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# Evaluation and impact of different biomarkers for early detection of hepatocellular carcinoma

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## Abstract

Worldwide, hepatocellular carcinoma (HCC) is a frequent complication of liver diseases and remains a major cause of cancer-related mortality. In addition, the prevalence of nonalcoholic steatohepatitis (NASH) as prerequisite of hepatocarcinogenesis, even in the absence of cirrhosis, is rising rapidly. The early detection of HCC has been crucial in improving the survival outcomes of those patients. However, in the mostly obese NASH population, diagnostic sensitivity of ultrasound-based HCC screening approaches is limited. On the other hand, biomarkers for HCC show promising potential to improve early detection, providing reproducible, investigator-independent results that can be used either alone or integrated with other biomarkers for scoring models. In the past, validation has been limited due to a lack of prospective longitudinal cohort studies. At present, large-scale retrospective phase-III- biomarker- development gives hope for the availability of biomarker-based screening approaches in the near future. This review focuses on the potential impact of biomarkers on surveillance strategies, potentially allowing for earlier HCC diagnosis.

**Keywords:** Nonalcoholic steatohepatitis, hepatocellular carcinoma, alpha fetoprotein, AFP-L3, des-gamma-carboxy-prothrombin, Gender, Age, GALAD-score, Glypican-3, microbiome



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## INTRODUCTION

Globally, hepatocellular carcinoma (HCC) is the sixth most frequent malignancy and the second most common cause of cancer-related death<sup>[1]</sup>. The global incidence of HCC has tripled since the 1980s<sup>[2]</sup>. Despite the availability of numerous locoregional therapies and a plethora of novel systemic treatment options, the overall survival of HCC remains relatively poor<sup>[3]</sup>. Furthermore, incidence of HCC in the western world is rising with hepatic steatosis being seen as the major risk factor [Figure 1]. Nonalcoholic fatty liver (NAFL; i.e., hepatic steatosis without significant inflammation) and nonalcoholic steatohepatitis (NASH; i.e., hepatic steatosis associated with hepatic inflammation and hepatocellular ballooning) in particular have increasingly been recognized as risk factors for HCC. Previously, these cases have frequently been classified as cryptogenic cirrhosis<sup>[5]</sup>. Moreover, a significant proportion of patients develops HCC in the absence of liver cirrhosis predominantly in the case of predisposing chronic hepatitis B or NASH<sup>[6]</sup>. Nevertheless, multiple HCC surveillance guidelines do not sufficiently recognize NASH as major risk factor of hepatocarcinogenesis. Additionally, ultrasound for detection of smaller lesions, such as in early stage HCC, lacks sensitivity and is further impaired when there is underlying cirrhosis, steatosis, or obesity. To address those aforementioned insufficiencies, the definition of patients at risk has to be more concisely defined and further prospective trials have to elucidate whether ultrasound alone has potential to detect a sufficient proportion of HCC at stages when curative treatment options are still available. Several recent trials clearly indicate that biomarker-based surveillance algorithms have potential to complement or even surpass ultrasound as a surveillance strategy. This review aims to provide an overview of current biomarkers with utility in HCC detection and how they could be implemented into current HCC early detection programs.

## EARLY HCC DIAGNOSIS DETERMINES PATIENT PROGNOSIS

In patients participating in HCC early detection programs, an initial diagnosis is made in less advanced stages, which results in a clear survival benefit. Successful surveillance, however, requires a reliable screening method and a definition of the risk population based on medical needs.

Targeted HCC monitoring anticipates offering curative intended therapeutic procedures, such as liver resection or transplantation, to the highest possible proportion of patients upon their initial HCC diagnosis. Unfortunately, HCC is diagnosed in these early stages only in a minority of patients<sup>[7]</sup>. In a representative German singlecenter cohort, between 1998 and 2009, only 23.5% of over a thousand HCC patients were in an early stage of HCC [Barcelona Clinic Liver Cancer (BCLC) 0/A] at initial diagnosis. Accordingly, less than half of the patients received curative therapies and the median overall survival was only 16 months<sup>[8]</sup>.

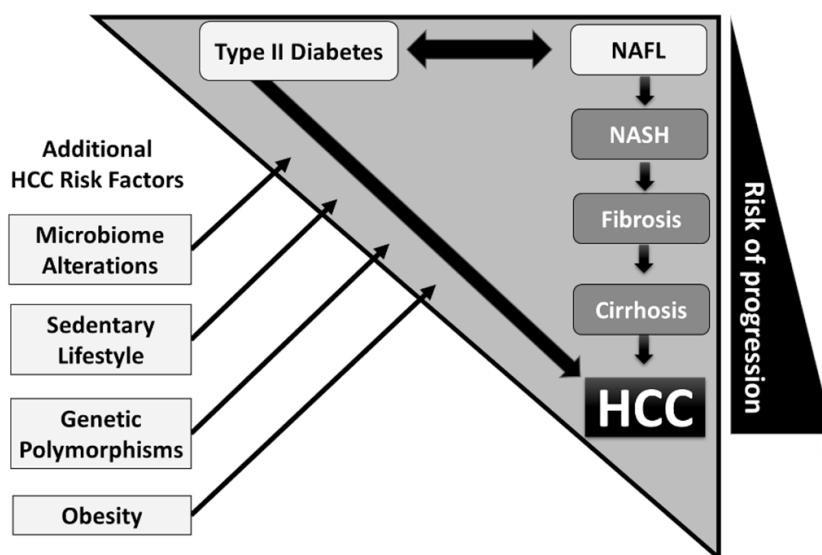
The above-mentioned data are consistent with large international trials where only 10%-23% of the patients were curatively treatable at first diagnosis<sup>[9-12]</sup>. Consequently, only 10%-39% of those patients survived at least one year after diagnosis<sup>[9-14]</sup>.

Since HCC is better suited for early detection programs than most other cancers, and since the risk population to be screened could be well defined, the currently available data is particularly discouraging. International guidelines recommend regular examinations of symptom-free risk patients according to certain criteria.

## INTERNATIONAL GUIDELINES PROVIDE DIFFERENT HCC SURVEILLANCE

### RECOMMENDATIONS

Globally, there are marked regional variations in the algorithms for HCC early detection [Table 1]. The guideline of the German Society for Digestive and Metabolic Diseases (DGVS) recommends that patients with liver cirrhosis of any etiology, as well as patients with chronic hepatitis B or NASH in the absence of cirrhosis, undergo liver ultrasound. Optionally, the liver tumor marker  $\alpha$ -fetoprotein (AFP) can be



**Figure 1.** Triangle of hepatocarcinogenesis in metabolic syndrome: hepatocellular carcinoma (HCC) risk in nonalcoholic steatohepatitis (NASH) significantly increases with progression of liver fibrosis. Independently, type II diabetes mellitus promotes progression from nonalcoholic fatty liver (NAFL) to NASH, but needs to be recognized as an individual predisposing HCC-risk factor since peripheral insulin resistance may promote hepatocarcinogenesis even in the absence of cirrhosis<sup>[4]</sup>. Microbiome alterations, sedentary lifestyle, genetic polymorphisms, and obesity represent additional factors aggravating HCC risk in the NASH population

**Table 1. Overview of international HCC surveillance recommendations**

Society	Risk group	Procedure
DGVS <sup>[15]</sup>	Liver cirrhosis of all etiologies: chronic HBV and NASH	US with or without AFP every 6 months, US- quality standards required
AASLD <sup>[16]</sup>	Liver cirrhosis of all etiologies; chronic HBV depending on ethnical background, age, and genetic background	US with or without AFP every 6 months
EASL/EORCT <sup>[17]</sup>	Liver cirrhosis of all etiologies at CTP stage A and B or CTP stage C if listed for oLT; chronic hepatitis B or active hepatitis; chronic HCV with advanced (F3) fibrosis	US every 6 months
APASL <sup>[18,19]</sup>	Liver cirrhosis, chronic HBV and/or HCV	US and AFP every 6 months
JSH <sup>[20]</sup>	High risk: Liver cirrhosis of all etiologies; chronic HBV and/or HCV	US, AFP, AFP-L3 and DCP every 6 months
	Very high risk: Liver cirrhosis with chronic HBV and/or HCV	US, AFP, AFP-L3 and DCP every 3-4 months

DGVS: German Society of Gastroenterology and Metabolic Diseases; EASL: European Association for the Study of the Liver; AASLD: American Association for the Study of Liver Disease; APASL: Asian Pacific Association for the Study of the Liver; JSH: Japanese Society of Hepatology; HBV: hepatitis B virus; NASH: non-alcoholic Steatohepatitis; HCV: hepatitis C virus; US: ultrasound; AFP: alpha fetoprotein; AFP-L3: Lektin reactive  $\alpha$ -Fetoprotein; DCP: Des-gamma-carboxy prothrombin also known as Protein-Induced-by-Vitamin-K-Absence-or-Antagonist-II (PIVKA II); CTP: Child Turcotte Pugh

determined. The sonographic examinations should be carried out according to quality criteria of the German Society for Ultrasound in Medicine (DEGUM)<sup>[15]</sup>.

While the guidelines of the the American Society for the Study of the Liver (AASLD) and the European Association for the Study of the Liver (EASL) do not recommend an AFP determination at all, or only recommend it on an optional basis, the Asian Pacific Association for the Study of the Liver (APASL) proposed a combination of regular ultrasound (US) and AFP determination. All the aforementioned guidelines recommend those examinations every 6 months. The Japanese Society for Hepatology (JSH) also recommends US in combination with complimentary determination of three different biomarkers, namely AFP, lectin-reactive- $\alpha$ -fetoprotein (AFP-L3), and des-gamma-carboxy-prothrombin (DCP). In contrast to all other guidelines, JSH discriminates between high risk and very high risk groups and therefore recommends screening every 6 months and every 3 months, respectively, as depicted in [Table 1](#).

The limitations of ultrasound scanning have been explicitly recognized in previous AASLD guidelines which state “performance characteristics have not been well defined in cirrhotic livers” and “some patients, particularly the obese, are not good candidates (for surveillance) despite their risk”<sup>[21-25]</sup>. To address this insufficiency, a recent large meta-analysis, which included thirty-two studies comprising 13,367 patients, characterized sensitivity of imaging with or without AFP for detection of HCC in cirrhotic patients. The authors concluded that US alone has a low sensitivity to detect early stage HCC in patients with cirrhosis and thus the addition of AFP to US significantly increased the sensitivity of early HCC detection<sup>[26]</sup>. The latest AASLD guidelines emphasize the importance of determining whether other serum biomarkers (specifically AFP-L3 and DCP) might complement AFP and US in the surveillance setting<sup>[16]</sup>.

### **NASH AS MAIN RISK FACTOR OF INCREASING HCC INCIDENCE**

The global prevalence of nonalcoholic fatty liver disease (NAFLD) is estimated at 25%<sup>[27]</sup>. In up to one third of all NAFL patients, progression to NASH can occur, which in turn may lead to higher-grade fibrosis on the basis of inflammation and, in the final stage, to cirrhosis and HCC<sup>[28,29]</sup>. A recent meta-analysis conducted by Younossi *et al.*<sup>[13]</sup> even reported a global prevalence of NASH among biopsied NAFLD patients that was almost double the previous studies’ findings at 59.1%. According to the latest epidemiological studies, NASH is regarded as the main cause of the steadily increasing incidence of HCC in western industrialized nations<sup>[30,31]</sup>. An analysis of the American liver transplant registry from 2002 to 2007 showed that the prevalence of HCC patients waiting for a liver transplant increased by 15.6% in the past 15 years, from 6.4% in 2002 to 22% in 2017. NASH became the fastest growing cause of HCC. The proportion of NASH as the cause of HCC increased 8.5-fold during the study period, from 2.1% in 2002 to 17.9% in 2017<sup>[31]</sup>. It should be noted here that many cases of HCC arise due to predisposing NASH in the absence of cirrhosis, a population that has so far not been internationally screened for the purpose of early detection of HCC. About 20% of all NASH-associated HCC cases occurred in the absence of cirrhosis<sup>[32]</sup>.

A German single-center study from 2015, which included 1,119 HCC patients of all etiologies, showed that there are relevant differences between patients with HCC with underlying NASH compared to those with other hepatopathies. Patients with NASH-related HCC were older than those with other predisposing hepatopathies (67.6 years *vs.* 65 years) when they were first diagnosed. In addition, the NASH-HCC cohort showed a higher prevalence of obesity (31.1% *vs.* 14.7%) and diabetes mellitus type II (T2DM) (66.7% *vs.* 37.85%) [Figure 1]. Interestingly, NASH-associated HCC shows a trend towards a higher frequency of multifocality (80% *vs.* 69.7%) with overall larger lesions (6 cm *vs.* 4.8 cm) and a tendency towards an increased rate of extrahepatic metastasis at time of initial diagnosis. It is also important to note that the NASH-HCC patients had better global liver function despite a higher tumor burden when diagnosed for the first time. It can be postulated that later HCC diagnosis may be the result of less intensive screening efforts due to lesser extent of hepatic deterioration in NASH patients compared to other liver diseases<sup>[33]</sup>.

### **DO BIOMARKERS ENHANCE HCC EARLY DETECTION?**

For clinical routine, the diagnostic significance of ultrasound of the liver as part of the HCC screening may exhibit distinct limitations. This includes the comparability with preliminary examinations due to changing investigators and ultrasound devices. Predominantly in the cirrhotic or steatotic liver, small lesions are detected in a limited extent. Determination of HCC biomarkers such as AFP have the advantage of being independent of the investigator. Laboratory tests are subject to strict quality guidelines and deliver reproducible results. For this reason, the addition of AFP is recommended in some guidelines to supplement the US. In the high-risk cohort suffering from chronic viral hepatitis B or C relevant for HCC screening, however, the hepatic inflammation may lead to a false positive AFP value elevation which significantly attenuates the specificity, especially in this scenario<sup>[34,35]</sup>. In contrast, if the AFP cut-off values are set higher to improve specificity, 40% of the early stages of HCC are not detected by AFP<sup>[36]</sup>.

In order to achieve a sensitivity superior to the combination of US and AFP while preserving high specificity in early HCC detection, a large number of studies examined the suitability of different biomarkers, such as Glypican-3, AFP-L3, and DCP for HCC surveillance<sup>[37-40]</sup>. The focus during recent years has been on validation of AFP, AFP-L3, and DCP which are already available as certified laboratory tests.

## AVAILABLE HCC-BIOMARKERS AND DIAGNOSTIC MODELS

### AFP and AFP-L3

The overall AFP, which is usually determined in the clinical routine, consists of the three different isoforms: AFP-L1, AFP-L2, and AFP-L3. AFP is a fetal glycoprotein that can be produced later in life when the hepatocytes are in the process of malignant transformation<sup>[41]</sup>. According to various studies, the sensitivity of AFP in HCC detection is between 39% and 65% and the specificity varies between 76% to 94%<sup>[42]</sup>.

AFP-L3 is a AFP variant that binds to the lectin molecule “Lens culinaris agglutinin” and, in contrast to the overall AFP, is HCC-specific. While the AFP-L3 fraction is produced exclusively by malignant transformed hepatocytes, an AFP-L1 elevation, the non-glycosylated AFP main fraction, can also be caused by viral hepatitis and in this scenario is responsible for an incorrectly increased total AFP level<sup>[43,44]</sup>. With a cut-off of 15%, sensitivities between 75%-96.6% and specificities of 90%-92% have been described for AFP-L3<sup>[45,46]</sup>.

### Des-gamma-carboxy prothrombin

Des-gamma-carboxy prothrombin (DCP), also known as Protein-Induced-by-Vitamin-K-Absence-or-Antagonist-II (PIVKA II), is a precursor of prothrombin and is formed in the context of hepatocarcinogenesis due to an impaired vitamin K metabolism. Here, the carboxylation of prothrombin is so impaired that the serum concentration of the DCP increases. Sensitivities between 48% and 62% and specificities between 81% and 98% have been described for the DCP, making DCP a more specific marker than AFP, albeit with lower sensitivity<sup>[47-49]</sup>.

### Combination of AFP, AFP-L3 and DCP

Various clinical trials have clearly demonstrated that there were no correlations among the results of AFP, AFP-L3, and DCP. Thus, some HCC cases can be positive for only one marker at a time while negative for the others. In clinical practice, this means that the combination of the above biomarkers leads to a gradual increase in sensitivity.

Especially when AFP remains the only available marker in clinical routine, the complementary use of AFP-L3 and DCP represents an additional diagnostic option. In a retrospective Japanese single-center study with 270 AFP-negative HCC patients, it was demonstrated that the majority of patients with positive AFP-L3 and/or DCP findings were correctly recorded<sup>[50]</sup>.

### GALAD model

The aforementioned triple combination of the biomarkers AFP, AFP-L3, and DCP demonstrated superior detection of HCC compared to their individual utilization with no significant decrease in specificity in an Asian patient cohort<sup>[51,52]</sup>. For further optimization, a statistically based model called GALAD score was developed a few years ago and was extensively validated in several international studies. It is a diagnostic algorithm based on rigorous statistical analysis. The formula is calculated on the measured absolute values of the three markers instead of defining cut-off levels. Thus, they are considered as continuous variables rather than categorical. Gender and age information are also included since older age and male sex represent independent HCC risk factors<sup>[53,54]</sup>. A GALAD point value of -0.63 serves as a cut-off value for optimal sensitivity and specificity regardless of the BCLC stage. Using this model in a British cohort, the GALAD model achieved an overall AUROC (area under the receiver operating characteristic curve) of 0.97 in detection of all BCLC stages, and early stage HCC (BCLC 0/A) was detected with an AUROC of

0.92 and a corresponding sensitivity of 86% and specificity of 89%<sup>[55]</sup>. A subsequent international multi-center validation study comprising 6,834 patients of different etiologies of HCC achieved an AUROC for GALAD of consistently > 0.90, confirming the efficacy of this model<sup>[56]</sup>. A following German single-center study observed that the GALAD score, even in BCLC 0/A stage HCC, achieved an AUROC of 0.92, again demonstrating superiority in HCC detection compared to the triple biomarker combination of AFP, AFP-L3 and DCP without considering gender and age<sup>[57]</sup>.

To further address the inadequate performance of US-based HCC early detection in NASH, where obesity and sound artifacts due to steatosis further attenuate the diagnostic performance, the utility of the GALAD score in the detection of NASH-associated early HCCs had been tested specifically in this high-risk collective. In a retrospective German multi-center cohort (8 centers) study, the GALAD score was able to detect NASH-HCC patients with an AUROC of 0.96, significantly better than the performance of the biomarkers alone [AFP (AUROC 0.88), AFP-L3 (AUROC 0.86), or DCP (AUROC, 0.87)]. Even for NASH patients in early HCC stages (within the Milan criteria), the GALAD score achieved an AUROC of 0.91. Furthermore, in a prospective Japanese cohort study, it was demonstrated that the average GALAD score in those patients who developed HCC during the observation period was already significantly elevated up to 1.5 years before the initial diagnosis of HCC. The GALAD scores of these HCC patients rose above the cut-off value of 0.63 approximately 200 days before first diagnosis<sup>[58]</sup>.

This implies that the GALAD model is quite suitable for early detection of HCC of all etiologies, even in NASH. However, a phase IV multi-center prospective study has yet to test whether the GALAD Score can be used in the future as an integral part of HCC screening algorithms in patients at risk.

### Osteopontin

Osteopontin (OPN) is an integrin-binding phosphoprotein that is overexpressed in a variety of cancers including lung, breast, colon cancer, and HCC<sup>[59,59]</sup>. At a low level, it is also secreted by biliary epithelial cells. OPN mediates cell signaling that controls inflammation as it is the case in hepatitis, HCC tumor progression, and metastasis<sup>[60]</sup>. Hepatocarcinogenesis results in elevated OPN levels compared to those patients with chronic liver disease in the absence of HCC<sup>[61]</sup>. In a meta-analysis investigating the efficacy of OPN in HCC detection, the sensitivity and specificity of elevated OPN levels have been reported between 75%-87% and 62%-82%, respectively<sup>[62]</sup>. In a phase III validation study, OPN outperformed AFP for HCC detection with an AUROC of 0.73 [95%CI: 0.62-0.85] vs. AUROC of 0.68 [95%CI: 0.54-0.82], respectively. The combined utilization of AFP and OPN resulted in a sensitivity of 82% and specificity of 77% for HCC detection; however, the number of patients at BCLC stage 0/A was limited in this study<sup>[63]</sup>.

### Glypican-3

Glypican-3 (GPC-3), is a heparin sulfate proteoglycan playing a pivotal role in cell proliferation and tumor suppression, representing a potential biomarker for the diagnosis of HCC. GPC-3 binds to growth factor receptors and is involved in cell proliferation and tumor suppression<sup>[64]</sup>. In healthy hepatocytes it is absent, but during hepatocarcinogenesis it is upregulated, and it is assumed to participate in the canonical Wnt- signaling growth pathway<sup>[65,66]</sup>. GPC-3 is present in approximately 33% of HCC patients that were seronegative for both DCP and AFP<sup>[67]</sup>. A meta-analysis found that GPC-3 had a sensitivity of 55.1% and a specificity of 97%<sup>[68]</sup>. AFP and GPC-3 in concert achieved a sensitivity of 76% even at early stage HCC. In light of these findings, GPC-3 has been proposed to be a complementary serologic biomarker to AFP due to the ability of GPC-3 to accurately distinguish between patients with small, well differentiated HCC and those with underlying cirrhosis<sup>[69]</sup>.

## HCC SURVEILLANCE BASED ON COMBINATION OF BIOMARKERS AND ULTRASOUND

### Combination of AFP and ultrasound

The latest HCC guidelines have recently shown a tendency to omit biomarker-based diagnostics in favor of sole ultrasound examinations. In a large American meta-analysis of US, the sensitivity and specificity of US in HCC detection was analyzed with and without additional AFP determination in an HCC high-risk patient group (32 trials/13,367 patients). Ultrasound alone detected an HCC across all stages with a sensitivity of 84% when carried out in accordance with regional guidelines, but there was a dramatic drop in sensitivity to 47% in early stage HCC. The combination of US with AFP was able to improve the sensitivity in the early detection of HCC to at least 63%<sup>[21]</sup>. This clearly indicates that ultrasound alone has low sensitivity in detecting early stage HCC in patients with cirrhosis. Hence, the addition of AFP to ultrasound may significantly increase the sensitivity of early HCC detection in future.

### Combination of GALAD and ultrasound

Recently, the GALAD model has also been validated in an American US cohort study [single-center cohort of 111 HCC and 180 controls and a multi-center cohort of 233 early HCC and 412 cirrhosis patients from the Early Detection Research Network (EDRN) Phase 2 HCC Study] and the performance has been shown to be clearly superior to sonography for HCC detection. Here the AUROC of GALAD for HCC detection was 0.95, which was clearly superior to the AUROC of ultrasound (0.82). The combination of GALAD and ultrasound (GALADUS score) achieved an AUC of 0.98, clearly superior to US or GALAD used solely<sup>[70]</sup>. These very promising data indicate that a combination of ultrasound and biomarker-based scores can significantly improve the performance of current surveillance strategies.

## MECHANISMS OF NASH-RELATED HEPATOCARCINOGENESIS AS POTENTIAL TARGETS FOR SURVEILLANCE

Understanding the sequence from NAFLD to HCC and the impact of additive risks such as type II diabetes mellitus [Figure 1], genetic polymorphisms, and stool microbiome are increasingly becoming the focus of current research.

Inflammation per se, which defines NASH, is a clinically relevant trigger of carcinogenesis, even without the basis of cirrhosis of the liver. While an altered lipid and glucose metabolism contributes to hepatic steatosis in the context of the metabolic syndrome, the interplay of genetic variations, mitochondrial dysfunction, altered immune response, and an imbalance of the microbiome cause a progression of simple fatty liver to NASH, and in the “worst case” scenario, HCC.

## GENETIC FACTORS

For certain gene polymorphisms, there is a direct relationship between the prevalence of NAFLD and the risk of progression to advanced NASH fibrosis.

The polymorphism of the patatin-like-phospholipase-domain-containing-3 (PNPLA3) gene leads to increased hepatic lipid accumulation and an alteration in retinol storage in the liver. Independent of potential disruptive factors such as body mass index (BMI), diabetes, and advanced fibrosis, there is a 3-fold increased HCC risk due to PNPLA3<sup>[71]</sup>.

The polymorphism of the transmembrane 6-superfamily member 2 (TM6SF2) gene manifests itself as a transport disorder of pre-VLDL (very low-density lipoprotein) particles. There is a correlation with the extent of steatosis and progression of fibrosis in NASH, regardless of obesity, diabetes, and PNPLA3 genotype. The direct role of this TM6SF2 variant in hepatocarcinogenesis has not yet been fully elucidated; it is possibly the profibrogenic effect that indirectly promotes progression to HCC<sup>[72]</sup>.

Genetic variability is not completely explained by these common aforementioned variants and many of the phenotypic differences potentially result from gene-environment interactions. NAFLD development and progression are also modulated by epigenetic factors, in particular microRNAs (miRNAs). At the post-transcriptional level, they control many complementary target mRNAs. Their dysregulation have a high predictive value in NAFLD development and progression<sup>[73,74]</sup>. Epigenetic changes, which cause aberrant DNA methylation, as well as the expression of various micro-RNAs (e.g., miR-21, miR-29, miR-23, miR-155, miR-221, miR-222, miR-106, miR-93, and miR-519) are additive drivers of carcinogenesis. There is a direct influence on the most relevant tumor-associated signal cascades [transforming growth factor beta, wingless and INT-1 (Wnt)/ $\beta$ -catenin, mitogenactivated protein kinase, and phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin]<sup>[75]</sup>.

### GUT MICROBIOME ANALYSES AS FUTURE PREDICTORS OF HCC RISK

Distinct changes or shifts in the composition of intestinal bacteria have been described for different intestinal metabolic and inflammatory diseases<sup>[76-78]</sup>. Shifts of certain bacterial strains may also affect the formation of different bacterial-derived metabolic active components such as short-chain fatty acids, bile acids, ethanol, cytokines, or other inflammatory metabolites that may affect the host and possibly promote cancer-related risk factors or diseases<sup>[79]</sup>. The microbiota of an obese host has an impact on bodyweight and gut permeability, and it affects the release of gut-derived components that may also influence the progression of inflammatory mechanisms and promote the formation of cancer. Mouse models of fecal microbiota transfer (FMT) from obese to lean animals could show that microbiota affect bodyweight<sup>[80]</sup>. FMT is a potential therapeutic tool that has been used to treat *Clostridium difficile* infections<sup>[81]</sup>. In mouse studies, FMT was shown to be a useful treatment to increase abundances of beneficial bacterial groups such as Christensenellaceae and *Lactobacillus* to alleviate the progression of NASH-development<sup>[82]</sup>.

The modulation of the gut microbiota by FMT to treat or study different metabolism-related diseases has been taken into account for many years<sup>[83]</sup>. It has been shown that antibiotic treatments, in order to modulate the gut microbiota, may reduce the risk of hepatic carcinogenesis<sup>[84]</sup>. In a high fat diet mouse model of NASH, antibiotic treatments were associated with a reduction of toxic secondary bile acids<sup>[85]</sup>.

NAFL and especially its progressive inflammatory form NASH are often related to the formation of HCC. Here, nutrition, metabolic disturbances, and related comorbidities such as diabetes may influence the composition of the gut microbiota. Changes in the abundances of different bacterial groups have been described within different patient groups.

The metabolism of certain bacterial groups affects the mucosal barrier, hepatic inflammation, fibrogenesis, and tumorigenesis<sup>[86]</sup>. The gut microbiota has an impact on energy balance, altering the uptake of calories derived from food and even alcohol<sup>[87]</sup>. Emerging data indicate that certain characteristic changes in the gut microbiome are associated with NAFLD and even with cirrhosis, which is a main driver of HCC-development<sup>[76,88,89]</sup>. It has not only been shown in NAFLD-related HCC, but also in viral hepatitis-related HCC (hepatitis B) that makes a modification of specific gut microbiota, a potential therapeutic option for HCC<sup>[90]</sup>.

In a study comparing NASH and NASH-HCC patients with or without cirrhosis, alterations in bacterial groups regulating bile acid metabolism had an impact on hepatic fibrogenesis and liver injury. Alterations of the bile acid pool was accompanied with increased abundances of different bacterial strains, especially *Lactobacilli* and *Bacteroides* which were associated with changed liver stiffness and liver injury<sup>[91]</sup>. In NASH patients, the abundance of bile salt hydrolase (an enzyme involved in deconjugation of bile acids) expressing bacteria is shifted, which leads to increased bile acid levels as well as an altered composition of the bile acid pool tending to an increased amount of secondary conjugated bile acids<sup>[92]</sup>. Changes within the



bile acid composition can be related to advanced fibrosis in NASH-HCC which indicates its important role in fibrosis-related tumorigenesis<sup>[93,94]</sup>. In obese children, an increased abundance of *Lactobacillus* strains was associated with NAFLD and NASH, while in colon cancer increased *Lactobacillus* abundance was related to an anti-tumor effect<sup>[95]</sup>.

## FUTURE PERSPECTIVES

In its current state, HCC biomarker research is far from fulfilling its promises. Therefore, it has to be subject of future investigations to elucidate the role of technologies that might complement current biomarker-based surveillance strategies. The identification of a subset of patients at the highest risk is critical to concentrate the effort and resources of regular HCC screening<sup>[96]</sup>. Chromosomal aberrations, epigenetic abnormality, and changes of gene expression are involved in hepatocarcinogenesis. Besides microbiome analysis, omics profiling (e.g., transcriptomics, proteomics, metabolomics) has been derived using several candidate HCC risk biomarkers which could refine HCC screening by enabling individual risk-stratified patient management. High-throughput omics technologies have been widely applied, aiming at the discovery of candidate biomarkers. Different types of biomolecules have been explored as sources of information to predict HCC risk. Transcriptomic dysregulations in chronic hepatopathies capture the functional molecular status supporting carcinogenesis. Circulating nucleic acids, proteins, and metabolites could serve as measures of molecular HCC risk. Large amounts of data on genetic and epigenetic abnormalities, gene expression profiles, and proteomics are available. Here, bioinformatics and network medicine increasingly play a pivotal role to organize and analyze the accumulated data<sup>[97]</sup>. Those analyses may facilitate the identification of a distinct niche of application for each individual biomarker.

## CONCLUSION

HCC surveillance, in line with guidelines, significantly improves survival outcomes due to a higher proportion of patients diagnosed in an earlier stage of HCC, allowing for curative treatment options. According to most international guideline, recommendations have considered ultrasound as the method of choice for screening. US alone lacks sensitivity in the detection of small lesions, particularly in advanced cirrhosis and obesity<sup>[98]</sup>, factors that are compounded by varying skill levels of investigators and available technology. Nevertheless, numerous guidelines omitted the additional determination of AFP, despite the fact that previous trials clearly demonstrated that the utilization of AFP or biomarker-based scores such as GALAD complimentary to ultrasound resulted in a significant improvement in sensitivity while preserving high level of specificity. During the past decade, several biomarkers such as AFP-L3, DCP, osteopontin, glypican-3, and others were evaluated, however, only few markers reached longitudinal retrospective phase III development. Widely lacking are large multinational phase IV prospective screening trials confirming the benefit of the markers or their combinations for surveillance.

Facing increasing global HCC mortality, NASH has become a major risk factor for HCC development, even in the absence of cirrhosis<sup>[5,99,100]</sup>, therefore the definition of the population at risk has to be redefined for future HCC diagnostic and treatment guidelines to address this epidemiological shift. Nevertheless, cost-effectiveness analyses do not support surveillance in the entire population with NAFLD who do not have cirrhosis or advanced fibrosis. Here, continued efforts of risk stratification with factors such as gender and age in conjunction with NAFLD-associated HCC risks such as PNPLA3 or TM6SF2 gene polymorphisms or distinct alterations of the microbiome and bile acid metabolism may facilitate a more concise definition of populations at HCC risk within this cohort.

Furthermore, diabetes mellitus needs to be recognized as an individual predisposing risk factor for HCC development in the presence and even in the absence of concomitant NASH.

In the near future, the combination of standard of care ultrasound and biomarker-based screening approaches seem to be the next step to increase sensitivity and specificity of HCC surveillance.

Among the efforts of optimizing screening algorithms, the education of gate keepers such as primary care physicians, health care associates, and also the population at risk per se is another pivotal factor to increase the rate of HCC early detection.

## DECLARATIONS

### Authors' contributions

Conception or design of the work: Best J, Bechmann LP

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