Circulating Biomarkers in The Diagnosis and Management of Hepatocellular Carcinoma **[Au: Title has been edited to meet article's guidelines: max 90 characters including spaces; feel free to change as you see fit.]**

Philip Johnson[1†], Qing Zhou[2,3], Doan Y Dao[4] and Y. M. Dennis Lo[2,3]

[Au: Please check author name formatting, considering that this is how the names will appear in PubMed.]

[1]Department of Molecular and Clinical Cancer Medicine, Institute of Systems, Molecular and Integrative Biology, University of Liverpool, UK

[2]Centre for Novostics, Hong Kong Science Park, New Territories, Hong Kong SAR, China

[3]State Key Laboratory for Translational Oncology and Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, New Territories, Hong Kong SAR, China

[4]Center of Excellence for Liver Disease in Vietnam, Division of Gastroenterology and Hepatology, Johns Hopkins School of Medicine, MD, USA

[†]Email: Philip.Johnson@liverpool.ac.uk

Abstract

Hepatocellular carcinoma (HCC) represents one of the most prevalent and lethal causes of cancer-related death worldwide. The treatment of HCC remains challenging and is largely predicated on early diagnosis. Surveillance of high-risk groups using abdominal ultrasonography, with or without serum analysis of alpha-fetoprotein (AFP), can **[Au: OK? 'May' carries potentially ambiguity and house style dictates to use either 'can' or 'might'. This rule will be applied throughout at first instance of 'may'.]** permit detection of early, potentially curable tumours, but remains insensitive. Reviewed here are two current approaches that aim to address this limitation. The first is the re-emergence of old biomarkers such as AFP, empirically derived, and now applied within statistical models. The second, circulating nucleic acid biomarkers, which include cell-free DNA (e.g. circulating tumour DNA, cell-free mitochondrial DNA and cell-free viral DNA) and cell-free RNA, applies modern molecular biology-based technologies and machine learning techniques closely allied to the underlying biology of the cancer. Taken together, these approaches are likely to be complementary. Both hold considerable promise for achieving earlier diagnosis as well as offering additional functionalities including improved monitoring of therapy and prediction of response thereto.

[Au: We have 3 levels of headings H1, H2 and H3 that can be 38, 39 and 80 characters in length (including spaces). Your headings have been edited to these limits.]

[H1] Introduction [Au: I have added [H] markers throughout to aid our production team during the layout. Please do not delete.]

Worldwide, hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related deaths (around 800,000 cases per year) and, in the western world it is the most rapidly increasing cause of cancer-related mortality [1,2] **[Au: Perhaps you could consider also citing an even more recent paper (2020) on global burden: DOI: 10.1053/j.gastro.2020.02.068]**. Less than 20% of individuals with HCC survive more than one year after diagnosis [3]. These grim figures testify to the magnitude of the problem, the aggressive nature of the disease and the limited available therapeutic options. Potentially curative therapies, including surgical resection, liver transplantation or local ablation, are currently predicated on early diagnosis achieved by surveillance of patients at high risk (generally those with chronic liver disease at the stage of advanced fibrosis or cirrhosis) using regular ultrasonography, with or without the tumour marker alpha-fetoprotein (AFP) measurement [4,5]. The concept of HCC surveillance and the practical means of delivery are surrounded by controversy **[Au: Please reference this statement. Why is it controversial?]**. Thus, many argue that in the absence of any definitive controlled trial showing benefit **[Au: We tend to use the term 'significant' in a statistical context and usually with an accompanying P value. Perhaps you could write 'notable' instead?]**, surveillance should not be adopted [6]. Indeed, even amongst those who support surveillance, the added benefit of serum biomarkers to the conventional ultrasonography is equally contentious.

Circulating biomarkers have three frequently quoted roles namely, for clinical diagnosis, monitoring of disease progression and assessment of prognosis. For instance, **[Au: We try to period, or delete if not necessary to understanding.]** circulating nucleic acid biomarkers have been demonstrated to be feasible and applicable to the early detection of HCC [7,8]. Analysis of circulating tumour DNA enables the implementation of precision oncology for advanced stage patients and the non-invasive detection and monitoring of the minimal residual diseases [9]. Prediction of responsiveness to systemic agents remains an ambition; there has, as yet, been very limited success. The changing epidemiology of HCC also has major implications for the role of biomarkers. Much of the rising incidence of HCC is attributable to obesity and associated nonalcoholic fatty liver disease (NAFLD) [10]. In the setting of obesity,

ultrasonography, already offering limited sensitivity in advanced nodular liver disease, becomes even less sensitive and serum biomarkers are likely to find an important complementary role here [11].

In this Review, we first consider the re-emergence and repurposing of so-called old biomarkers that were largely discovered empirically. Their 're-emergence' and any novelties that might lie in the analytical approach. We then move on to consider liquid biopsy as an approach more strongly related to the biology of the cancer and more in line with current concepts of translational research. Despite much promising research few biomarkers have emerged over the past 20 years that have real clinical impact, and it is the re-emergence of long-standing biomarkers, particularly AFP that are receiving more attention. Although the role of AFP, which has been used in clinical practice for more than 60 years, continues to be highly controversial, it remains the one all new approaches are initially compared and contrasted with. We therefore start with a brief review of this protein and then move on to consider newer ideas that might supplant or replace it, including detection of mutations, epigenetic alterations and viral DNA associated with the tumoral genome. **[Au: could you please include here a few examples of content]**

[H1] AFP biology, function, and controversy

Alpha-fetoprotein in humans is encoded by the *AFP* gene located on the q arm of chromosome 4 (4q25) and is a member of the albuminoid gene superfamily [12] **[Au: Please reference this statement.]**. AFP is the most abundant plasma protein found in human foetal serum. AFP levels decrease towards the end of the first trimester of pregnancy and albumin levels increase reciprocally to the extent that AFP is often regarded as 'foetal albumin'; and indeed, the two molecules share a considerable degree of sequence homology [12] **[Au: Please reference this statement.]**. Normal adult serum levels are usually achieved by the age of 8 to 12 months and only rise again in the presence of liver disease, specifically primary tumours of the liver (including hepatoblastoma in children and hepatocellular carcinoma in adults), in germ cell tumours and, to a lesser degree, in patients with benign liver disease[13] **[Au: they should also rise during pregnancy, right?]** .

[H2] The clinical role of AFP in HCC

As HCC tends to arise in an already damaged (usually fibrotic and/or cirrhotic liver, a diagnostic test is required to discriminate not between patients with HCC and healthy individuals, but between patients with HCC and patients with chronic liver disease without HCC. **[Au: Please reference this statement.]** AFP serum levels can be modestly raised in patients with chronic liver disease alone (i.e., not complicated by HCC) and they likely reflect cancer-permissive tissue milieu and might serve to estimate the risk of developing HCC [13]**[Au: Please reference this statement.]**. These observations, clearly, have an effect on the sensitivity of the test for HCC. However, as a result of several reviews and meta-analyses the underlying figures are now fairly clear. The area under the receiver operating characteristic curve (AUROC) was evaluated between 0.80 and 0.85 to indicated HCC from controls (supported by two meta-analyses with pooled sensitivities of about 0.6)[14] **[Au: I think here it'll be worthy to mention that these values are indicative of HCC and also what is the threshold (something like: 0.5 suggests no discrimination, 0.7-0.8 is acceptable, 0.8-0.9 excellent)]**. In patients with early, potentially curable disease (Barcelona-Clinic Liver Cancer (BCLC) stage 0 and A), Marrero et al. showed the similar results [15]. **[Au: Xu et al was published in 2013, whereas Marrero et al in 2009. Therefore, Marrero couldn't have confirmed Xu. Perhaps it's the other way around?]** The evidence that adding AFP to routine ultrasonography increases the sensitivity of detection of early HCC has been considered weak[16] **[Au: could you please cite here paper(s) that speak of potentially weakness of AFP to increase sensitivity?]** but the most recent meta-analysis (2018) [17] **[Au: From when exactly specifically. Are you referring to re #14 or #15?]** does suggest some benefit so that many international guidelines now suggest ultrasound 'with or without' AFP for surveillance [17,18]. Evidence that AFP levels are raised several years before clinical diagnosis of HCC further supports such a contention [19].

[H2] AFP as a predictor of drug response

The modest effect of systemic therapies in advanced HCC [20], or to improve on sorafenib (for more than a decade the standard of care) **[Au: Is it still considered the standard of care? Or it was previously standard of care for 10 years?]** has been the subject of much speculation. Among the many suggested reasons is the lack of effective biomarkers and/or enrichment strategies, which might identify specific populations that might benefit. After a decade of intensive research, no biomarkers predictive of response to sorafenib or any other drug has been identified [21,22]. The only exception has been AFP [22]. A randomised controlled trial (59

participants) **[Au: how many patients?]** in patients failing sorafenib therapy showed no overall benefit of ramucirumab in HCC [23], but a post-hoc analysis suggested that a subgroup, defined by AFP levels, had better survival **[Au: by defined, do you mean the patients were grouped as in AFP high and AFP low? Which group had better survival? How many patients in the said subgroub? Also, please reference this statement.]**. A subsequent prospective trial (292 participants) **[Au: how many patients?]** confirmed that patients with AFP levels >400 ng/ml did indeed experience prolonged survival of 1.2 months **[Au: How long was the survival difference?]** compared to placebo [24-26] **[Au: perhaps here you can elaborate a little by explaining that prolonged survival was observed in patients with AFP response (≥20 decrease from baseline) and that AFP > 400ng/ml was evaluated as an appropriate criterion for ramucirumab. I think it will add clarity to the sentence and help readers understand the value of this observation.]**.

[H2] AFP and liver transplantation

The retrospective US Scientific Registry of Transplant Recipients analysis, including 6,817 patients listed with the diagnosis of HCC, showed that patients with down-staged AFP levels from >400ng/ml to ≤400 ng/ml had a better intent-to-treat survival than patients who failed to reduce AFP levels after loco-regional treatment [27]. The Zurich Consensus stated that AFP had an added prognostic value in patients with HCC, and that this marker combined with imaging criteria might be useful to make decisions regarding the indication for liver transplantation [28]. Douvoux et al. [29] demonstrated that AFP levels at listing, in combination with the usual criteria of tumour size and number according to the Milan Criteria, significantly **[Au: P value? If not possible then replace with 'notably', 'markedly', 'importantly', or any other word you find fit.]** improved prediction of HCC recurrence compared with the Milan criteria alone ($p<0.001$). In 2021 **[Au: please state a specific timeframe, or period, or delete if not necessary to understanding]**, an international team of investigators proposed the inclusion of AFP Response (AFP-R) into the Milan selection criteria or other models for liver transplantation selection [30] **[Au: Reference added at the end of Reference list and will be properly included at the end of the editorial assessment. Please don't delete this comment]**. AFP-R measures the difference between the maximum and final pre-liver transplantation AFP levels. This incorporation of AFP-R seems to allow safe expansion of the currently used morphometric HCC selection models.

[H2] Combinatorial approaches

Amidst the controversies surrounding serological approaches to diagnosis and surveillance, AFP has attracted almost personal degrees of opprobrium ranging from 'an obituary' [31], 'time to quit' [32], the 'demise of a bright star' [33] and queries as to why 'AFP won't go quicker into that dark night' [34]. Why does AFP evoke such strong opinions? Perhaps the fact that the function of AFP in adult humans remains unknown, that AFP was discovered by serendipity and that there is no consensus as why it should be related to HCC, makes it an unattractive marker in the current environment of rational translational research. There would therefore seem to be a strong evidence-based case for AFP to act as a basis and/or backbone for surveillance and diagnosis on to which can be added some further markers (i.e., referred as combinatorial approaches) to increase its performance in terms of sensitivity and specificity.

[Au: It isn't very clear the message you're trying to convey here. Is there a strong evidence-based case? Is there a potentially strong case? Do the following scores make a strong case for AFP? Could you please expand a little and also introduce the following subsections? Could you name one or two additional markers you're referring to, as an example? Alternatively, you could delete this sentence entirely if not necessary to understanding.]

[H3] The GALAD score

Japanese investigators have, for several decades **[Au: is there a more recent report to cite?]**, combined AFP with two additional markers, des-carboxy-prothrombin (DCP) and AFP-L3, an isoform of AFP, for diagnosis and surveillance [35]. DCP (also known as protein induced by vitamin K absence or antagonist-II), is an immature form of prothrombin [36,37]. Elevated serum DCP values (>=7.5 ng/ml) **[Au: in the serum?]** have been shown to be associated with a 5-fold increased risk of developing HCC and on this basis DCP has received Food and Drug Administration (FDA) approval for risk assessment [38,39] **[Au: Please reference this statement.]**. AFP-L3, a glycoprotein normally produced by foetal liver, is one of three AFP glycoforms that can be separated on the basis of their lectin binding characteristics, most readily with Lens culinaris agglutinin (LCA) **[Au: It's *Lens culinaris* lectin, or agglutinin, right? If this is correct then it should be restated as: '…most readily with agglutinin, a *Lens culinaris* lectin.]**[40]. In adults, an increase in AFP-L3 levels seems more specific for HCC,

than an increase in total AFP levels [41]. It is usually presented as a percentage of the total AFP with a reference range of <10% [42] **[Au: Please reference this statement.]**.

A statistical model called GALAD (Gender, Age, AFP-L3, AFP, DCP) formally combines the three serum biomarkers with age and gender to produce an algorithm with better performance **[Au: Could you provide with an example what do you mean by better? How much is better?]** (AUROC: GALAD: 0.9662 vs. AFP-L3:0.8430, AFP: 0.8775, DCP:0.9030) than its individual constituents [43]. The GALAD model is of the form:

Z=-10.08 + 0.09 x age + 1.67 x sex + 2.34 log (AFP) + 0.04 x AFP-l3 + 1.33 x log (DCP). For males, sex=1; for females, sex=0

The model offers remarkably good performance as indicated by AUROCs of > 0.9 even for small tumours [44]. The performance of the model **[Au: 'It' as in 'the model' or the 'model's performance'?]** has been independently validated internationally [45-47] (Table 1) by a multicentre North American study (291 patients) **[Au: how many participants]** coordinated by the Mayo Clinic [48] (Figure 1). This latter study is of particular importance as it suggested that the model might prove to be better than ultrasonography in the surveillance setting **[Au: better as in 'instead of' an ultrasound? Or in combination with an ultrasound?]**. Furthermore, the model is not influenced by the aetiology of the HCC or, in cases of chronic viral hepatitis C, by whether or not sustained virological response (SVR) **[Au: neither is the ultrasound, right?]**. On the basis of these findings the GALAD score was awarded 'breakthrough status' by the FDA in 2020 [49] **[Au: Please reference this statement.]** . However, despite these encouraging results, the reported performance measures were all based on case-control studies, which might not be the optimal way of testing potential biomarkers as case-control studies can overestimate biomarker performance. Judgment, therefore, should be reserved until such times as the results are confirmed in large prospective studies, the first of which shows promising results [50].~~is currently underway~~. **[Au: Could you please mention the ClinicalTrial.gov pages or registry numbers if available? The ClinicalTrial.gov pages should be added as new references.]** 1

[H3] The BALAD score

The BALAD score, introduced by Toyoda et al., combines five clinical variables (Bilirubin, Albumin, AFP-L3, AFP, DCP), to assist in the assessment of prognosis in HCC [51]. When the same dataset was re-assessed and externally validated using rigorous statistical methodology[52]

the performance of the model (now referred to as BALAD-2) was very similar, showing the power of clinical intuition and experience **[Au: The latter statement is a little vague. What do you mean by 'paying testament...experience'?]**. Th model can be used to place patients with HCC in one of four classes of risk **[Au: In which classes are you referring to? BCLC has five stages.]** that accurately define prognosis. The model is plausible in two factors which define prognosis in HCC, i.e., the liver function and the tumour related facts. The 'BA' relates to liver function and 'LAD' to tumour characteristics **[Au: Does this mean that serum bilirubin and albumin levels, tumour size and number of tumour nodules are added to the algorithm as variables?]**. The model's discriminatory power across all aetiologies and types of HCC treatment (including resection, locoregional ablative therapies (i.e., radiofrequency ablation and percutaneous ethanol injection), transarterial chemoembolization, and so on) is remarkable [53]. **[Au: As stated it refers to ALL recognised forms of HCC treatment - as described in the reference 45. This sentence needs some clarity, especially for the nonspecialist reader. Which are the types of HCC treatment you are referring here? And how the model increased prediction in the context of HCC treatment? In all aetiologies?]**

[H3] Doylestown Algorithm/Doylestown Plus **[Au: Only 3 levels of subheadings are allowed. I've consider this subheading as H3.]**

The Doylestown Algorithm (DA) comprises log AFP, age, gender, alkaline phosphatase serum levels, and alanine aminotransferase serum levels. Through an internal validation cohort of 360 patients and external validation cohort of 2,700 patients, both cohorts with cirrhosis, Wang et al. showed that the DA significantly improved detection of HCC as compared with AFP alone ($p<0.0001$) **[Au: Please mention P value]** [54]. There was limited benefit in those with an AFP <20 ng/mL, but this limitation could be mitigated by the addition of fucosylated kininogen (Doylestown Plus) [55] **[Au: Could you please explain why, and perhaps how if necessary to understanding and not too technical.]** . In a study of 69 patients with early stage HCC and 93 cirrhosis controls **[Au: do you mean 93 patients with cirrhosis and without HCC as control group?]** , the Doylestown Plus had a higher AUROC than the DA and AFP alone [55].

[H3] HES algorithm **[Au: Same as above; I've consider this subheading as H3.]**

The HES (Hepatocellular Carcinoma Early Detection Screening) algorithm combines age, AFP, rate of AFP change **[Au: change of serum levels during disease progression, during treatment?]** during routine clinical follow up within 1 year, alanine aminotransferase serum levels and platelet count, and it was validated internally and externally, but only in a cohort of patients (n=38,431) **[Au: how many patients?]** with active HCV and cirrhosis in the US Department of Veteran Affairs [56]. At 90% specificity, the HES algorithm identified patients with HCC (n=4,804) with 52.56% sensitivity, versus 48.13% sensitivity for AFP alone, within 6 months prior to diagnosis[56].

[H1] Glypican-3

Glypican-3 (GPC3) is a proteoglycan attached to the cell surface by a glycosyl-phosphatidylinositol anchor, which is expressed by most HCCs but not in normal or cirrhotic liver [57] **[Au: Please reference this statement.]** . Immunostaining of GPC3 is widely used to confirm HCC diagnosis in diagnostic pathology [57]. GPC3 might offer a new target for the treatment of HCC and clinical trials are ongoing. Glypican-3 has been proposed as a serological marker for HCC and in recent (2019) **[Au: please state year published or delete if not necessary]** meta-analyses the pooled sensitivity was 55%, specificity was 58% (AUROC 0.7793) [58]. This relatively high specificity might have potential utility as a complementary biomarker to increase sensitivity of AFP, or other HCC biomarkers, alone or in combination.

[H1] Liquid biopsy

In addition to markers mentioned earlier, circulating nucleic acid markers, such as cell-free DNA (cfDNA), are also emerging as biomarkers for HCC under the general term of liquid biopsy. cfDNAs are DNA molecules released from cells into bodily fluids, including blood, urine, and cerebrospinal fluid, through apoptosis, necrosis, and active secretion [59] **[Au: Please reference this statement.]**. Although the presence of cfDNA in plasma was first reported in 1948 [60], it was not until 1989 that plasma fragments in patients with cancer were suggested to originate from cancer cells [61], a phenomenon that was confirmed in 1994 in acute myeloid leukaemia and pancreatic cancer [62,63]. From this point on, a subset of cfDNA released from tumour cells, circulating tumour DNA (ctDNA), has taken centre stage. At present, ctDNA-based liquid biopsy is being widely applied in cancer diagnosis, treatment guidance, and actual patient care [64]. Subpopulations of cfDNA molecules such as cell-free mitochondrial DNA

(mtDNA) and cell-free viral DNA, cfRNA, and extracellular verticals (EVs) are all informative biomarkers in oncology (Figure 2) **[Au: Please reference this statement.]**.

[H2] ctDNA for the early detection of HCC **[Au: Edit OK?]**

[H3] Genomic changes

Liquid biopsy has shown promising results for many cancer types, including HCC **[Au: specifically in HCC? Or in general?]** , that warrant further exploration of the potential to contribute to surveillance of high-risk populations (Figure 2; Table 2).

The feasibility of detecting HCC-specific mutations in plasma DNA has been confirmed in multiple studies [65-67]. However, due to the low amounts of ctDNA present in early-stage cancers, the task of accurately distinguishing between true mutations from polymerase chain reaction (PCR) and sequencing errors, is challenging [68] **[Au: Please reference this statement.]**. Many technologies have been developed to overcome this problem including droplet digital PCR [69], BEAMing (Beads, Emulsions, Amplification, and Magnetics) [70,71], and several next-generation sequencing strategies [72-74]. One example is the application of unique molecular identifier (UMI)-based methods that integrate a UMI into every DNA molecule, allowing the tracing of PCR duplicates back to the original DNA template. In 2021, a method termed SaferSeqS, which combines UMIs with strand-specific amplification to obtain mutations from both the Watson and Crick strands for further correction, reported a limit of detection (LOD) of below 0.001% [72], which raises the question, in terms of early cancer detection, what level of detection sensitivity is sufficient? Approximately 4 ml of plasma can be obtained from 10 ml total blood and around 40 ng DNA can typically be extracted [68]. One genome equivalent is equal to ~6.6 pg DNA. Thus, 4 ml plasma can contain ~6,000 genome equivalents (i.e. ~12,000 molecules per gene). If one mutation is present in those 12,000 molecules, the mutation allele frequency will be ~0.008% (1:12,000) [68]. In other words, the theoretical LOD is required to be below 0.008% to detect a mutation in 4 ml of plasma. However, if the mutation allele frequency presented in the plasma is naturally below 0.008%, detecting a single mutation in 4 ml of plasma would not be statistically robust.

One way to overcome this problem is by targeting a panel of mutations, which would also solve the problem of lack of prior knowledge of mutations in tissues when applying to HCC screening. One example is the HCC screening study conducted by a Chinese group **[Au: in which region this study was conducted?]**, which combined a set of plasma DNA genetic alterations with

two serum protein markers (AFP and DCP) for early detection of HCC [75]. Such a set of genetic regions included *TP53*, *CTNNB1*, *AXIN1* coding regions and the promoter region of *TERT*, and hepatitis B virus integrations. With this method, the authors identified early HCC cases from patients who were at high risk (Hepatitis B surface antigen (HBsAg) positive) but had a negative screening (normal liver ultrasonography and serum AFP levels) [75], demonstrating the advantages of liquid biopsy over routine screening procedures. Another advantage of liquid biopsy is that it enables pancancer diagnosis within a single blood draw. A composite panel of blood markers (i.e. CancerSEEK) including circulating proteins and mutations (i.e. a 61-amplicon panel covers hotspot mutations from 16 genes such as *TP53*, *CTNNB1*, *PIK3CA*, and *PTEN*) has shown promising results for the diagnosis of various types of cancer, including HCC, in a case control study of 1,005 patients with known cancers [7]. One limitation of the study was that the cases all represented symptomatic patients, and future study on asymptomatic populations would be needed. To overcome this limitation, this group of investigators applied a modified CancerSEEK approach (called the DETECT-A blood test) to almost 10,000 women with no prior history of cancer and validated the result with a PET-CT scan [76]. Ultimately, 26 patients were correctly identified via this liquid biopsy procedure. However, another 70 cancer cases were identified with standard-of-care screening, due to symptomatic presentation or by other means [76], indicating that more research is needed to establish the positioning of this new technology in the established hierarchy of screening.

Notably, there are several potential limitations of mutation-based liquid biopsy markers. For example, one report revealed that a substantial proportion of cfDNA mutations (81.6% in healthy individuals and 53.2% in patients with metastatic cancer (including breast cancer, non-small cell lung cancer and prostate cancer) **[Au: what type of cancer?]** were derived from clonal haematopoiesis (CH) [77]. Thus, the presence of CH-related mutations would confound the detection of tumour-derived mutations, resulting in false-positive calls when the appropriate control DNA (e.g., DNA from paired white blood cells) is not available. In addition to the CH confounding effect, mutations present in precancerous tissues would further pose a challenge to the detection of HCC. It was reported that HCC hotspot mutations related to the *TERT* gene promoter were found in around 6% and 20% of low-grade and high-grade dysplastic nodules from patients with cirrhosis, and mutations related to *CTNNB1* were found in 10-15% hepatic adenomas [78].

[H3] DNA methylation

In view of the limitations of genomic monitoring (Table 2), the potential of plasma epigenetic changes for early cancer detection has been increasingly investigated. The most well-studied epigenetic signal in plasma is methylation at CpG sites (i.e., 5-methylcytosine (5mC)) [79]. Approximately 28 million CpG sites are present in the human genome [79], providing valuable resources for biomarker discovery. Furthermore, changes in DNA methylation occur early on in tumorigenesis, making it an attractive prospect for early detection purposes [80]. Thus, methylation at CpG sites offer a rich and informative source for cancer detection, bypassing several restrictions associated with genomic monitoring. For example, methylation changes are tissue-specific [8], allowing such biomarkers to point towards possible sites of a cancer detected via liquid biopsy. Such tissue specificity might also result in methylation-based liquid biopsy being potentially less susceptible to false-positive results due to CH [8].

Whole-genome bisulfite sequencing is an analytical approach that indicates the overall feasibility of a genome-wide epigenetic approach for cancer detection using liquid biopsy [9]. More targeted and potentially more cost-effective approaches have been developed over the past few years, such as methylated CpG tandems amplification [81], cell-free methylated DNA immunoprecipitation-sequencing [82], and targeted bisulfite sequencing [8,83,84], to enrich specifically for DNA molecules originating from CpG sites. A flurry of studies confirmed the general applicability of such epigenomic approaches in HCC early detection [8,83,84]. For example, one study implemented a targeted bisulfite sequencing panel covering 401 CpG sites that were significantly (false discovery rate (FDR) at a significance level of 0.05 with the lowest p values) **[Au: please add P value]** differentially methylated between HCC tissue and whole blood to plasma DNA [84]. The authors constructed a diagnostic prediction model that distinguished HCC cases (training: n=715; validation: n=383) **[Au: how many participants?]** from healthy individuals (training: n=560; validation: n=275) with a sensitivity of 83.3% and a specificity of 90.5% in the validation set by only using 10 valuable CpG sites [84]. Moreover, this model was highly correlated with tumour burden and stage, particularly among patients with early-stage HCC, for whom no statistically significant difference in AFP values was observed [84], indicating a potential advantage of this model over AFP in HCC early detection. In 2019, the IvyGene Liver test, a companion diagnostic test designed based on the study mentioned earlier [84], indicated improved diagnosis power for HCC (sensitivity: 95% and specificity: 97.5%), which was given a breakthrough device designation by the FDA [85]. Pancancer early detection via ctDNA methylation patterns also showed some progress. In 2020, one group reported that targeted methylation analysis of cfDNA enabled simultaneous detection and tissue-of-origin

identification of multiple types of cancer across different disease stages [8]. The tissue-of-origin was correctly identified from over 50 types of cancer with an accuracy of around 93% [8].

[H3] 5-Hydroxymethylcytosine

With increased knowledge of **[Au: emerging as in increasing or as in early?]** the involvement of DNA demethylation in tumorigenesis and cancer progression [86], monitoring ten-eleven translocation (TET) enzymes-mediated DNA demethylation (e.g., 5-hydroxymethylcytosine (5hmC)) is an emerging area for liquid biopsy. Two groups confirmed the detectability of 5hmC in plasma through a selective chemical labelling method, in which 5hmC was labelled with biotin and enriched with streptavidin beads, followed by sequencing to determine the genomic distribution of 5hmC [87,88]. The potential of plasma DNA 5hmC is now moving towards early cancer detection. In 2019, one group profiled the genome-wide 5hmC distribution in 2,554 Chinese individuals with or without HCC [89]. By applying a 32-gene diagnostic model, the investigators accurately distinguished (AUROC: 0.884) early HCC (BCLC 0 & A) from non-HCC cases (including chronic hepatitis B virus infection, liver cirrhosis, and healthy individuals) [89].

[H2] ctDNA in minimal residual disease **[Au: Edit OK? Is this section under 'Epigenetic changes'? I've considered it is.]**

The rate of recurrent disease after HCC resection is around 50-70% [90]. Thus, predicting the presence of minimal residual disease (MRD, i.e., the residual cancer cells in a patient during or after treatment, or in remission) **[Au: perhaps here you could explain in one sentence what MRD is, for our nonspecialist audience? Something along the line: MRD refers to the remaining cancer cells in a patient during or after treatment, or in remission.]** and recurrence has important potential for clinical decision-making for patients with early-stage HCC (Figure 2). The principle is similar to early cancer detection as discussed above. One difference is that alterations present in the tissue can potentially be elucidated following the analysis of the resected tumour, allowing them to be specifically targeted during MRD monitoring in the plasma [91,92]. The utility of liquid biopsy in MRD monitoring has been tested at both genomic [91,92] and epigenetic [9] levels in HCC, and current results indicate that the detection of ctDNA is a risk factor of MRD and recurrence **[Au: Please reference this statement.]**. Moreover, one publication suggests that comprehensive ctDNA profiling might

enable the prediction of relapse before magnetic resonance imaging, and the performance seems to be superior to serum biomarkers AFP, AFP-L3, and DCP in HCC [91].

[H2] ctDNA in HCC precision oncology

Although plenty of efforts are underway in HCC early detection, over 50% of patients with HCC are still diagnosed at advanced stages [93]. Owing to the limited benefit from cytotoxic agents, advanced HCC represents one of the cancer types for which targeted therapy is recommended as a first-line treatment [90]. One problem, as mentioned earlier, is the lack of effective biomarkers for correct patient selection and early identification of acquired resistance (Figure 2). There is increasing evidence indicating that liquid biopsy greatly supplements tissue biopsy samples in precision oncology owing to its non-invasive nature and ability to provide a bird's-eye view of cancer heterogeneity [67]. Many efforts have been made in testing the utility of mutation-based liquid biopsy in precision oncology[64,94]. For example, the investigators of the TARGET study (Tumor characterization to Guide Experimental Targeted therapy), a molecular profiling programme that aims to test the utility of liquid biopsy for patient-therapy matching in a broad range of advanced cancers (but not including HCC) **[Au: including HCC or not?]**, demonstrated that actionable alterations (variant allele fraction > 2.5%) can be identified in 41 of 100 patients, 11 of which received matched molecular therapies and demonstrated responses [94]. In terms of HCC, ctDNA was used to identify suitable patients (i.e. patients with *RAS* mutations) in a Phase II study with 498 participants **[Au: how many participants?]** involving refametinib and sorafenib [95]. In another study, two patients with HCC and druggable somatic alterations in ctDNA, received matched targeted treatment, both of whom demonstrated response to the therapy [96] **[Au: Could you be more specific on the response? Did they respond to therapy? What kind of therapy was matched?]**. There is currently a paucity of research regarding patient selection conducted about first-line drugs for HCC, which requires further explorations.

As a non-invasive method, liquid biopsy represents a powerful tool for serial monitoring of acquired resistance during treatment. Its utility has been widely tested in non-small cell lung cancer and patients with colorectal cancer under anti-EGFR therapies to detect *EGFR*, *KRAS* and *NRAS* mutations **[Au: *KRAS*?]** that can confer resistance to treatment [64]. However, drugs administered to patients with HCC are typically multi-kinase inhibitors [90]. Unlike anti-EGFR treatment, one or two genetic changes do not directly cause drug resistance to these treatments, limiting the application of monitoring specific mutations for resistance prediction. In this case, measurement of ctDNA quantity to predict tumour burden might represent an alternative

method (Figure 2). Indeed, the frequency of HCC-specific mutations in plasma showed a good correlation with HCC tumour burden, and promising results were reported in the application of predicting drug resistance [97,98] presence of metastases [99], and patient survival [84].

[H2] Other liquid biopsy markers in HCC

Liquid biopsy is not restricted only to the genetic and epigenetic changes of ctDNA. Even the fragmentation of circulating DNA molecules is informative, referred to as fragmentomic features. Unlike cfDNA, which has a dominant length at ~170 bp that is associated with nucleosome structure, ctDNA has been reported to be more frequently detected in the shorter (<150 bp) size population [100-103]. Moreover, ctDNA demonstrated distinct fragmentomic markers, e.g., 5'-end motif, preferred ends, and nucleosome footprints [79]. Notably, one group by combining 5hmC profiling with cfDNA fragmentation profiles, distinguished early HCC cases (n=201) **[Au: how many patients?]** from patients with liver cirrhosis (n=2247) (AUROC: BCLC A: 0.944 & BCLC 0: 0.889), which outperformed AFP and/or DCP and individual features [104]. The biology and more potential diagnostic use of fragmentomic features in cancer were discussed in detail in one previous review [79]. In addition, cell-free viral DNA, cell-free mtDNA, cfRNA, and EVs have all been highlighted for their potential in HCC cancer care **[Au: Please reference this statement.]**.

[H3] Cell-free viral DNA

Around 50% of HCC cases develop from chronic hepatitis B virus (HBV) infection worldwide[105]. Random HBV DNA integrations occur in 80%-90% of HBV-related HCC [106-108] and they create unique junctional fragments at the integration site for each cell. The chimeric DNA fragments released by HCC cells during tumour turnover are, therefore, considered as ctDNA for HBV-associated HCC (Figure 2). Plasma chimeric DNA mainly originates from tumour tissues rather than adjacent non-neoplastic liver tissues [109]. Thus, the richness of plasma HBV-integrated DNA can be used to facilitate HCC diagnosis [109-111]. Moreover, studies indicated that the presence of plasma chimeric DNA is an independent risk factor of early recurrence [110], and that the methylation levels of integration sites can accurately discriminate HCC from non-HCC samples [111].

The potential of plasma viral DNA for early cancer detection has been tested in nasopharyngeal carcinoma. Plasma DNA from Epstein-Barr virus was successfully applied to screening nasopharyngeal carcinoma from a total of 20,174 community participants with a sensitivity and specificity of 97.1% and 98.6%, respectively [112]. With the observation that high viral load is associated with HCC development [113], tumour-derived HBV DNA may **[Au:OK?]** present a rich and informative source for early HCC detection whilst overcoming the low tumour burden restriction.

[H3] Cell-free mtDNA

The presence of cell-free mtDNA has been confirmed in multiple studies involving many types of cancer [114-118] **[Au: generally or specifically to HCC?]**. Unlike diploid nuclear DNA, the copy number of mtDNA can vary from 100 to more than 10,000 copies depending on cell type and pathological condition. In human HCC cells, the copy number of mtDNA seems to be markedly **[Au: Please mention P value if possible. Otherwise you can write instead 'markedly']** decreased [119]. Moreover, owing to the lack of protective histones and an inefficient DNA repair system, mtDNA has a higher mutation frequency than nuclear DNA. HCC exhibits numerous mtDNA mutations, with the accumulation of mtDNA mutations in tissue reflecting the degree of tumour differentiation [120]. To date, there is no consensus as to the alteration of plasma mtDNA levels in patients with HCC [100,114-116] and the detectability of HCC-specific mtDNA mutations in plasma [115,116]. More work is needed to validate the clinical utility of cell-free mtDNA level and mutations for HCC diagnosis and prognostication. Another upcoming direction in this area is the topology of cell-free mtDNA. It was reported that both linear and circular mtDNA were present in plasma, with liver-derived mtDNAs being mainly linear in patients undergoing liver transplantation [117]. The linear mtDNA proportion was well-correlated with liver DNA contribution in the plasma DNA [117], therefore rendering it a potential biomarker for monitoring liver function after transplantation.

[H3] cfRNA and extracellular vesicles

Another informative source of biomarkers is cfRNA that includes a large family of members, e.g., microRNA (miRNA), messenger RNA (mRNA), and long non-coding RNA (lncRNA). The presence of tumour-derived cf-mRNA in plasma was confirmed [121] and used for cancer detection, tumour tissue-of-origin prediction, and cancer subtype determination [122]. Aside from

cf-mRNA, cf-lncRNA and cf-miRNA are alternative biomarkers for HCC cancer care, and the utility have been evaluated in HCC diagnosis and survival prediction [123-125].

cfRNAs can be associated with EVs and, therefore, can be protected from degradation (Figure 2). EVs represent a group of lipid bilayer-delimited particles naturally released from cells, including apoptotic bodies, microvesicles, and exosomes [126]. miRNA is particularly enriched in EVs. Analysis of tumour-derived exosomal miRNA can reflect tumour-related information, and its utility was tested in recurrence [127] and survival prediction [128,129] in HCC. As proteins can also be carried by EVs, analysis of the protein cargo might be helpful to generate validated markers for EV classification and detailed characterization of EV subfamilies for diagnostic purposes. For example, novel chip-based enrichment platforms could facilitate high-throughput and high-purity isolation of EVs based on their protein cargos. After enriching HCC-associated EVs through targeting three HCC-associated surface protein markers (i.e. epithelial cell adhesion molecule (EpCAM), asialoglycoprotein receptor 1 (ASGPR1), and cluster of differentiation 147 (CD147)) with a novel HCC EV chip-based purification system, HCC EV-derived mRNA markers (e.g., AFP, GPC3, and albumin (ALB)) exhibited **[Au: such as?]** great potential for non-invasive early cancer detection (sensitivity: 94.4%; specificity: 88.5%) [130].

[H1] Future directions **[Au: I am not quite sure this works as a general conclusion as it only relates to one aspect of the Review. I propose this section as a subheading under 'Liquid biopsy'. Perhaps you could write a more general 'Conclusion' that sums up the main points of the article as a whole.]**

Despite much promising research few biomarkers have had emerged over the last 20 years that have real clinical impact. AFP as a biomarker has been used in clinical practice for more than 60 years remains the one to which all new approaches are initially compared and contrasted with. However, the role of AFP continues to be highly controversial. This mainly due to the unclear biology behind the relationship between AFP and HCC, which makes AFP an unattractive marker in the current environment of rational translational research. In the past decades, the combinatorial approaches (e.g., the GALAD score, the BALAD score, the Doylestown Algorithm/Doylestown Plus, and the HES algorithm) have achieved promising results for HCC surveillance and diagnosis, which combined AFP with some other markers. Although promising, the combinatorial approach described earlier should be treated with some

caution. There are likely to be numerous potential variables (such as different protein markers and clinic characters) **[Au: could you give an example or two?]** that could be combined in numerous ways using many different analytical and statistical methodologies. This discrepancy raises the possibility that we can end up with multiple models each claiming relevance to different geographical regions **[Au: regions as in geographically?]**, different aetiologies and different disease stages, yet all having much the same performance characteristics. Which ones should be chosen for clinical practice and how reliable any comparisons can be, will be the subject of much research over the coming decade.

Genomic and epigenetic changes of ctDNA, cfRNA, EVs, and other subsets of cfDNA molecules are all potential biomarkers for HCC (Figure 2, Table 2). Attempts were made to clarify the origin and clearance of circulating nucleic acid-containing sources to understand how they enter and exit the circulation [79]. Such insights might provide answers to several important questions, such as whether certain markers can be more clinically useful for certain subjects, and what is the biological basis of such inter-individual variation. Another question concerns the potential synergy of liquid biopsy biomarkers with other clinical modalities, e.g., imaging and protein biomarkers. Such synergistic integration might increase the sensitivity and specificity of the resulting testing protocol. One problem of current studies is that most tend to put the primary focus on one class of liquid biopsy biomarker. More effort is needed to generate data using multiple classes of liquid biopsy biomarkers to unlock even more diagnostic information from a single blood sample. To date, there are still no FDA-approved liquid biopsy assays for HCC, mainly owing to the lack of survival benefit analysed or reported by such assays. It is hoped that such information will be forthcoming in the next few years, ultimately positively effecting the care and outcome of patients with HCC.

[Please ensure that references are cited sequentially in the following order: main text, tables, figure legends and then boxes. The numbered references should be listed at the end of the article in the format: 1. Author, A. B. & Author, B. C. Title of the article. Nat. Cell Biol. 6, 123–131 (2001). (with journal abbreviation italic, and volume bold). If there are six or more authors to a reference, only the first author should be listed followed by 'et al.'. For more details on reference format please consult the Guidelines to Authors.]

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

Y.M.D.L. holds equities in Grail/Illumina, DRA, and Take2; and previously received research funding from Grail. Y.M.D.L. is a scientific cofounder of Grail. Y.M.D.L. receives patent royalties from Sequenom, Illumina, Xcelom, Grail, DRA, and Take2. The other authors declare no competing interest. [Au: The other authors declare no competing interest? Competing interests should cover everyone. The author statements here need to match that on our eJP manuscript tracking system. Please update this section, on manuscript and on eJP when you upload your revised version.]

Key points [Au: Key points have been edited to reach journal's guidelines: 30 words max each. One sentence per key point.]

1. The development of clinically useful biomarkers for hepatocellular carcinoma (HCC) management has been slow; alpha-fetoprotein (AFP), despite much controversy and many limitations, remains widely used.

2. Biomarkers predicting response to systemic therapy are urgently needed; AFP is the only biomarker to predict response, and only in a subset of patients receiving ramucirumab in the second-line setting.

3. Promising combinations of biomarkers in diagnostic, predictive and prognostic roles are largely based on case-control studies; judgment on these should be reserved until they are backed up by prospective studies.

4. The analysis of cell-free DNA (cfDNA) based on their genomic and epigenetic changes can serve as promising biomarkers for early HCC and minimal residual disease monitoring.

5. The analysis of genetic changes of circulating tumour DNA (ctDNA) enables deciphering tumour heterogeneity, facilitating precision oncology for patients with advanced-stage HCC.

6. Liquid biopsy can be beyond genomic DNA molecules, with cell-free mtDNA, cell-free viral DNA, cfRNA, and extracellular vesicles as potential biomarkers for HCC.

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Display items

Table 1: AUROCs of GALAD for early detection of hepatocellular carcinoma in different studies worldwide **[Au: Are the studies listed selected or all known? If selected, what is the selection criteria? This information can be note in the footnote for clarity.]**.

First author and country/region	Type of Study	Aetiology & severity of chronic liver disease	Sample size	Overall AUROC	AUROC small[a]	Year/Reference
Johnson (UK)	Prospective, case control study, multi-centre	Mostly hepatitis B, hepatitis C, alcohol associated liver cirrhosis, or NASH cirrhosis	670 (331 cases*, 339 controls# **[Au: healthy individuals? Same query throughout]**)	0.97	0.92	2014/43
Berhane (Japan)	Retrospective	Hepatitis B, hepatitis C, or alcohol associated liver disease cirrhosis	833 (394 cases, 439 controls)	0.93	0.89	2016/44
Caviglia (Italy)	Prospective, cross sectional study, single centre	Hepatitis C, hepatitis B, alcohol associated liver cirrhosis	98 (54 cases, 44 controls)	0.98	Not done	2016/47
Best (Germany)	Retrospective, single centre	Cryptogenic, NASH, hepatitis B, hepatitis C,	687 (285 cases, 402 controls)	0.98	0.93	2016/46

		or alcohol associated liver disease cirrhosis				
Yang (USA)[a]	Case control	Mostly hepatitis B, hepatitis C, alcohol associated liver disease, or NASH cirrhosis	291 (111 cases, 180 controls)	0.95	0.92	2019/48
Best (Germany)	Retrospective case control	NASH cirrhosis	356 (125 cases, 231 controls)	0.96	0.92	2020/45
Singal (USA)	Prospective, nested case control	Alcohol associated liver cirrhosis, hepatitis C, or NASH cirrhosis	87 (29 cases, 57 controls)	0.77	0.79	2021/131

[a]Early HCC

AUROC: area under the receiver operating characteristic; NASH: nonalcoholic steatohepatitis

*HCC subjects

#Participants have the condition showed in 3rd column.

Table 2. Clinical applications of circulating nucleic acid markers of patients with HCC.

Biomarker	Advantages	Limitations	Clinical Application	Example Studies
ctDNA				
Mutation	High sensitivity methods available; The mutation profile can be used to guide treatment decisions; Novel mutations identified might lead new targeted therapies.	Lack of well-defined hotspot mutations in HCC; Hampered by confounding signals from clonal haematopoiesis; Lack of tissue specificity; No predictive marker for first-line HCC treatment.	Early detection	Qu et al. 2019 [75]
				Cohen et al. 2018 [7]
			MRD monitoring	Cai et al. 2019 [91]
				Shen et al. 2020 [92]
			Precision oncology	Lim et al. 2018 [95]
				Ikeda et al. 2018 [97]
				Oh et al. 2019 [98]
				Alunni-Fabbroni et al. 2019 [99]
Methylation	Early event of HCC tumorigenesis; Global hypomethylation frequently happened in HCC; Tissue-specific; A rich source of tumour information.	Some methylation changes can occur in the pre-malignant stage; Lack of utility of drug guidance.	Early detection	Chalasani et al. 2020 [83]
				Xu et al. 2017 [84]
				Liu et al. 2020 [8]
			MRD monitoring	Chan et al. 2013 [9]
			Survival prediction	Xu et al. 2017 [84]
5hmC			Early detection	Cai et al. 2019 [89]

	An alternative source of epigenetic information.	Rare events cross the genome, need specific enrichment.		Chen et al. 2021 [104]
Other liquid biopsy markers				
Cell-free virus DNA	An alternative source of ctDNA; HBV integration happened widely in the early stage; Virus load is associated with HCC disease risk.	Only can be applied to virus-associated cancers; No large cohort study conducted in HCC.	Diagnosis	Chen et al. 2020 [109]
				Zhang et al. 2020 [111]
			MRD monitoring	Li et al. 2020 [110]
Cell-free mtDNA	Topological changes correlate with plasma liver contribution.	Detectability of HCC-specific mtDNA mutations in plasma is not agreed.	Liver function prediction	Ma et al. 2019 [117]
			Diagnosis	Li et al. 2020 [115]
				Liu et al. 2021 [116]
				Li et al. 2016 [114]
cfRNAs & EVs	miRNAs and non-coding RNAs are abundant in plasma; Reflect gene expression changes in the tumour, which have the potential for treatment guidance; Tissue-specific; EV RNA can be selected based on the protein cargo.	Unclear biological background; Highly variable due to the unstable feature; Some tissue-specific transcript markers are not disease-specific; Studies are limited to diagnosis and survival prediction.	Diagnosis	Jin et al. 2019 [123]
				Tan et al. 2019 [125]
				Sun et al. 2020 [130]
			Recurrence prediction	Sugimachi et al. 2015 [127]
			Survival prediction	Koberle, et al. 2013 [124]
				Jin et al. 2019 [123]
				Tan et al. 2019 [125]
				Qu et al. 2017 [128]
				Shi et al. 2018 [129]

ctDNA: circulating tumour DNA; EV: extracellular vehicle; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; miRNA: microRNA; MRD: minimal residual disease

Figure 1. GALAD Performance in cohorts of patients with HCC at Mayo Clinic, USA. Panel. A: The receiver operating characteristic (ROC) curve of GALAD Score for detection of HCC. Panel B: The ROC curve of GALAD score for detection of early-stage HCC.[37] (From Yang JD et al., 2019, with permission)

Figure 2: Overview of liquid biopsy in the management of HCC. Tumour cells can undergo different genetic, epigenetic changes. Cancer-associated DNA can enter the blood through apoptosis, necrosis, and secretion, which can be detected by analysing tumour-specific mutations, chromosome copy number aberrations, aberrations in DNA methylation, and HBV integration. Tumour-associated RNA, cell-free mitochondrial DNA and extracellular vehicles can also enter the blood and bring additional nucleic acid-containing information.

Cancer-associated cell-free nucleic acid-containing sources can reflect tumour burden and the contribution of different tumour sub-clones throughout the whole disease course. Such monitoring can be used for early cancer detection, MRD monitoring, patient selection, and prediction of drug response, presence of metastasis and survival.

cfDNA, cell-free DNA; ctDNA, circulating tumour DNA; cfRNA, cell-free RNA; EV: extracellular vehicle; HBV: hepatitis B virus; mtDNA: mitochondrial DNA; MRD: minimal residual disease

ToC blurb **[Au: A short description of the Review will appear in our Table of Contents, blurb OK? Please edit as you see fit (max. 40 words)]**

Surveillance of hepatocellular carcinoma, one of the most lethal solid cancers globally, remains insensitive towards detection of early-stage tumours. In this Review, the authors discuss HCC biomarkers that can improve early diagnosis, therapy monitoring and prediction of therapy response.