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Review

Alternative Biomarkers to Predict Tumor Biology in Hepatocellular Carcinoma

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Abstract. *Hepatocellular carcinoma (HCC) is the most frequent primary malignant liver tumor, with more than 800,000 new cases diagnosed each year and with high mortality, ranking fourth in the world in cancer deaths. The worst prognosis is related to the late diagnosis, in which the tumor is at an advanced stage and curative treatments are not efficient in terms of increasing overall survival. Currently, screening and monitoring tests based on current guidelines have limited accuracy, which points to the need for the development of new biomarkers that improve HCC detection as well as its early diagnosis. This review will discuss the five phases of development of a biomarker, from its discovery to its application in clinical practice, and indicate the main biomarkers per development phase. Potential emerging technologies such as "Radiomics", "Proteomics" and "Metabolomics" will also be discussed, which should serve as tools for the elucidation of tumor heterogeneity, as well as provide data for future studies on HCC biomarkers.*

Hepatocellular carcinoma (HCC) is the most frequent primary tumor of the liver, and its incidence is increasing, currently occupying the sixth position among the incidences of all cancers, and the fourth position among cancer-related deaths (1). The prevalence of HCC is higher in men than in women (1). As for the geographical distribution of the disease, HCC has the highest incidence in developing countries, with more than 80% of cases reported in sub-Saharan Africa and East Asia as a result of the hepatitis B endemic in these regions; while the incidence has been decreasing in some Eastern countries such as China and Japan due to vaccination programs and treatment of viral hepatitis. Cases of HCC have increased in Western countries, such as the United States, related to the aging population of patients with hepatitis C and the emergence of non-alcoholic steatohepatitis (NASH) as a chronic liver disease (2, 3). The pre-existence of cirrhosis is estimated in 80% of patients diagnosed with HCC (4), but currently any etiological agent leading to chronic liver lesions and cirrhosis is considered a risk factor associated with the development of HCC, the most common being hepatitis B, hepatitis C, high alcohol consumption and NASH (5).

Once diagnosed, the prognosis of the HCC patient depends, among other factors, on the intention of the treatment: curative or palliative. Among the curative treatments, surgical resection, local ablative therapies and liver transplantation may be indicated, the latter being the most indicated for early stage patients and the one offering the best overall survival (6, 7). Other factors that directly influence the patient's prognosis, such as number of lymph nodes involved, presence of metastasis, tumor size and history of hepatitis, the analysis of these factors being important in the definition of survival rates and individualized therapeutic plans (8).

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The development of biomarkers as complementary diagnostic methods and in the follow-up after treatment, has been the object of study in the last two decades, but before their widespread adoption, there are validation phases (9, 10). In this review, the development stages of the current biomarkers will be addressed and the most promising ones in the early detection of HCC will be highlighted. Emerging technologies that may assist in the diagnosis of HCC in the coming years, such as artificial intelligence, are also pointed out.

Current Recommendations

The diagnosis of HCC is made by imaging examinations or liver biopsy, but for early detection of HCC, screening of asymptomatic patients by monitoring tests should be performed. Therefore, the American Association for the Study of Liver Diseases (AASLD) recommends monitoring every 6 months by abdominal ultrasonography, with or without alpha-fetoprotein (AFP) dosage, in all adults with cirrhosis, except in cirrhotic patients with Child-Pugh C Score who are not on the transplant list (6). Active surveillance then becomes an early detection tool for HCC, which is directly associated with improved survival rates of patients, as demonstrated by the meta-analysis of Singal *et al*. (11).

Despite the surveillance recommendations, there are flaws in this process (12). In a meta-analysis, Singal tried to characterize the performance of abdominal ultrasound in the detection of HCC and obtained only a 63% sensitivity in the detection of early-stage tumors, without any statistically significant improvement in sensitivity when associated with the serum AFP examination (13). Lack of screening and follow-up contribute to the detection of HCC in more advanced tumor stages in one-third of the cases; however, the low sensitivity in detecting the tumor in currently available tests represents the most common cause of late diagnosis, suggesting the need for more effective investigation strategies (12).

In Brazil, the Diagnostic and Therapeutic Guidelines for hepatocellular carcinoma were published by the Ministry of Health in 2012, and recommend screening for HCC by abdominal ultrasonography every 6 months, associated or not with AFP dosage, among patients diagnosed with hepatic cirrhosis who may benefit from curative treatments (patients classified as Child-Pugh A-C without comorbidities) (14). The diagnosis can be preferably made by radiological methods such as computed tomography, magnetic resonance imaging or contrast ultrasonography, reserving the anatomopathological diagnosis to non-cirrhotic patients and to cases in which the radiological methods are inconclusive (14). Screening patients in risk groups is the best way to ensure an early diagnosis and the possibility of healing, and increase the overall survival of these patients (15).

Development of Biomarkers

A biomarker can be defined as an objective and quantifiable feature of a biological process (16, 17). In practice, we can define it as a clinical sign, a parameter observed by the physician, which can be precisely measured and which can be reproduced, and can be used in cancer screening, in measuring therapeutic response or in defining prognosis (17). In HCC, biomarkers are widely applied, whether in decision making for a therapeutic choice or in the selection of patients most likely to get the best results from different treatments (18). The development of potential biomarkers for HCC faces challenges, mainly due to the tumor heterogeneity in this cancer, which makes its clinical applicability difficult (16, 18). Recently, gene expression analysis of known potential biomarkers of HCC was used to correlate their expression in tumoral tissues with tumor prognosis (16).

The expansion of studies on biomarkers as monitoring tools led to the establishment of the Early Detection Research Network (EDRN) by the National Cancer Institute (NCI), with the objectives of coordinating research among the biomarker development and validation laboratories, in order to maintain rigor and promote collaboration among them, generating greater efficiency in the studies (10, 19). With the objectives of EDRN as a basis, Pepe *et al*. categorized the development of biomarkers into five phases, from their discovery to their application, as a monitoring tool in the early detection of cancer (10).

Phase 1. Phase 1 development usually begins with pre-clinical studies, comparing tumor tissue with non-tumoral tissue (10). The objective is to identify unique characteristics of tumor tissues that may lead to a candidate biomarker (10). Techniques such as immunohistochemistry, western blot and more recent technologies such as gene expression profiles based on mass spectrometry have been used to identify genes or proteins in tumor tissues when compared to control tissues (10). Thus, phase 1 aims to identify and prioritize potential biomarkers (establishing the best case selection and control for the development of studies), establish reliable and reproducible assays, determine how well the biomarker in question distinguishes case and control, analyze data obtained and conduct other confirmatory studies with new tissue samples (10).

Phase 2. Phase two consists of clinical trials executed with samples obtained in a non-invasive manner (10). The choice of samples, as well as their sizes, needs to be carefully considered, and even if blood bank samples are used, it is recommended that the final conclusions be obtained from studies with population samples (10). The clinical trials should be performed to show the ability of the biomarker to distinguish between the samples with and without cancer,

thus allowing it to be considered promising for screening (10). An ideal biomarker for early detection of cancer should be highly sensitive, which indicates that the main objective of phase 2 is estimate the true-positive and false-positive rates in the trial and the ability of the biomarker to distinguish between samples with and without cancer, in addition to optimizing procedures to ensure the reproducibility of the trial in other laboratories (10). Phase 2 has other important objectives, such as evaluating the variations in the levels of the biomarker in question based on clinical-epidemiological factors of the individuals, such as age, gender and also based on tumor characteristics, such as histology, tumor staging and prognosis (10).

Phase 3. The main objective of phase 3 is to evaluate the ability of the biomarker to identify the disease before clinical diagnosis and thus, define a criterion for a positive test (10). Analyzing the group of cases, a biomarker whose levels change months before the diagnosis of the disease has more potential to become a useful tool in early detection when compared to a biomarker whose levels change days before the diagnosis (10). In this way, this capacity is evaluated and a criterion is defined to classify the test as positive (10). In addition, this phase aims to explore the impact of variables such as demographic factors, characteristics associated with the disease and clinical information of individuals on the discriminatory ability of the biomarker and, in case the biomarker performs better in certain subgroups, assist in the development of prospective studies of phase 4 (10).

Phase 4. Phase 4 consists of a prospective study, in which the objective is to describe the characteristics of the tumor detected by the screening test and these characteristics with possible benefits offered by early detection (10). The samples in this phase should be chosen based on a population that is possibly the target of the screening, that is, a group at risk for development of the disease, which will improve the performance of the biomarker when compared to phase 3 (10). Based on the results obtained, the receiver operating characteristics of the screening test are determined, such as the true-positive rate (proportion of those tested positive versus those that have the disease) and the false-positive rate (proportion of those who tested positive versus those that do not have the disease) (10).

Phase 5. Phase 5 assesses whether screening reduces mortality from the disease (10). Even when there is early detection of the disease, there may be no benefits for the specific population (10). The reasons for this may be related to ineffective treatment of the detected tumors, difficulties in implementing screening programs or overdiagnosis (10). This phase also considers cost-benefit information, assesses compliance with screening and compares the different approach protocols in the treatment of screened diseases in relation to their effects on mortality and cost (10).

Biomarkers for HCC per Development Phase

Table I presents the main biomarkers per study development phase for HCC as well as their applications and initial results.

Phase 1. MicroRNAs. MicroRNAs (miRNAs) are post-transcription gene regulators, which silence messenger RNA and thus, play a homeostatic role by adjusting protein translation, while their deregulation is related to tumor progression (20). The potential of 19 circulating microRNAs as biomarkers for HCC has been studied by Huang *et al*. (21). In this meta-analysis, when the ability of the marker to differentiate between HCC patients and control group individuals was evaluated, the sensitivity and specificity of miRNA-21 was 86.6% and 79.5, respectively, while miRNA-122 obtained a sensitivity of 68% and specificity of 73.3% (21). Huang *et al*. have studied the ability of miRNA to differentiate between patients with HCC and hepatic cirrhosis (22). They obtained 16 miRNAs with a statistically significant difference between groups, namely miRNA-15a, miRNA-21, miRNA-29a, miRNA-30c, miRNA-486-3p, Let-7g, miRNA-122, miRNA18a, miRNA-338-3p, miRNA-126, miRNA-222, miRNA-223, miRNA-26a, miRNA192, miRNA-27a and miRNA-124 (22). In the recent study by Wu *et al*., serum levels of miRNA-199a in patients with HCC were significantly lower than those in patients with hepatic cirrhosis, pointing to its potential as a marker for the diagnosis of HCC (23). Thus, the study of microRNAs becomes a useful tool for the development of HCC markers (20).

Metabolomics. Metabolomics is a field of study that aims to provide information about endogenous metabolites in a biological sample, and with these data one can map biochemical changes in a disease and thus develop potential predictive biomarkers (24). Intracellular and extracellular metabolic changes, as well as metabolic pathways, are associated with tumor progression and reprogramming of the microenvironment caused by tumor growth (25).

Recent advances in the Metabolomics field can provide several new types of markers for HCC (26). Wang *et al*. have proposed an analysis of metabolic profiles based on high efficiency liquid chromatography (UPLC) and mass spectrometry (MS) of samples of HCC patients with cirrhosis of the liver and healthy individuals (27). The metabolic profile showed 100% sensitivity and specificity in differentiating HCC patients from those with hepatic cirrhosis, and 79.3% sensitivity and 100% specificity in the diagnosis of HCC. In addition, the analysis of the profiles indicated 13 potential biomarkers suggestive of metabolic disorders, such as phospholipid and organic acid alterations, in patients with

Table I. *Biomarkers available in the studies of HCC.*

Phase	Biomarker	Application	Results	Author
PHASE 1	miRNA 21	Differentiation between HCC patients and control group	SN: 86.6% SP: 79.5%	Huang *et al.* (21)
	miRNA 122	Differentiation between HCC patients and control group	SN: 73.3% SP: 79.5%	Huang *et al.* (21)
	miRNA	Differentiation between HCC and CH patients	16 miRNA as potential biomarkers	Huang *et al.* (22)
	miRNA 199a	HCC diagnosis in CH	Significantly lower serum values in HCC patients	Wu *et al.* (23)
	Metabolomics	Differentiation between HCC and CH patients	SN: 100% SP: 100%	Wang *et al.* (27)
		HCC diagnosis	SN: 79.3% SP: 100%	Wang *et al.* (27)
		Differentiation between HCC and CH patients	Potential in early diagnosis	Luo *et al.* (28)
	Proteomics	HCC diagnosis in patients with AFP<20 ng/ml	HCC detection rate: 89%	Wang *et al.* (31)
	Radiomics	Recurrence Forecast	AUC predictive: 0.83	Zhou *et al.* (35)
PHASE 2	hs-AFP-L3	HCC diagnosis in patients with AFP<20 ng/ml	SN: 41.5% SP:85.1%	Toyoda *et al.* (45)
	Dkk1 + AFP	HCC diagnosis	SN: 80% SP: 87%	Li *et al.* (48)
	Gpc3 + CD34	Differentiation between patients with HCC and benign hepatic lesions	SN: 82%	Enan *et al.* (54)
	AFU	Differentiation between patients with HCC and CH or HC	SN: 90% SP: 97.5%	Montaser *et al.* (57)
	Scca-IgM	HCC diagnosis	SN: 89% SP: 50%	Pozzan *et al.* (59)
	GP73	HCC diagnosis	SN: 78.3% SP: 85.4%	Jiao *et al.* (63)
PHASE 3	OPN	HCC diagnosis in CH or CH and AFP patients <20 ng/ml	SN: 78.26% SP: 80.45%	Zhu *et al.* (66)
	OPN + AFP	HCC diagnosis	SN: 88.12% SP: 74.21%	Zhu *et al.* (66)
	OPN	HCC diagnosis	SN: 79.21% SP: 79.24%	Zhu *et al.* (66)
	MDK	HCC diagnosis	SN: 86% SP: 75.4%	Zhang *et al.* (69)
		HCC diagnosis	SN: 88.5% SP: 80.6%	Zekri *et al.* (70)
		Serum values in HCC and AFP patients <20 ng/ml	Significantly higher serum values in 59.18% of patients	Vongsuvanh *et al.* (68)
	DCP	HCC diagnosis	SN: 66% SP: 88%	De *et al.* (72)
	GALAD	HCC diagnosis	SN: 91.6% SP: 89.7%	Berhane *et al.* (75)
PHASE 5	AFP+US	HCC diagnosis in CH	SN: 97%.	Tzartzeva *et al.* (78)
	AFP	Use of AFP cut-off values	Improved diagnostic accuracy and posttreatment follow-up	Zhang *et al.* (81)
		Prediction of liver transplant results	AFP predictive global survival after liver transplantation	Merani *et al.* (82)
		Prediction of recurrence after liver transplantation	Preoperative predictive global survival AFP	Hameed *et al.* (84)

AFP: Alpha-fetoprotein; AFU: alpha-1-fucosidase; AUC: area under the curve; CHC: hepatocellular carcinoma; DCP: descarboxiprothrombin; DKK-1: Dickkopf-1; GALAD Scores: acronym for Gender, Age, AFP-L3, AFP, DCP; GP-73: Golgi protein 73; GPC-3: Glipican-3; hs-AFP-L3: alpha-fetoprotein fucosilate highly sensitive; MDK: midkine; miRNA: micro RNA; OPN: Osteopontine; SCCA-IgM: immunocomplex of squamous cell carcinoma antigen with immunoglobulin M; SN: sensitivity; SP: specificity; US: ultrasonography.

HCC (27). Luo *et al.* have characterized the serum metabolic profiles of 1448 individuals using UPLC and MS-based methods and a panel was defined after selecting the appropriate biomarkers (28). The ability of the panel to discriminate between HCC and cirrhosis patients was compared with that of the AFP values, and was found to be more efficient (AUC 0.866 *vs.* 0.682), revealing its role in early stage HCC screening (28). The analysis of serum metabolites values is a promising tool for predicting metabolic changes at tissue levels due to the tumor installation (25).

Proteomics. Proteomics is an area that studies the complete set of proteins expressed in a cell, tissue or biological fluid (29). Proteomics, besides determining protein expression profiles, also identifies structure, location, activity, modifications and interactions of proteins in physiological or pathological conditions (29). In HCC, proteomics has the potential not only to elucidate the mechanisms of installation and progression of HCC, but also to provide a basis for the development of biomarkers for the disease (29). Wang *et al.* have proposed a useful algorithm to predict HCC that associates AFP with 4 other clinical variables, being age, sex, alkaline phosphatase and alanine aminotransferase (30). More recently, they added to the algorithm the biomarker fucosilated kininogen, to improve HCC detection in early stages and in patients with AFP<20 ng/ml (31). As a result, they obtained AUC greater

than that of AFP alone (0.977 *vs*. 0.828), and 89% HCC detection rate in patients with AFP<20 ng/ml (31).

Gao *et al*. have conducted a proteogenomic HCC characterization in patients with hepatitis B (32). They found two enzymes, PYCR2 and ADG1A, which can be potential biomarkers for the prognosis of HCC, and observed lower expression of hepatitis B virus proteins and receptors in patients with HCC suggesting that metabolic changes are related to the advanced stage of the disease and worse prognosis, and may serve as a basis for future studies on HCC treatment (29). In general, proteomic data can provide information that helps in the clinical, biological and therapeutic understanding of HCC (29).

Radiomics. Radiomics is an emerging field that is based on the conversion of images from exams such as computed tomography and magnetic resonance imaging into high dimension data, which are analyzed by artificial intelligence to reveal hidden pathophysiological characteristics that, when added to the patient's characteristics, function as a prognostic or diagnostic tool (33). Regarding HCC, "*omics*" although at embryonic stage, it has presented promising results (34). Zhout *et al*. published a study with 215 patients to predict early recurrence of HCC based on the radiomic characteristics of the patients and obtained a predictive AUC of 0.83 in a combined model, showing that the Radiomics signature may be promising in predicting early recurrence (35). Zheng *et al*. have reported that the combination of factors obtained from the *Radiomics* model with clinical variables of the patients has good performance in predicting microvascular invasion after HCC resection (36). Radiomics has shown promise in diagnosis, choice of therapeutic treatment, screening evaluation and prognosis of HCC (37).

Polo-like Kinase Proteins

Polo-like kinase proteins (PLK) are characterized by an N-terminal serine/threonine kinase domain, highly conserved at one or two polo boxes in the C-terminal region, which are crucial for subcellular location, specific phospho-peptide binding and centrioles duplication (38). PLK-4 shows cytoplasmic expression in most human tissues. The protein is located in centrioles and microtubules, and regulates the duplication of centrioles during the cell cycle. Also, tissue expression of PLK-4 is very variable among human tissues, and depends on the number of cells with a high concentration of centromeres, which is directly related to the cellular function of tissues during cell division (39). It is considered a prognostic marker in colorectal cancer (favorable), lung cancer (unfavorable) and pancreatic cancer (unfavorable) (39). In HCC, low expression of PLK-4 is possibly adversely associated with global and disease-free survival in preclinical models (38, 40-43).

Phase 2

AFP-L3. AFP-L3 is an AFP isoform and its percentage relative to the total AFP has been used as a marker for HCC detection, with a cut-off value of 10% (44). In the study by Toyoda *et al*., hs-AFP-L3 (highly sensitive AFP-L3) was tested in HCC patients under surveillance with AFP values below 20 ng/ml (45). As a result, for cut-off value of 5% of hs-AFP-L3 relative to total AFP, sensitivity 41.5% and specificity 85.1% in diagnosis of patients with AFP<20 ng/ml were obtained (45). In that study, the relationship between the percentage of hs-AFP-L3 and prognosis was also evaluated, showing that the measurement of hs-AFP-L3 before treatment can help predict prognosis and define therapies (45). Thus, it has been shown that hs-AFP-L3 may be useful in the early detection of HCC in patients with AFP<20 ng/ml, having improved sensitivity when combined with Des-carboxy-prothrombin, and may be part of pretherapy as a diagnostic predictor (45).

Dickkopf-1 (DKK-1). DKK-1 is a glycoprotein that acts as an inhibitor of the Wnt/beta-catenine signaling pathway (46). Shen *et al*. have shown significantly higher serum DKK-1 levels in HCC patients when compared to healthy individuals with chronic hepatitis B or liver cirrhosis (47). In the meta-analysis of Zhenije *et al*., the combination of DKK-1 and AFP showed better accuracy in the diagnosis of HCC when compared to the single use of these biomarkers (48).

Glipican-3 (GPC3). GPC3 is a member of the proteoglycan heparan sulfate family, comprised of 6 subtypes of glypicans (GPC1-6), which have several functions, the main one being to regulate Wnt, Hedgehog, bone morphogenic protein (BMP) and fibroblastic growth factor signaling (49). GPC3 is present in different stages and different tissues during embryonic development, which indicates its involvement in morphogenesis (50). In adult liver, no GPC3 expression is detected (51) while microarray analysis of tissue samples revealed increased GPC3 expression in 63.6% of HCC patients (52). The mechanisms involving GPC3 in the development of HCC have been described by Zhou *et al*., and include stimulation of Wnt signaling, interaction with growth factors, stimulation of macrophage recruitment and promotion of epithelium-mesenchymal transition (EMT) (53). The literature indicates GPC3 as a highly specific biomarker, and the combination of markers has shown improved sensitivity in the distinction between HCC and non-malignant hepatocellular lesions, as Enan *et al*. pointed out by combining GPC3 with CD34 and obtaining 82% sensitivity (54). Nault *et al*. have studied the serum levels of GPC3 in patients with hepatic cirrhosis, obtaining significantly higher values in patients with advanced HCC when compared to patients without HCC or in patients with early stage disease (55).

Alpha-1-fucosidase (AFU). AFU is a lysosomal enzyme that can be detected in the serum of healthy patients, but its activity is increased in patients with HCC, cirrhosis and chronic hepatitis (56). In the study of Fawzy *et al*., the AFU values of 80 patients (40 with HCC and 40 with chronic liver diseases) and 40 healthy individuals were measured and compared (57). The results showed significantly higher serum AFU values in the HCC group when compared to the other two groups ($p<0.001$), and for AFU cut-off value of 2.3005 micromol (l^{-1}) (min^{-1}) the sensitivity and specificity delivered were 90% and 97.5%, respectively (57). The study also found a decrease in AFU values in patients who underwent local therapies for HCC [radiofrequency ablation (RFA) or trans-arterial chemoembolization (TACE)] (57). Thus, AFU has shown promise as a biomarker for the diagnosis of HCC and for the follow-up of therapeutic results (57).

Squamous cell carcinoma antigen (SCCA). SCCA is a serine protease inhibitor, physiologically found in squamous epithelium, but also expressed in epithelial cell neoplasms (58). Caterina *et al*., have studied the serum levels of the immunocomplex SCCA with IgM (SCCA-IgM) in patients with HCC and found sensitivity and specificity values of 89% and 50%, respectively, regarding the diagnosis of HCC (59). In the meta-analysis of Liu *et al*., both SCCA and SCCA-IgM showed moderate diagnostic accuracy in HCC, but combining SCCA-IgM with AFP there was an increase in diagnostic accuracy and early detection of HCC (60).

Golgi protein-73 (GP73). The GP73, is a transmembrane protein of the Golgi apparatus expressed in the normal liver, is mostly secreted by cells of the biliary epithelium and has insignificant expression in the hepatocytes (61). It was isolated for the first time in 2000, when its expression was observed in liver tissue cells of patients with cirrhosis and chronic hepatitis but not in normal liver cells in *in vitro* experiments (61). Later, it was demonstrated that serum levels of GP73 are significantly higher in HCC patients compared to healthy individuals (62). In the study of Jiao *et al*., 180 patients with HCC, 61 with cirrhosis of the liver, 99 with chronic hepatitis, and 103 healthy individuals, were submitted to blood sampling (63). It was shown, that the serum levels of GP73 were significantly higher in patients with HCC when compared to those in the other three groups (63). From the analysis of clinical and pathological variables, the presence of lymphatic metastasis was associated with high GP73 levels in patients with lymph node metastasis (63). As for HCC diagnosis, GP73 showed, for the optimal cut-off value of 117.53 ng/ml, a sensitivity of 78.3% and a specificity of 85.4%. However, AFP showed sensitivity and specificity values of 51.1% and 99%, respectively, for the optimal cut-off value of 176.91ng/ml, which shows that GP73 is more efficient in the early detection of HCC compared to AFP (63). The combination of GP73 with AFP presented the largest AUC, and obtained better sensitivity and specificity values (63).

Phase 3

Osteopontine (OPN). OPN is a multifunctional protein that is involved in several pathological processes such as inflammation and carcinogenesis in several tissues (64). In HCC, the plasma levels of OPN have been considered as a potential biomarker (65). Zhu *et al*. have conducted a study with 322 patients (105 with chronic hepatitis, 116 with cirrhosis and 101 with hepatocellular carcinoma) (66). Serum samples from patients were obtained before any invasive procedure, such as surgery or biopsy, and before any non-surgical oncologic treatment, such as chemotherapy or radiotherapy. The results showed significantly higher OPN values in patients with HCC (median 39.84 ng/ml, IQR=15.55-91.81) when compared with the values of patients with chronic hepatitis (median 10.18 ng/ml) and liver cirrhosis (10.93 ng/ml) (66). In the diagnosis of HCC in the risk group (patients with chronic hepatitis and liver cirrhosis) and AFP-negative individuals (with cut-off value of 20 ng/ml), OPN showed sensitivity of 78.26% and specificity of 80.45% (66). In the diagnosis of HCC, OPN reached higher values of AUC when compared to AFP [AUC: 0.851 (95%CI=0.807-0.888) *vs*. AUC: 0.68 (95%CI=0.629-0.734)], and in the optimal cut off value of OPN (14.64 ng/ml) the sensitivity was 79.21% and the specificity 79.24% (66). When AFP and OPN were combined, the value of AUC increased to 0.876 ($p<0.0001$), higher than AFP and OPN alone, and reached 88.12% and 74.21% of sensitivity and specificity, respectively (66). Regarding the early detection of HCC, patients with tumors smaller than 2 cm presented significantly higher OPN values (mean=29.67 ng/ml, IQR=14.69-47.97) when compared to those with chronic hepatitis ($p<0.0001$) and those with hepatic cirrhosis (p=0.0029), and the performance of OPN was higher than AFP in these patients (66). These results suggest that OPN is promising in the early detection of HCC, although the number of patients in this group was low and other studies need to confirm it (66).

Midkine (MDK). MDK is a growth factor that binds to heparin which is encoded by the MDK gene on chromosome 11. It plays a key role in carcinogenic activities such as proliferation, migration and angiogenesis, its serum values are increased in most cases of HCC, and can play a key role in AFP-negative patients and patients with tumors in the early stages (67, 68). In the most recent meta-analysis of Zhang *et al*., 10 studies were selected representing a total of 1730 individuals (753 with HCC and 977 without HCC); the sensitivity and specificity of MDK for the diagnosis of HCC was 86% (95%CI=83.3-88.4%) and 75.4%

(95%CI=72.6-78.1%), respectively (69). This meta-analysis, however, presented significant heterogeneity as well as limitations that may have affected the results. In a study by Zekri *et al*., single or combined biomarkers were compared; for optimal MDK cut-off values, sensitivity was 88.5% and specificity 80.6% ($p<0.001$), and their serum levels were significantly higher in HCC patients (70). Vongsuvanh *et al*. investigated the role of MDK in AFP-negative patients with HCC (AFP<20 ng/ml), and found that in 59.18% of these patients the levels of MDK (MDK>0.44 ng/ml) were high, which indicated the possible importance of MDK complementary to AFP (68). It is, therefore, necessary to develop more rigorous studies with larger samples to obtain future evidence on the role of MDK in the diagnosis of HCC (70).

Des-carboxyprothrombin (DCP). DCP, also known as vitamin K-induced protein, is a protein produced by hepatocytes in situations of vitamin K deficiency (71). Multiple mechanisms raise the levels of CPD in patients with HCC, including genetic alterations in the hepatocytes, changes in their cytoskeleton or unregulated entry of vitamin K (71). In the systematic review of Ji De *et al*., the results showed 66% grouped sensitivity and 88% grouped specificity of serum DCP in the diagnosis of HCC (72). The study of Lok. *et al*. who compared the accuracy of AFP and DCP in the early diagnosis of HCC, showed that these biomarkers were complementary, achieving better sensitivity results when combined (73). However, further studies are needed to determine whether this combination of biomarkers improves early detection of HCC.

GALAD score (Gender, Age, AFP-L3, DCP). The GALAD Score is a tool based on parameters such as gender, age and the serological biomarkers AFP, DCP and AFP-L3 (74). Yang *et al*. have compared GALAD Score and liver ultrasonography (74). They obtained 91% sensitivity and 85% specificity in HCC detection (74). When the analysis was restricted to initial stage HCC patients, the AUC of the GALAD Score remained high and was higher than the ultrasound (0.92 *vs*. 0.82, $p<0.001$) (74). Thus, despite the limitations of the study, the GALAD score showed potential as a complementary parameter compared to ultrasound, being particularly important in the detection of HCC in patients with high liver dysfunction and obesity, conditions that increase the risk of obtaining false-negative abdominal ultrasound (74). Berhane *et al*. compared the GALAD Score with the AFP, AFP-L3 and DCP single biomarkers and showed 91.6% sensitivity and 89.7% specificity for the GALAD Score in HCC diagnosis, significantly higher than the single biomarkers, indicating the GALAD Score as a tool capable of detecting tumors in which curative treatments are more applicable (75).

Phase 4

To date, there are no phase 4 biomarkers that have completed the tests for early diagnosis of HCC. Progress is hampered by the lack of suitable phase 3 samples for validation (76).

Phase 5

Alpha-fetoprotein (AFP). Although not all HCCs secrete AFP and other liver diseases can show increased levels of this marker, AFP is the most commonly used biomarker in HCC surveillance (77). Tzartzeva *et al*. have analyzed studies on HCC surveillance performance in patients with hepatic cirrhosis and obtained results indicating best sensitivity in early stage HCC detection when AFP was associated with ultrasound (mean sensitivity of 97%) (78). AFP can correlate with alanine aminotransferase (ALT) and aspartate aminotransferase (AST), especially in patients with hepatitis C, which implies reduced specificity of this biomarker (79). However, Liu *et al*. have studied whether the relationship between AFP, AST and ALT could result in an index that would be useful in HCC and, despite the limitations of the research, this relationship was able to provide information regarding the diagnosis, therapeutic outcome and prognosis of HCC (80). AFP cut-off values vary according to guidelines around the world (81). The meta-analysis of Zhang *et al*. examined these values and concluded that the AFP cut off of 400 ng/ml shows the best accuracy in HCC diagnosis, while the cut off of 20 ng/ml can be used for follow-up after treatment, as it presents high sensitivity (81). However, future studies should focus on the dynamics of changing AFP values as HCC progresses. The potential of AFP values in predicting transplant outcomes has been studied by Toso *et al*. (82). They have shown that AFP values can be used as a predictor of post-transplant global survival and can be included in the selection criteria along with morphological variables (size and number) (82). Duvoux *et al*. have proposed an HCC recurrence prediction model that incorporates AFP values and compared its accuracy with the Milan criterion. Establishing the relationship between AFP levels and tumor behavior, such as presence of vascular invasion, and consequently with prognosis, they pointed out that the adoption of AFP, a simple and reliable tool, to the selection criteria of patients for transplantation can refine this choice (83). Metha *et al*. have demonstrated that the serum levels of AFP are an important biomarker in predicting post-transplant recurrence (84). In patients with preoperative serum AFP>1000 ng/ml, the disease-free survival was 53%, while in patients with AFP<1000 ng/ml it was 80% (84). Halazun *et al*. have published a model that incorporates the AFP response concept, which is the difference between the highest and final value of serum AFP before transplantation while in the waiting list, to the criteria for patient selection. AFP response to treatment is an important tool that can improve patient selection and outcomes after transplantation (85).

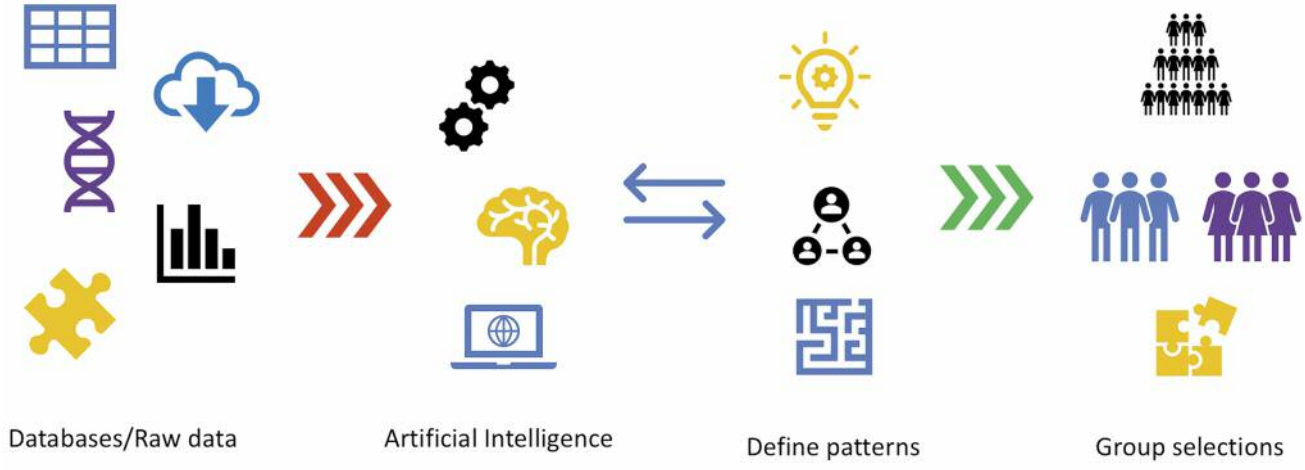

Figure 1. "*Machine learning*" *application algorithm*.

Future Directions

Artificial intelligence (AI) can be defined as the study of algorithms that give machines the ability to reason and perform cognitive functions such as problem solving, object and word recognition, and decision making (86). One of the subfields of AI is machine learning (ML). ML allows machines to learn from the data and make predictions through pattern recognition (87). In the case of supervised ML, data are analysed using an ML algorithm with reinforcement learning to potentially identify clinically applicable patterns, and from a set of predictors (such as the clinical characteristics of a patient) to be able to predict outcomes (such as HCC recurrence) (87).

ML algorithms can be static or incorporate new information that allows constant improvement of the forecasting tool, allowing the algorithm to perfect itself (87). Thus, ML can be used to build a forecasting tool for a given population of HCC patients to identify those most likely to benefit from the various treatments and determine the most important variables to distinguish these patients, as presented in Figure 1. ML considers all the variables available in a given database as "Big-data" and incorporates the interrelationships between them in the outcome prediction tool (87).

Conclusion

New biomarkers predictors of tumor biology have been investigated to adequately select patients for each treatment offered. To date, the response to initial treatment and the biological behavior of the tumor are effectively used in clinical practice in HCC patients. Artificial intelligence technologies, capable of taking into account clinical-laboratory and biological tumor data, need to be implemented, so that information from the so-called Big-data, is correctly interpreted for anticipating the chances of tumor recurrence in HCC. Several molecules related to HCC can contribute to the complete understanding of the disease, and indirectly estimate the response to the indicated treatment.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

This manuscript was written under the role assignment specified below. PA as corresponding author, and RF, were primarily in charge of manuscript drafting and contributed equally to it. VM, MAR, FGF conducted literature review and participated in study design. RMMV, FDST, LAS supervised drafting of the manuscript and gave final approval of the version to be published. All Authors read and approved the final manuscript.

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