors and those with cirrhosis (47).

Other markers used in combination with GPC3 include HSP70 (heat shock proteins) and GS(glutamine synthase). HSP70 belongs to a class of genes which are abundantly overexpressed in advanced HCC compared to initial HCC and in initial HCC compared to precancerous lesions (48). Furthermore, a study by Osada et al. showed a gradual increase in GS immunoreactivity from precancerous lesions to early and advanced HCC suggesting that GS has a role in HCC (49).

❖ Cytokeratin 19

CK19 (cytokeratin 19) is a novel HCC biomarker associated with poor prognostic factors in HCC patients due to high risk of MVI and distant metastasis, as well as worse treatment outcome (50).

❖ Golgi Protein 73

GP73 (Golgi protein 73) is a transmembrane protein localized in the Golgi complex. Although it is absent in normal hepatocytes, abundantly overexpressed in HCC patients, compared with cirrhotic patients. GP73 could be used as a marker in early-stage (51).

❖ Osteopontin

OPN (osteopontin is a glycoprotein, an extracellular matrix proteinexpressed in HCC cells and other various types of malignancies (52). The low specificity can be explained by its relationship with more than 30 types of cancers. Therefore a combination with AFP is necessary to optimize its performance (53).

❖ Squamous cell carcinoma antigen

SCCA (squamous cell carcinoma antigen) is a serine protease inhibitor. It is found in squamous epithelium.it might play a role as a biomarker for response to treatment as there is an inverse correlation with the treatment response for HCC. Finally, the combination of AFP and SCCA should be investigated in future studies to validate the diagnostic role of SCCA as a predictor for the risk of HCC in patients with chronic liver disease (54).

❖ Annexin A2

Annexin A2 is a calcium-dependent, phospholipid-binding protein. It is present in the cell surface, and it seems to be implicated in the development and metastasis of HCC. It has been used as a serological biomarker for diagnosis and prognosis of early-stage HCC patients with higher sensitivity and specificity than AFP (55).

❖ MicroRNAs

miRNAsare small non-coding endogenous RNAs that have been implicated in various biological roles at the cellular level including apoptosis and oncogenesis.There are several types of miRNAs being tested as diagnostic and prognostic markers for HCC.As a

prognostic factor, low level and down-regulation of miRNA-542 and miRNA-139 are associated with poor prognosis as vascular invasion, larger tumor size and metastatic disease(56).However, the miRNA expression profiles in HCC patients could vary significantly according to the tumor stages. Subsequently, it was difficult to distinguish between patients with different tumor stages. This was a limitation of the diagnostic utility of miRNAs as serological biomarkers.In conclusion, miRNAs are the promising biomarkers in the field of HCC diagnosis, prognosis, and potential therapeutic targets. However, they do not yet fit for the routine clinical setting (57).

❖ Long non-coding RNAs

lncRNA(Long non-coding RNAs) are a unique class which are defined as transcripts of more than 200 nucleotides that present in genome-wide analysis of mammalian transcriptome. Accumulating evidence showed that dysregulatedlncRNA had been involved in the pathogenesis of HCC. Lately, lncRNA has been recognized as important regulators for carbohydrate and lipid metabolism; this has led to discovering a novel biomarker “lncRNAFtx” which stimulateHCC progression and glycolysis. Therefore, lncRNAFtx may act as a prototype for further research in targeted therapy for HCC (58).

❖ Circulating Tumor Cells

One of the most adverse prognostic features of HCC is the presence of vascular invasion which leads to hematological spread and distant metastasis of malignant cells. Therefore, detection of CTCs (circulating tumor cells) has strategic clinical value in predicting HCC recurrence and monitoring treatment response (59).

❖ Cell Free DNA

Dysregulated levels of cfDNA (cell free DNA) have a role in diagnosis, monitoring of treatment response, and even outcome prediction for cancer diseases. Furthermore, single-nucleotide polymorphism of cfDNA such as Ser249 p53 mutation which is commonly found in the plasma DNA, unfortunately, is detected in HCC and non-HCC individuals.

The possibility of using novel biomarkers to predict tumor behavior to targeted therapies is appealing.No biomarker combination is reliable enough to diagnose a lesion as HCC

without confirmatory histological or radiological features. The diagnostic accuracy, particularly for early-stage HCC, can be improved by combining two or more biomarkers to reach an acceptable (> 80%) sensitivity with a modest decrement in specificity. For this purpose,the combination of different biomarkers improves the sensitivity and specificity (60).

3. AIM OF THE STUDY

HCC is one of the most common cause of death from cancer worldwide (1), and is characterized by a low patients survival, despite a moderate range of options therapeutics. This dramatically poor outcome is due to the clinically silent nature of tumor, which cause a diagnosis too late in most case, namely when HCC is at advanced stage. As consequence, an early diagnosis is essential to guarantee a complete and durable recovery. In this view, the challenge for research in the setting of HCC has being the finding of additional tools for the diagnosis, that associated to the imaging tecniques, can help to detect early and easy-to treat tumors, and to discriminate between neoplastic and non neoplastics nodules, that very often complicate the diagnosis usually performed on a cirrhotic liver.

A number of biomarkers, easily measurable on serum, have been proposed and constitute a great attractive, being without risks, performable everywhere, and allowing a strict monitoring without discomfort for patients. PIVKA-II is one of these new markers, and the present study has been designed to evaluate the role of PIVKA-II as diagnostic and on treatment monitoring tools in a real-world cohort of patients affected by HCC.

4. PROJECT DESCRIPTION

4.1. Study design

This study is a prospective, monocentric, exploratory, longitudinal, cross sectional study on patients with HCC, both primary and recurrent. Two control groups, consisting of patients with liver cirrhosis and hepatitis C without HCC complete the study population. Patients have been recruited among patients attending the outpatient clinics of Department of Medical and Surgical Sciences, AOU Sant'Orsola Malpighi, in a real-life context.

4.2. Study population

The study population (protocol approval by local Ethics Committee AOU Sant'Orsola Malpighi 123/2017/O/Tess) consists of 65consecutive patients with HCC, 111 liver cirrhosis (LC) and 111 with chronic hepatitis C (CHC) regularly followed up at the Department of Therapeutic Innovation Chronic Viral Hepatitis (ITEC) of AOU Sant'Orsola Malpighi among patients which have meet the following inclusion /exclusion criteria:

Inclusion criteria

- Adult age (≥ 18 years);
- Males and females;
- Written informed consent to participate to the study;
- Subjects who present only one of the following conditions:

a) HCC (instrumental and or histological diagnosis) primary or recurrent, on liver disease of different etiologies;

b) Liver cirrhosis (in stage A and B of Child Pugh) without HCC of multiple etiologies;

c) Chronic hepatitis C, naives.

Exclusion criteria

- ❖ Inability to give informed consent;
- ❖ Pregnancy or nursing;
- ❖ HIV infection;
- ❖ Any other neoplastic disease in addition to HCC;
- ❖ HCC known in medical history in patients with cirrhosis or chronic hepatitis;
- ❖ Patients with HCC in systemic treatment with antiangiogenetics;
- ❖ Patients receiving coumarin anticoagulant drugs;
- ❖ Concomitant diseases such as to interfere with the laboratory analysis of the marker PIVKA-II, in the Investigator's opinion.

4.3. STUDY OBJECTIVES

Primary Objective:

This study has been designed to evaluate the role of PIVKA-II as diagnostic HCC marker, through the comparison between PIVKA-II serum levels of HCC patients, in a consecutive cohort of patients diagnosed with HCC by imaging techniques or histologyand the two control groups consisting of patients with liver cirrhosis (LC) and chronic hepatitis C (CHC) without HCC. Additional objective has been the evaluation of PIVKA-II as marker of treatment moitoring, and as predictor of tumor recurrence.

Secondary Objectives:

a)To establish the possible correlation between the serum levels of PIVKA-II and the clinical and instrumental features of HCC (number and size of nodules, tumor histology, if primary or recurrent), in order to determine the potential role of PIVKA-II in detect early or late the stage of HCC;

b) To compare the use of PIVKA-II and AFP levels as diagnostic marker of HCC, alone or in combination, to define if the use of multiple markers improves the diagnostic performance and accuracy;

c)To determine the efficacy of PIVKA-II in predicting the response to HCC treatment and the occurrence of relapses in follow-up, through longitudinal determination of the marker levels, in the year following the treatment.

4.4. Methods

After providing informed consent, serum and plasma aliquots of enrolled patients were stored at -80 ℃. Subsequently the samples were thawed and the test was performed to dose this PIVKA-II marker, using serum-immunological methods. Specifically, this protein was quantified by means of a microparticlechemiluminescent assay (CMIA), on an automatic Architect platform that guarantees a highly standardized, controlled and reproducible system.
In detail, the analytical procedure has been performed according to manufacturer's instructions, and in detail:

1. The sample is incubated with a Tris-HCl preservative / diluent solution and paramagnetic microparticles coated with an anti-PIVKA-II monoclonal antibody. The principle is that the PIVKA-II protein, if present in the sample, will bind to the microparticles coated with anti-PIVKA-II antibody.

2. After washing, to eliminate the unbound portion, the anti-prothrombin antibody labeled with acridinium is added to create a reaction mixture.

3. Another washing cycle follows and the Pre-Trigger solution (containing 1.32% w/v of hydrogen peroxide) and the Trigger solution (containing 0.35N of sodium hydroxide) are added to the reaction mixture.

4. The resulting chemiluminescent reaction is measured as its light unit (RLU). There is a direct correlation between the amount of PIVKA-II in the sample and the light unit detected by the ARCHITECT instrumentation. A calibration curve is constructed from a system of calibrators, from which the concentration values of PIVKA-II in the tested samples are easily extrapolated.
For ensuring the validation and accuracy of the data obtained, quality controls have been added at each analytical session.

The measuring interval of PIVKA-II is defined as the range of values in mAU/mL which meets the limits of acceptable performance for both imprecision and bias for an undiluted sample. This range is 5.06 mAU/mL (Limit of Quantitation [LoQ]) to 30000.00 mAU/mL.
Its output range (calibration) is between 0.00-30000.00 mAU/mL.The systemautomatically performs a 1:10 dilution of the specimens with a PIVKA-II value exceeding 30000.00 mAU/mL.Central 95% (mAU/mL): European Population (17.36mAU/mL -50.90mAU/mL), Asian Population (11.12 mAU/mL- 32.01 mAU/mL).

4.5. Visits and Evaluations

After obtaining the informed consent for participation in the study, the patients who were eligible were divided into 3 groups(A, B, C) according to the following schedule:

A) Patients with instrumental and/or histological diagnosis of HCC, primary or recurrent, on liver disease of different etiologies: plasma and serum samples (about 12 cc) were collected at the time of diagnosis and immediately after treatment (24 hours after the end of the procedure) with a curative therapeutic approach (surgical resection, RFA, percutaneous alcoholization) and for non-curative approach (TACE). After treatment, samples were collected every three months thereafter, until one year from treatment, or alternatively, until occurrence of recurrence.

B) Cirrhotic patients (in stage A and B of Child Pugh) without HCC of multiple etiologies, as control group: during one of the scheduled visits for their regular monitoring,a single blood sample was collected and stored.

C) Patients with virus C infection as control group: during one of the scheduled visits for their regular monitoring, a single blood sample was collected and stored.

4.6. Confidentiality of the information collected

The data have be collected anonymously, each patient was encoded by an alphanumeric code. The owner of the personal data is the University Hospital of Bologna, PoliclinicoSant'Orsola-Malpighi, and the person responsible is the Investigator in charge of

the study, Prof. PietroAndreone. The samples have been manipulated only by the he person in charge of the study and coworkers authorized and involved in the study. Similarly, the access to the data has been guaranteed only to person in charge, and Regulatory Authorities.

5. STATISTICAL MANAGEMENT AND DATA ANALYSIS

5.1.Sample size

Since this is a descriptive study for the evaluation of laboratory parameters, not formulated on a specific hypothesis, a formal estimate of the sample size has been not carried out. 65 HCC patients, 111 patients with C virus infection, 111 patients with liver cirrhosis were enrolled.

5.2. Statistical Methods

Given the on normality of variables under evaluation, differences between the clinical groups were analyzed using non parametric Mann-Whitney U test, or Kruskal-Wallis for multiple unmatched groups Correlations between variables were performed using non-parametric Spearman rank correlation. Receiver-operator characteristic (ROC) curve analysis was performed in order to evaluate the diagnostic accuracy as HCC marker of PIVKA-II and Alpha-fetoprotein. The Youden-index was used to select the optimal cut-off. A p value <0.05 was considered to indicate statistical significance with all tests being two-sided.Data handling and analysis were performed with SPSS software for windows, version 17.0 (SPSS Inc., Chicago, IL, USA) and Prism Software,version 13.

6. ADMINISTRATIVE PROCEDURES

This study was conducted in accordance with the principles of Good Clinical Practice [ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996 Directive 91/507 / EEC; D. M. 15.7.1997], to the Helsinki declaration and to the national regulations concerning the conduct of clinical trials. The investigator, signing the protocol, agrees to adhere to the procedures and instructions contained therein and to carry out the study according to GCP, the Helsinki Declaration and the national regulations governing clinical trials. To participate in the study, before being subjected to any specific study procedure, each patient provided written informed consent. Any change in the protocol has been made in the form of amendment. No other modification of the protocol during the study period is allowed. The investigator is responsible for archiving and storing the essential documents of the study, first, during the conduct of the study and after the completion or interruption of the study itself, in accordance with what and for the time required by current legislation and the Good Clinical Practice (GCP).

7. RESULTS

❖ PATIENTS

A total of consecutive 287 patients at different stages of liver disease, fulfilling the inclusion/exclusion criteria,were included in the study. The main demographic and clinical features of study population are reported in Table (n°8). HCC and LC groups showed a similar distribution of risk factors of liver disease: in both the groups the viral aetiology accounted for the majority of enrolled patients, and a multifactorial complex liver disease has been described in about a third.

According to scores for assessing stage of disease and prognosis (CHILD and MELD), no patients presented signs of the most severe liver disease in both the groups: no patients had child C cirrhosis, or Meld > 25. Among the HCC group, the BCLC staging system were 0 in 24 (37%), A in 24 (37%), B in 17 (26).

TABLE 8: BASELINE CLINICAL AND DEMOGRAPHY FEATURES OF PATIENTS ENROLLED

	HCC	LC	CHC

	N=65	N=111	N=111
Age, years: median [range]	66 [48-81]	63 [20-83]	59 [28-84]
Male/female: N [%]	53/12 [81/19]	67/44 [60/40]	41/70 [37/63]
Liver Disease Etiology N [%]			
• Hepatitis C infection	23 [35]	46 [42]	104 [94]
• Hepatitis B (± Delta Coinfection)	9 [14]	11 [10]	
• Alcohol abuse	8 [12]	5 [4]	
• NAFLD	4 [6]	9 [8]	
• Cryptogenetic disease	4 [6]	2 [2]	
• Multifactorial disease			
Viral+Alcohol	*2 [3]*	*14 [13]*	*1 [1]*
Metabolic+Alcohol	*6 [9]*	*5 [4]*	
Viral +Metabolic	*3 [5]*	*13 [12]*	*6[5]*
Metabolic+Autoimmune	*1 [2]*	*1 [1]*	
• Mixed etiology*	5 [8]	5 [4]	
CHILD SCORE			
• A	53 [82]	102 [92]	110 [99]
• B	12 [18]	9 [8]	1 [1]
• C			
Diabetes N [%]	25 [38]	29 [26]	8 [7]
AFP, ng/mL: median [range]	5.6 [1-43857]	3 [1-109]	3 [1-33]
Meld score [range]	9 [3-16]	8 [1-22]	5 [0-9]

*more than three risk factors

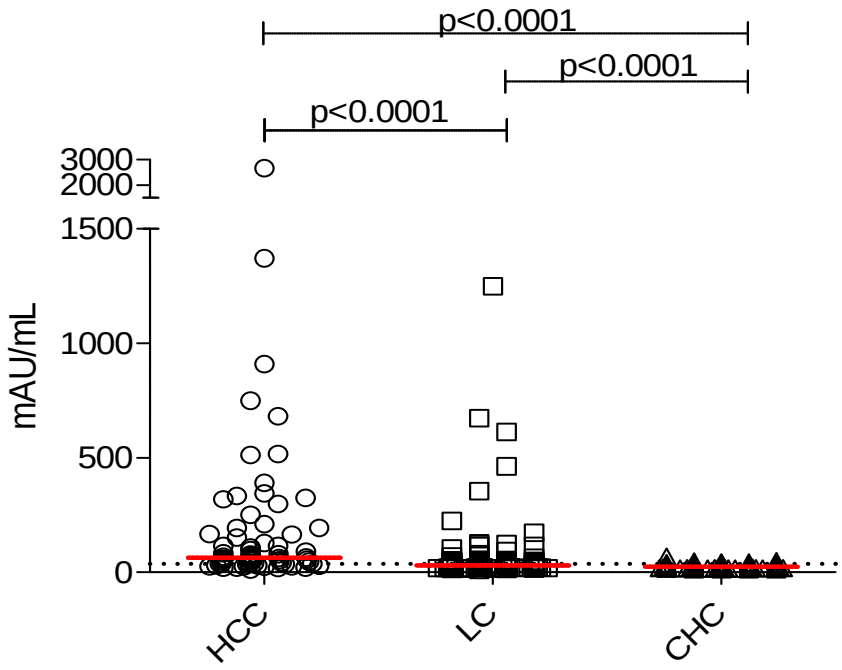
❖ PIVKA-II LEVELS DISTRIBUTION

Considering the overall population of patients included in the study, PIVKA-II levels were spread in a wide range, from 11 to more than 2000 mAU/mL.

When the patients were stratified by clinical group, the values distribution displayed a progressive increase of PIVKA-II values according to stage of liver disease, with significantly higher PIVKA-II levels in patients with HCC than in those without, either patients with LC and CHC(median values HCC: 63.75, range: 12–2675, vs median values LC: 30.95, range: 11.70-1251 and median values CHC: 24.89, range: 12.98-67.68, Figure 1). Furthermore, patients with LC showed higher PIVKA values than CHC (Figure 1).

Figure 1

Cross sectional PIVKA-II distribution

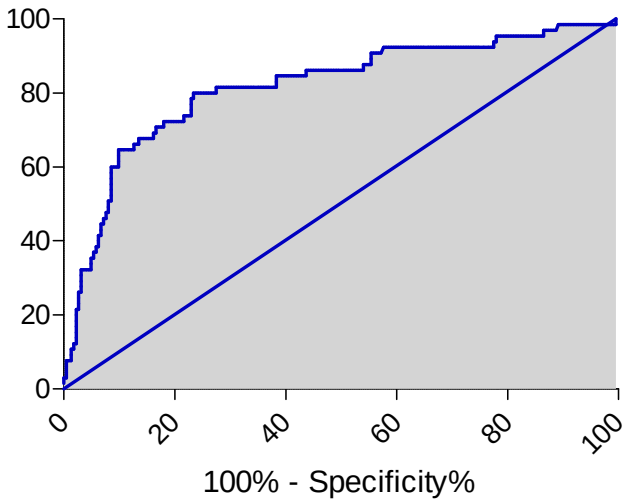


In order to assess and compare the diagnostic performance of PIVKA for the HCC diagnosis, receiver-operating characteristic curve (ROC) analysis was performed. The area under curve (AUC) was 0.817 (95% CI 0.752-0.881), and a value of 37 mAU/mL was

identified as the cut-off with able to optimize the sensitivity and specificity via the Youden-Index (Figure 2). This value yielded 79 and 76 % of specificity and sensitivity, respectively. More in detail, when the established cut-off was applied to the study population, a moderate positive predictive value (PPV) but a good negative predictive value (NPV) were found (Figure 3).

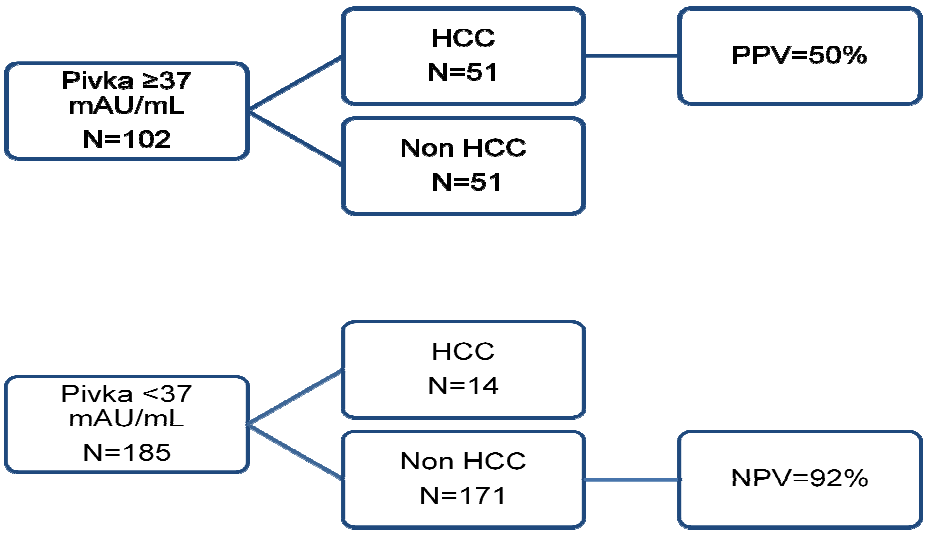
Figure 2

PIVKA Receiver Operating Characteristic (ROC) curve



Area	0.8168
Std. Error	0.03289
95% confidence interval	0.7523 to 0.8812
P value	< 0.0001

Figure 3



Predictive values of the PIVKA cutoff for HCC diagnosis.

❖ PIVKA-II/AFP COMPARISON

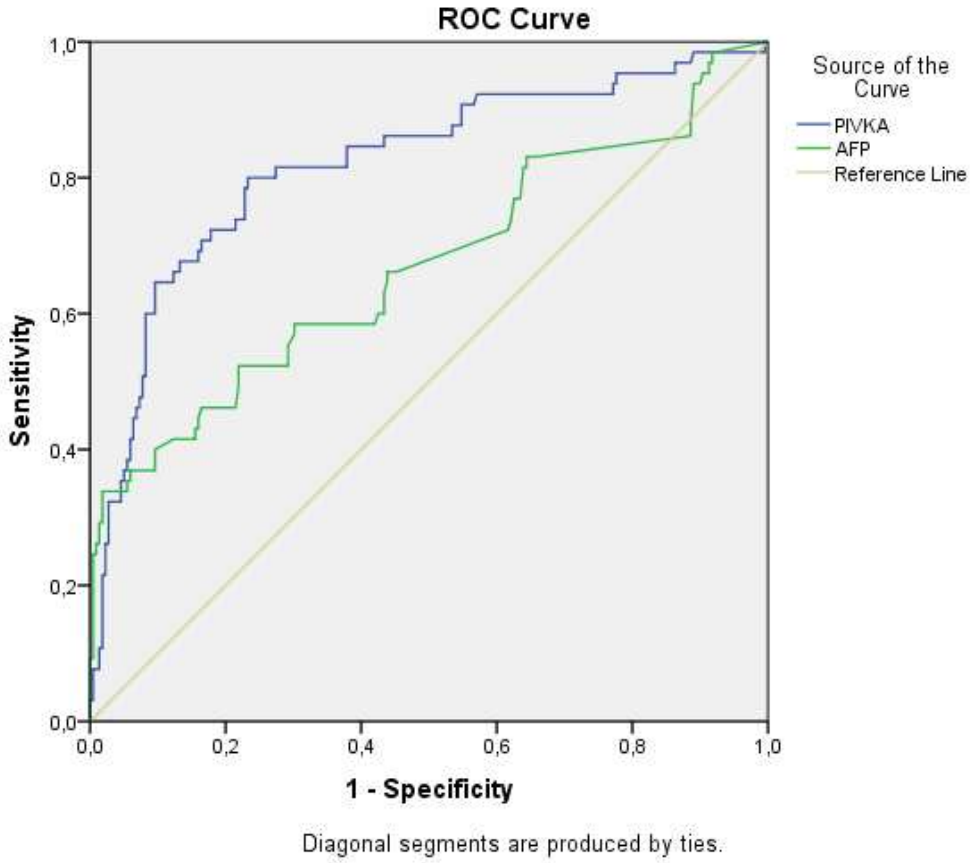
Then the diagnostic potential of PIVKA-II was compared to that of AFP, the most widely clinically used biomarker to date for HCC surveillance. Interestingly, the two biomarkers showed different behaviors in the entire population, showing a low degree of correlation (Spearman r=0.15, p=0.013, data not shown), and when only the HCC group was considered, the two biomarkers did not display correlation at all (r=0,016, p=ns), suggesting a complete differential expression of two biomarkers from the enrolled patients.

The comparison of the two biomarkers as diagnostic tools for HCC highlighted a better performance of PIVKA-II than AFP: the area under the curve was 0,670 (95% CI 0.585-0.754, p<0.0001), significantly different from AUC of PIVKA (0.817) (Figure 4). For AFP, the best cut-off of 16.4 ng/mL yielded 98% of specificity but only 34% o sensitivity.

The application of the defined cut-off on the sample population produced values 83% and 85% of PPV and NPV, respectively (Figure 5).In other words, elevated levels of PIVKA-II were expressed by the majority of patients with HCC, but also by a quarter of patients with liver disease non HCC, thus losing strength in terms of specificity.AFP shows a robust specificity, being upper the defined threshold about exclusively in patients with HCC, but it is abnormally expressed only from a third of patients with HCC.

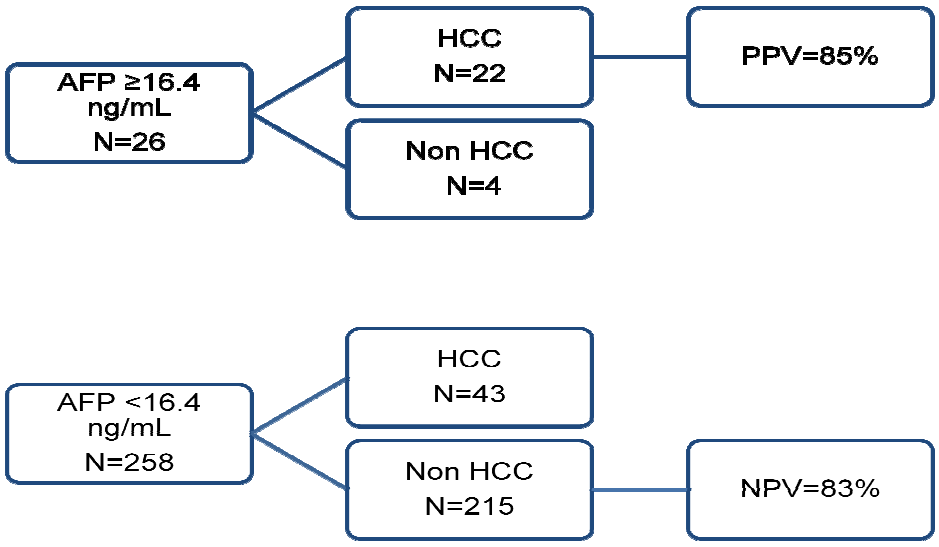
Interestingly, the combination of both PIVKA-II and AFP resulted in the best diagnostic effectiveness, reaching 100% and 85% of PPV and NPV, thus improving the diagnostic capability of the two markers alone (Figure 6). However, being the two biomarkers increased in different patients, only a minimal fraction of HCC patients would be defined by this combined approach (17/65=26%).

Figure 4: Comparison between PIVKA and AFP



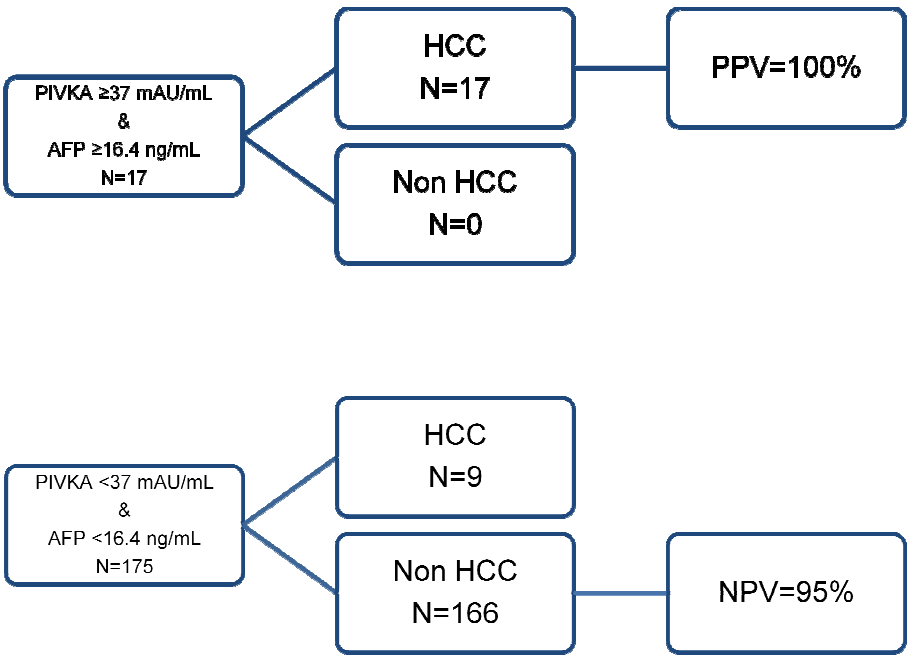
	PIVKA	AFP
Area	0.8168	0.6695
Std. Error	0.03289	0.04290
95% confidence interval	0.7523 to 0.8812	0.5824 to 0.7536
P value	<0.0001	<0.0001

Figure 5



Predictive values of the AFP cutoff for HCC diagnosis.
3 missing data

Figure 6



Predictive values of the AFP and PIVKA combined cutoffs for HCC diagnosis.

❖ PIVKA-II LEVELS AND HCC STAGE

In order to establish if PIVKA-II levels are associated to the HCC stage, patients were stratified according to BCLC stage, and to its main parameters.PIVKA-II values were significantly increased in BCLC-2(B) than in BCLC-1(A) (median 211.48, range: 12.23-2674.98 mAU/mL *vs* median 59.28, range: 19.27-1371.87 mAU/mL, respectively; p=0.0013) and BCLC-0 (median 211.48, range: 12.23-2674.98 mAU/mL *vs* 55.13, range: 25.92-299.61 mAU/mL, respectively; p=0.02). PIVKA-II levels of BCLC-0 and BCLC-A were not statistically different (Figure 7A). In terms of number of nodules, patients with multiple nodules showed PIVKA-II levels higher than patients with single nodule (median 151.0, range: 12.23-2675 mAU/mL, vs median 57.47, range: 19.27-750.5 mAU/mL, respectively; p=0.0474) (Figure 7B). Finally, PIVKA-II values seemed to be not affected by the nodule size, being the two variables not correlated.Summarizing this part, a more advanced stage of HCC associates to higher levels of PIVKA-II.

Figure 7A

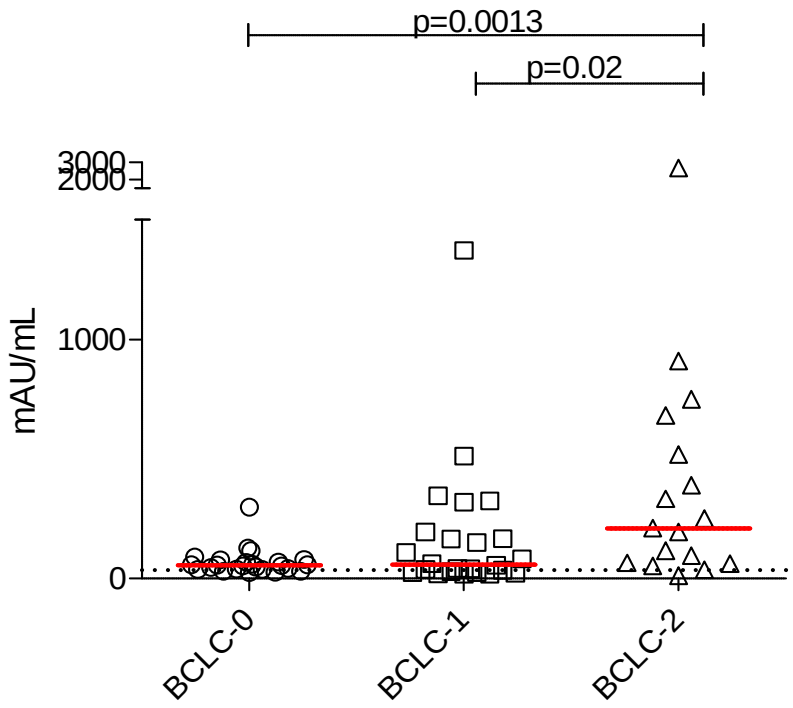
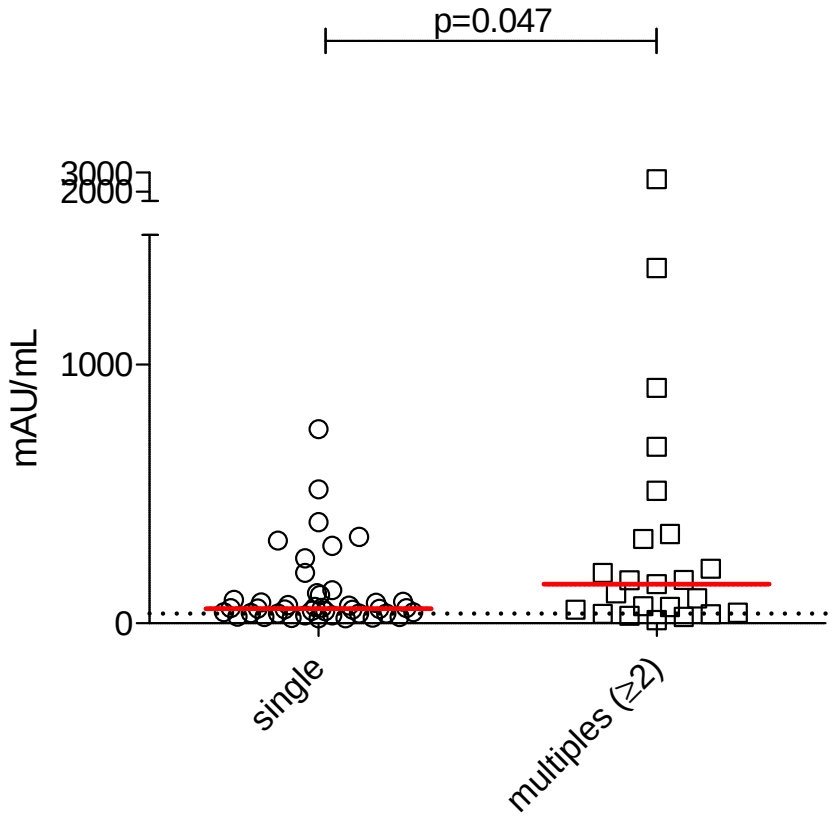


Figure 7B

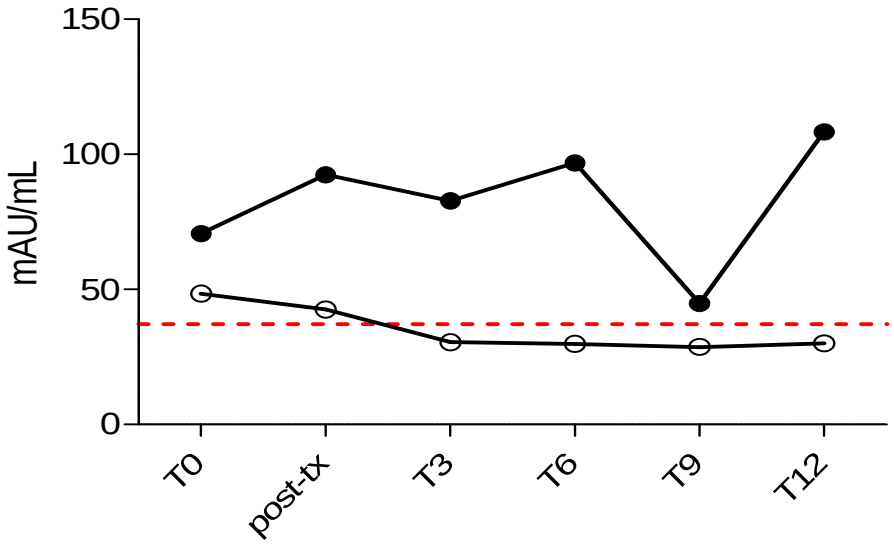


❖ PIVKA-II LEVELS AND HCC OUTCOME

Focusing on HCC group, time course analysis of Pivka-II levels were evaluated in order to assess if baseline values can predict subsequent clinical outcome.Among the 65 patients of the study, 24 (37%) were de novo diagnosis, while the remaining 41 had recurrent HCC (63%). Baseline value were not different between these two groups (median de novo: 56.19, range 12.23- 1372 mAU/mL; median recurrent: 70.69, range 19.83- 2675 mAU/mL; p=ns).After diagnosis, all patients underwent a curative treatment for liver cancer. In the subsequent year of follow-up, 14 maintained a status tumor-free, while 41 had HCC recurrence. Among the remaining patients, 5 withdrawn consent from the study, 5 were drop-out for different reasons, and thus did not complete the study. Interestingly, at

baseline no difference were found in the PIVKA-II levels between patients who would have recurrence compared to patients without recurrence (median recurrence: 77.22, range 21.84- 2675 mAU/mL; median no recurrence recurrent: 48.45, range 21.72-1372 mAU/mL; p=ns). In the time course analysis, patients without recurrence maintain stable levels of PIVKA-II, that did not display relevant changes, although a slight decrease in the median values was appreciated (p=ns). Patients with recurrence showed higher PIVKA-II levels than the counterparts with no recurrence, that tend to increase during follow-up, apart a decrease at month 9. Also these changes were not statistically significant (Figure 8).

Figure 8
Time course analysis



8. DISCUSSION

The proposal has been designed to delineate the role of PIVKA-II as diagnostic HCC markers, through the values comparison between HCC and liver disease without HCC.
This poor outcome of HCC makes mandatory the attempt in optimizing the diagnosis, that should be early as much possible, to give more chances to get a complete recovery.
The possibility to have a number of biomarkers able to help HCC identification has attracted a robust number of research in this setting. The advantages of using biomarkers in routine clinical practice are quite evident: a protein measurable on serum, mainly if an automatic platform is available, can make the screening of risk population a procedure easy to perform everywhere, not requiring sophisticated facilities, or particularly staff's skills; it is reproducible, and relatively low-cost. The rationale of this approach is not to substitute the imaging approach, and in particular US, which has been defined asthe most appropriate testto perform surveillance, according to most recent guidelines; rather to help the diagnosis and positively impact on subsequent outcome. Serological tests for HCC underinvestigation include AFP, DCP also knownas PIVKA-II, and several others. The AFP is the most widely biomarker for HCC, but has suboptimal performance, failing to detect a significant number of HCC, since not all HCC express AFP. Furthermore, the rate of detection of additional HCC not visualized by US is somewhat negligible (2).
PIVKA-II has been proposed as s a promising biomarker of HCC, and a number of evidence are accumulating to assess its diagnostic capability alone or in combination with other biomarkers, in the HCC detection and for surveillance post treatment.
The results of the present study show that patients with HCC present higher levels of PIVKA-II, than patients with liver disease without HCC. The increase in the PIVKA-II values is progressive, according to stage of disease and retraces the natural history of hepatic disease. This result confirm previous data obtained on similar cohorts (45-61-62)underlying the strength of this evidence. This study in comparison to other experiences presents some advantages: first, this is a real-life study population. Patients have been recruited among patients regularly followed up at one single clinical centre, on the basis of few elegibility criteria, with the intent of non creating "ideal" patients. This pre-requisite is responsible for the number of multifactorial disease, and comorbidities characterizing the study population. Indeed, the study has intended to evaluate the diagnostic performance of PIVKA-II in a "true" population, consisting of patients of all ages, with different anamnesis

and different conditions. On the other hand, the recruitment at one single clinical tertiary clinical center has guaranteed the uniformity for study procedure, and for criteria of HCC definition. Another “plus” of the study is the ethnicity of our populations, which consisted of Caucasian subjects. To date, the large majority of PIVKA-II data has been obtained on Asiatic cohorts (40). Due to this more consolidated experience, PIVKA-II is currently included in evidence-based Japanese and Asia Pacific Association for the Study of Liver (APASL) clinical guidelines (63).

The cut-off which has been identified as best cut-off for detect HCC in comparison to other condition is slightly higher than that proposed by Asiatic guidelines, while is almost overlapping to those found in a case-control study on French population (45), and in an Italian study (64). This observation reinforces the need to better investigate European cohorts, as they can greatly differ from Asiatic patients.

Furthermore, our study confirmed that AFP and PIVKA-II are independent biomarkers, being completely unrelated (64). More important, these results show that PIVKA-II presents a better diagnostic performance. Many evidences suggest that PIVKA-II ad AFP should be used in combination, with the purpose to increase sensitivity and specificity. This study is in line with these observations, but the AFP at the cut-off identified by roc analysis, which is only slightly higher than that used in clinical practice, is expressed only from a third of this study cohort. Therefore, the combined use increase the sensitivity and specificity but still a number of patients remains excluded from detection.

An important finding is the association between PIVKA-II values and stage of HCC, expressed by BCLC score, for its implications for outcome and prediction of survival and recurrence. On the other hand, if this aspect limits the use of biomarker in the context of early stage remains to be clarified. No significant differences were appreciated after treatment for HCC. It should be underlined that a more robust follow-up is needed to define the prediction capability of PIVKA-II.

In conclusion, PIVKA-II appear to be superior to AFP in screening for HCC in our cohort of patients; levels of PIVKA-II reflect the severity of the HCC, the combination of PIVKA-II; AFP is inadequate as screening test, and may be considered combined to PIVKA-II, although still suboptimal. These data need to be validated in larger cohorts, and on more long follow-ups.

❖ FIGURE LEGENDS (RESULTS)

Figure 1: PIVKA-II levels mAU/mL in 65 patients with HCC, 111 patients with liver cirrhosis (LC) and 111 patients with Chronic Hepatitis C (CHC). Each symbol represents anindividual patient, with red horizontal bars showing the median values per patient group.

Figure 2: Receiver Operating Characteristic (ROC) curve for PIVKA-II as diagnostic biomarker for HCC.

Figure 3: Predictive values of the PIVKA-II cutoff 37 mAU/mL on identification of HCC patients.

Figure 4: Receiver Operating Characteristic (ROC) curve for PIVKA-II and AFP as diagnostic biomarker for HCC. Blu line= PIVKA-II; Green line= AFP.

Figure 5: Predictive values of the AFP cutoff 16.4 ng/mL on identification of HCC patients.

Figure 6: Predictive values of combination PIVKA-II/AFP at established cut-offs on identification of HCC patients.

Figure 7: PIVKA-II levels mAU/mL in 65 patients with HCC, divided for BCLC score.
BCLC-0=0 (24 patients); BCLC-1= A (24 patients); BCLC-2= B (17 patients). Each symbol represents anindividual patient, with red horizontal bars showing the median values per patient group (7A). PIVKA-II levels mAU/mL in 65 patients with HCC, divided for number of nodules (7B).Single nodule= 42 patients; Multiple nodules= 23 patients.
Each symbol represents anindividual patient, with red horizontal bars showing the median values per patient group.

Figure 8: Time course analysis of PIVKA-II kinetics after diagnosis. X axis: time in months
Y lines: median PIVKA-II values mAU/mL in patients with recurrence (filled dots), and with no recurrence (empty dots).

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