Chronic infection with the Hepatitis B virus (HBV) is one of the most common causes of liver disease worldwide. Chronic HBV infection is currently incurable because of the persistence of the viral template for the viral transcripts, covalently closed circular (cccDNA). Detecting changes in cccDNA transcriptional activity is key to understanding fundamental virology, determining the efficacy of new therapies, and deciding the optimal clinical management of HBV patients. In this review, we summarize surrogate circulating biomarkers that have been used to infer cccDNA levels and activity in people with chronic hepatitis B. Moreover, we outline the current shortcomings of the current biomarkers and highlight the clinical importance in improving them and expanding their use.

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Surrogate Markers for Hepatitis B Virus

Covalently Closed Circular DNA

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Abstract

Chronic infection with the Hepatitis B virus (HBV) is one of the most common causes of liver disease worldwide. Chronic HBV infection is currently incurable because of the persistence
of the viral template for the viral transcripts, covalently closed circular (cccDNA). Detecting changes in cccDNA transcriptional activity is key to understanding fundamental virology, determining the efficacy of new therapies, and deciding the optimal clinical management of HBV patients. In this review, we summarize surrogate circulating biomarkers that have been used to infer cccDNA levels and activity in people with chronic hepatitis B. Moreover, we outline the current shortcomings of the current biomarkers and highlight the clinical importance in improving them and expanding their use.

**Keywords**

covalently closed circular DNA

viral persistence

biomarkers

liver cancer

liver disease

**Main concepts and learning points**

- Hepatitis B virus covalently closed circular DNA is the key molecule underpinning infection persistence (and therefore the associated liver disease and cancer risk), as well as current definitions of HBV cure.

- Markers of cccDNA levels are needed to identify the intrahepatic state of HBV patients and understand efficacy of therapeutics.
• Circulating markers are preferred due to practicality and uptake.

• Major markers currently used to infer transcriptionally-active cccDNA levels in patients include: circulating HBV DNA; HBV surface protein; HBV e antigen; HBV core-related antigen; and serum HBV RNA.

• Existing markers are not ideal and only perform within narrow situations, highlighting the need to develop novel markers or combinations of existing ones.

Introduction

Chronic Hepatitis B infection affects almost 300 million people worldwide and is a major cause of cirrhosis and liver cancer, together leading to ~1 million deaths annually\(^1\)\(^-\)\(^2\). Currently there is no cure for hepatitis B: current treatments only suppress viral replication and do not directly target the underlying persistent HBV template, covalently closed circular (ccc)DNA. The persistence of cccDNA even decades after the onset of effective suppression of HBV DNA replication by nucleos(t)ide analogues (NA) is the reason for the almost universal rebound of HBV replication after discontinuation of NAs and consequently indication for lifelong treatment\(^3\). In addition to the persistence of the cccDNA itself, its transcriptional activity is a major factor determining the course and outcome of HBV infection. Silencing of cccDNA transcriptional activity can occur in the natural course of infection or be induced by antiviral treatment, leading to an inactive chronic HBeAg-negative HBV infection phase (carrier state) associated with an excellent long-term clinical prognosis\(^4\)\(^-\)\(^6\).

The current lack of a cure and continual threat of disease progression contributes significantly to the stigma, discrimination, and other non-medical impacts of chronic hepatitis B\(^7\). New HBV biomarkers helping to describe the level of cccDNA persistence and
its transcriptional activity both in the natural course of the infection and under effective antiviral treatment would be a major step forward in our attempt to individualize care in patients with chronic HBV infection. In this review, we summarize both established and new circulating biomarkers that have been developed to infer cccDNA levels in people with chronic HBV. We then expand on the implications on both clinical management and patients, highlighting gaps in the field to be addressed by future studies.

The central role of cccDNA in HBV infection and cure

Formation of cccDNA and the regulators of its transcriptional activity

Within 8-12 hours of viral infection of a hepatocyte, the HBV relaxed circular DNA (rcDNA) genome is converted by host DNA repair enzymes into the stable episomal cccDNA form. Active transcription from cccDNA (which acts as the template for all viral RNA transcripts necessary for nascent viral production) can be detected within 12 hours of its formation.

The transcriptional activity of cccDNA varies over the course of an infection and is regulated by numerous genetic and epigenetic factors, derived from both the host cell and the virus. HBV cccDNA is complexed with host histones: changes in post-translational modifications of these histones (e.g. activating acetylated H4/H3 histones or transcriptionally repressing H3K9me3 and H3K4me3 histones) have been reported to play a role in regulating cccDNA transcription. Moreover, transcription from cccDNA has been shown to be controlled by multiple hepatocyte-enriched transcription factors (including hepatocyte nuclear factor 4, retinoid X receptor α, and peroxisome proliferator-activated receptor α).

Viral factors can also influence cccDNA transcriptional activity via trans or cis mechanisms. The HBV x protein (HBx) is a known transcriptional transactivator and is essential for
efficient viral replication\textsuperscript{16,17}. HBx regulates cccDNA transcription by facilitating the ubiquitination (and subsequent degradation) of the cellular transcriptional silencing complex SMC5/6\textsuperscript{18}. Interestingly, replication-deficient integrated HBV DNA forms can code for HBx that is also active in this transactivation function\textsuperscript{19}, which may lead to complex trans-complementation dynamics as HBV DNA integrations increase in frequency with clonal expansion during the late stages of HBV infection\textsuperscript{20,21}.

Some groups have suggested the viral capsid protein HBV core antigen (HBcAg) complexes with cccDNA\textsuperscript{11} (possibly from the incoming virus\textsuperscript{22}) and activates its transcription\textsuperscript{23-26}. However, these studies contradict findings by other groups that have not observed a difference in viral transcription in HBV mutants deficient in HBcAg expression or the transfection of HBV DNA in the absence of input capsids\textsuperscript{27-29}. As such, this contentious point remains under active investigation.

Changes to the cccDNA itself can regulate transcriptional activity. The HBV genome has 3 CpG islands, which can be methylated to reduce transcriptional activity in \textit{in vitro} studies\textsuperscript{30}. Indeed, high levels of cccDNA methylation have been observed in patients with lower levels of active cccDNA\textsuperscript{27,31}. Finally, variations in the viral sequence (particularly basal core promoter regions, splice variants, and different HBV genotypes) can lead to altered levels of viral transcription\textsuperscript{32-34}.

The stability of cccDNA

In non-dividing cells, cccDNA is highly stable: it is likely that cccDNA survives for the entire lifetime of a cell. In cell culture and animal models of HBV infection where liver turnover is low, no clear reduction in cccDNA levels is detectable over time\textsuperscript{35-37}. However, if infected
cells undergo mitosis, cccDNA is efficiently lost. While the specific mechanics of this loss are still controversial (with some studies suggesting complete loss of cccDNA in daughter cells\textsuperscript{38,39} and others suggesting a proportion of cccDNA survives mitosis\textsuperscript{37,40}), it is likely that loss during mitosis drives the majority of the cccDNA loss during an infection\textsuperscript{41-43}.

Consistent with the above, suppressing new cccDNA synthesis in HBV patients using NA therapy leads to slow reduction of cccDNA (~1 log reduction after 1 year, but only ~2 logs after 10 years\textsuperscript{44-46}) reflecting the low background turnover of the liver\textsuperscript{47,48}. However, even in cases where marked liver turnover occurs (e.g. HBeAg-seroconversion, HBsAg seroconversion, or clearance of an acute HBV infection), cccDNA can persist at low but detectable levels\textsuperscript{44,49}. The persistence of cccDNA is possibly resultant from the heterogeneity of turnover across the liver and that some hepatocytes are more likely than others to undergo mitosis\textsuperscript{50-52}.

The role of cccDNA in current definitions of cure

The major aim in the field is to therapeutically induce (with finite-term treatment) a state where i) reactivation of the infection is unlikely; ii) ongoing liver disease progression is slowed or reversed; and iii) the risk of liver cancer is greatly reduced or eliminated. Various clinical endpoints to define these states have been suggested\textsuperscript{53} (Table 1).

The cure states in which cccDNA are completely cleared from the liver (sterilizing and complete cure) are theoretical and aspirational. No reports to date (even in model infection systems) have shown that this can be achieved after an HBV infection has been established. From first principles, reaching such endpoints would reduce or eliminate the risk of viral
reactivation and the ongoing risk of liver cancer and disease, but little is known about how specifically to induce these states (or if they can be achieved at all).

Given the difficulty of achieving these ambitious states, a consensus endpoint for the field is to induce a functional cure — the equivalent to a patient that has cleared spontaneously cleared a chronic HBV infection. This state is linked with lower disease progression, reduced risk of liver cancer, and low risk of viral reactivation. However, as cccDNA is still present in the liver, the potential of viral reactivation exists this state, particularly in response to immunosuppression of B-cells. Moreover, underlying low-level replication (controlled by an active antiviral immune response) may be driving the increased risk of liver cancer associated with this state (though HBsAg-negative patients have a lower risk compared to HBsAg-positive patients). These risks are present (likely to a greater extent) in a partial cure state, where HBsAg is still circulating.

Although it has been defined as optimal treatment endpoint, the mechanism underlying functional cure is not fully understood. Both animal models and patient studies have shown that cccDNA levels are lower after functional cure compared to HBsAg-positive states, but still detectable. Some of these cccDNA molecules have been shown to carry mutations or be replication-defective (though not all), others suggest a suppression of transcriptional activity, and still others show low-level viral replication and targeting by the immune response. Together, this suggests that a reduction of transcriptionally-active cccDNA through multiple approaches could facilitate a functional cure.

Long term suppression of HBV DNA after treatment (defined as “partial cure” in the field) could be achieved by eliminating or transcriptional silencing of cccDNA without achieving a state of functional cure because HBsAg can be encoded by the replication-deficient
integrated HBV DNA (indeed, this form appears to be the major source of HBsAg in HBeAg-negative patients\textsuperscript{63,64}). The state encompasses patients who are at high and low risk of viral reactivation and disease progression, and should not be uniformly classed as a failure of treatment. Therefore, there is the clear need to establish markers that distinguish between transcriptional HBV activity derived from cccDNA and integrated HBV DNA.

Measuring cccDNA levels and its transcriptional activity
To determine if a patient is likely to achieve these cure states (e.g. via novel therapeutic strategies), transcriptionally-active cccDNA levels must be accurately measured. However, several challenges need to be overcome. Firstly, cccDNA resides in the nuclei of hepatocytes and is therefore difficult to access to measure. Technical limitations in cccDNA quantification techniques are low sensitivity, specificity (distinguishing between other forms of HBV DNA present in much higher abundance than cccDNA), and precision. Moreover, the majority of the assays that directly quantify cccDNA do not distinguish between replication-competent and -defective cccDNA, nor can they determine the transcriptional activity of cccDNA.

Circulating markers of transcriptionally-active cccDNA
To address these key issues, research has been focused on surrogate markers for cccDNA, circumventing the challenges in accessing and directly quantifying this form in the liver. These biomarkers include standard HBV serology markers (HBV DNA, HBsAg, and HBeAg) as well as more recently developed and specialized candidates, including HBV core related antigen (HBcrAg) and circulating HBV RNA (Figure1, Table 2). It is important to note that
some therapies will affect these serum biomarkers independently of their effect on cccDNA levels, reducing their specificity in clinical trials or patient care.

The remainder of this review will describe these well-studied standard HBV markers and the contexts in which they have been useful in reflecting intrahepatic cccDNA levels. We also include a detailed scientific description, recent clinical data, and clinical implications of two new major candidates for cccDNA surrogate markers, HBcrAg and HBV RNA.

Circulating HBV DNA

Transcriptionally-active intrahepatic cccDNA levels likely correlate well with circulating HBV DNA, which is contained within virions that can only be produced by hepatocytes containing cccDNA. However, this is not a viable marker for patients on long-term NA therapy, which suppresses viral replication (but very weakly affects cccDNA levels).83

Clinical value of HBV DNA

HBV DNA levels in serum of treatment patients are a hallmark of hepatitis B. They are highest in the acute and immune tolerant phase (HBeAg-positive infection) and decrease during transition to HBeAg-negative infection. The level of HBV DNA is correlated with the likelihood of onward transmission84 as well as the risk of developing hepatocellular carcinoma (HCC) and liver cirrhosis. On treatment, HBV DNA levels reflect the effectiveness of antivirals, most directly that of NA, which inhibit the viral polymerase. Moreover, the increase of HBV DNA on treatment often reflects resistance development against the NA, signalling the need for clinical intervention (e.g. switching NA agent). Given that novel treatments developed to induce a cure of HBV will likely be used in combination with NA
therapy, measuring cccDNA using circulating HBV DNA has limited utility in many pre-clinical trials (though this may still be useful for monitoring in trials involving NA naïve patients).

HBsAg

Thus, viral proteins (such as HBsAg) have been investigated as potential correlates to cccDNA transcriptional activity. Analyses of HBeAg-positive NA-naïve patients has shown high correlation between HBsAg and HBV DNA levels\(^{85}\). These have supported similar high correlation to HBV DNA titres and HBsAg levels in pregnant mothers from Taiwan\(^{86}\), Mainland China\(^{87}\) and Australia\(^{88}\). Given these studies took place NA treatment-naïve individuals, HBV DNA titres likely closely reflect cccDNA levels. As HBsAg levels do not change with NA treatment (unlike HBV DNA titres), this marker holds promise as a candidate surrogate for transcriptionally-active cccDNA levels in these particular sub-populations.

In HBeAg-negative patients however, the correlation between HBsAg and HBV DNA disappears and they become decoupled\(^{86,88}\). This is likely due to the marked reduction of cccDNA in HBeAg-negative phases\(^{44}\), reduction in cccDNA transcriptional activity\(^{89}\), and the extensive clonal expansion of hepatocytes containing integrated HBV DNA\(^{20,21}\) (that can encode a transcriptionally-active HBs ORF\(^{63,90-93}\)). Indeed, integrated HBV sequences appear to be a major source of HBsAg in HBeAg-negative patients\(^{63}\).

Some researchers have investigated novel approaches to address these shortcomings. For example, we have hypothesised that integrated HBV DNA may express a different ratio of large, medium, and small HBsAg forms compared to cccDNA\(^{94}\), though this has yet to be confirmed. With the existing commercially-available assays however, HBsAg levels are a poor surrogate for active cccDNA levels in HBeAg-negative patients.

*Clinical value of HBsAg*
Due to its stability and strong association with decreased complications of HBV infection, it is currently the ideal end-point of upcoming treatment strategies. As with serum HBV DNA levels, HBsAg levels are highest in acute and HBeAg-positive phases and decrease (slightly) during the transition to HBeAg-negative phases.

HBsAg loss is considered a functional cure from HBV infections. While it may appear tautological to suggest that lower HBsAg levels reflect loss of HBs (functional cure), there is value in measuring HBsAg in other associated aspects to achieve this state. For example, baseline or changes in HBsAg levels on treatment have been reported to predict the probability of HBsAg loss in NA and pegylated interferon-based treatments. Moreover, HBsAg levels can reflect the extent of therapeutic target engagement of various (e.g. those targeting HBV RNAs with small interfering RNA, Table 3); however, its potential to predict long term response is still unclear. Importantly, HBsAg levels strongly determine the likelihood of response to NA treatment withdrawal, an approach that is currently under investigation for inducing functional cure and sustained response in HBeAg-negative chronic hepatitis.

HBcAg
HBV capsids particles are highly expressed by HBV infected cells (10-100 fold higher levels than virions) and are composed of 240 copies of HBcAg each. cccDNA is the only source of HBcAg possible, as the integrated HBV genome is rearranged such that the HBV core promoter is separated from the core ORF. Moreover, as with other viral proteins, secretion of capsids are not affected by NA therapy. Thus, HBcAg has the potential for
being a sensitive and specific marker for transcriptionally-active HBV cccDNA in all patients.

Indeed, HBcAg levels correlate closely with cccDNA levels in both animal models\textsuperscript{104} and HBV patients\textsuperscript{105}. However, the use of HBcAg as a surrogate marker is limited due to the poor sensitivity of detection assays. As host anti-HBc antibodies can sterically hinder detection of circulating HBcAg by ELISA and are present in every chronic HBV infection, the use of HBcAg as a surrogate for cccDNA is fundamentally limited. Moreover, owing to its close sequence similarity to HBeAg (explained below), no commercial or experimental assay is available to selectively quantify HBcAg, and so no use in clinical practice has been established.

HBeAg

HBeAg (in which the “e” neither stands for “envelope” nor for “early”\textsuperscript{106}) is not a component of the virion but a secreted protein with immunomodulatory properties. HBeAg is quantifiable using FDA-approved commercial serological assays based on a widely-accepted standard. The HBeAg status defines phases of chronic hepatitis B which are designated as HBeAg-positive or -negative infection or hepatitis\textsuperscript{4}.

HBeAg is translated from the pre-core mRNA, which is slightly longer than pgRNA due to a 5’ extension\textsuperscript{106,107}. Thus, it represents a transcription product from HBV cccDNA. HBeAg can become undetectable following the selection of HBeAg-negative HBV variants to a major species, and/or
through the emergence of anti-HBe antibodies resulting in HBeAg seroconversion\textsuperscript{4}.

\textit{Clinical value of HBeAg}

HBeAg in serum can be considered a measure for immune control over the HBV infection. A seroconversion from HBeAg-positivity to -negativity reflects active immune response against the virus infected cells leading to a reduction in cccDNA levels, transcriptional activity and HBV DNA levels\textsuperscript{89}. HBeAg seroconversion can therefore also be an intermediate milestone of HBsAg loss (i.e. a functional cure).

However, even if HBeAg-seroconversion occurs, it is not necessarily permanent: reversion to a HBeAg-positive state is common after treatment cessation\textsuperscript{108}. Moreover, HBeAg-negative HBV variants may subsequently emerge which are often accompanied with a re-increase in HBV replication and in inflammatory activity (thus, HBeAg cannot be used as a measurement for active cccDNA). In view of this, a long term follow-up of HBeAg-negative patients is still mandatory.

\textbf{HBV core-related antigen (HBcrAg)}

A novel circulating marker HBV infections is quantitative HBcrAg. The current commercial HBcrAg assay, the ChemiLuminescent Enzyme Immunoassay (CLEIA) (Lumipulse\textsuperscript{®} G HBcrAg, Fujirebio) is currently available only for research use. The marker HBcrAg is composed of the three components hepatitis B core antigen (HBcAg), HBeAg and the pre-core related antigen (PreC or “p22cr”).
Circulating HBcrAg levels were demonstrated to monitor relatively accurately intrahepatic cccDNA levels (correlation coefficients of 0.6-0.7 in most reports, though lower in some studies\textsuperscript{109,110}). Moreover, HBV DNA and pgRNA levels in liver were shown to be significantly higher in HBcrAg-positive compared to HBcrAg-negative individuals, suggesting that HBV replication is higher in HBcrAg-positive livers and that HBcrAg correlate with the course of the disease and treatment response\textsuperscript{111,112}.

**Potential clinical value of HBcrAg**

HBcrAg levels can distinguish different clinical phases of CHB\textsuperscript{113,114}, with the limitation that its accurate quantification may be hampered by BCP/PC variants that are often present in HBeAg-negative hepatitis B infections. HBcrAg has also been demonstrated to potentially discriminate HBeAg-negative hepatitis from (inactive) infections, which might be helpful in view of the often-fluctuating disease courses in HBeAg negative disease\textsuperscript{112,114-118}. Elevated HBcrAg levels in HBeAg-negative, treatment-naïve CHB patients were shown to be a strong predictor of cirrhosis development\textsuperscript{119}. Moreover, HBcrAg levels may also predict the risk of HCC\textsuperscript{112,120}, particularly in Asian patients with HBV genotypes B or C\textsuperscript{121}.

Cessation of long-term nucleos(t)ide analogue (NA) treatment is an approach to increase immune control for patients in the HBeAg-negative chronic Hepatitis B phase. Detection of residual HBcrAg in patients receiving long-term NA treatment, in combination with circulating HBV RNA, were shown to be associated with severe ALT flares following NA treatment withdrawal\textsuperscript{122}. In a recent study including 1216 patients from different clinical trials in Europe and Asia upon NA treatment cessation, a combination of HBsAg and HBcrAg
levels was shown to be useful to identify patients unlikely to achieve HBsAg loss after NA treatment cessation.  

Serum HBV RNA  
Serum HBV RNA can be quantified using reverse transcription quantitative PCR. Assays that detect various regions of the HBV genome (such as the precore, X, core, and s regions) have been described, which affect recognition of different RNA species (e.g. those derived from integrated HBV DNA versus those encoded from cccDNA). The inter-correlation of these assays has not yet been assessed and there is no international HBV RNA standard available, though ongoing standardisation efforts are underway.  
Relatedly, the origin and composition of serum HBV RNA are still under debate. HBV RNA in serum or in cell culture supernatant is found in various structures that protect it against degradation (virus-like particles as well as in naked capsids).  
HBV cccDNA is the template for HBV RNAs including the preCore/core (preC) RNA, the pregenomic (pg)RNA, the preS1 RNA, the preS2/S RNA and the X RNA. The pgRNA and precore RNA are over-length molecules of approximately 3.5 kb in size, and therefore can only be transcribed from cccDNA, and not integrated HBV DNA. HBV RNA molecules transcribed from cccDNA also include the 5' truncated RNA with 3' poly-A tail and the HBx RNA. Additionally, over 20 splice variants of the pgRNA and preS2/S mRNA have been described to be present in the supernatant of transfected cell lines and in patient sera. The quasispecies of serum HBV RNA have been shown to reflect the genome of cccDNA, adding to the argument that serum HBV RNA could be an accurate surrogate marker for cccDNA.
However, some HBV RNA species may not be derived from cccDNA. Sub-genomic HBV RNAs encoding HBx or HBsAg may also be transcribed from integrated HBV genome (e.g. one found in the PLC/PRF/5 hepatoma-derived cell line\textsuperscript{141}). Thus, additional research needs to be carried out to understand the relative contribution of integrated HBV DNA molecules to the HBV RNA signal.

\textit{Potential clinical value of serum HBV RNA}

Serum HBV RNA levels show distinct variations across different disease stages. For example, they are significantly lower in inactive HBV carriers compared HBeAg-positive or HBeAg-negative CHB patients\textsuperscript{142,143}. HBV RNA levels in serum can vary according to HBeAg status, disease status, liver inflammation, HBV genotype as well as to basal core promoter and pre-core mutants\textsuperscript{44,131,144}.

In patients achieving suppression of HBV DNA under the detection limit during NA treatment, HBV RNA often remains detectable, and its kinetics can be used as predictors for HBeAg seroconversion in HBeAg-positive patients\textsuperscript{124,142,145}. Accordingly, HBV RNA at 3 and 6 months after the initiation of NA treatment was the strongest predictor of HBeAg seroconversion compared to HBV DNA levels, HBsAg, HBeAg, HBcrAg, ALT and sex, age and HBV genotype\textsuperscript{145}. For patients receiving treatment with pegylated interferon, greater reductions in serum HBV RNA were observed in patients achieving HBeAg seroconversion compared to those patients who did not\textsuperscript{146}. Together with HBcrAg, serum HBV RNA is also a prognostic biomarker for predicting HBV DNA and ALT relapses and following cessation of NA treatment\textsuperscript{122,147}. A lower cut off for HBV RNA levels for predicting response to NA treatment cessation has, however, not yet been defined.
Together, this suggests that serum HBV RNA probably reflects cccDNA transcriptional activity, and may serve as a surrogate marker to assess the efficacy of drugs targeting HBV RNAs (e.g. interferon, siRNAs and antisense oligonucleotides). On the other hand, HBV RNA in serum can also be affected by therapeutic approaches targeting the transport structure of HBV RNA, e.g. core protein assembly modulators (CpAMs, also referred to as capsid assembly modulators). Indeed, the CpAM NVR 3-778 plus PEG-IFNα (but not the NA entecavir) caused a decrease of serum HBV RNA levels, though CpAMs do not cause substantial changes in cccDNA levels in themselves\textsuperscript{147-149}. IFN-α treatment lowered HBV RNA levels in liver and serum of humanized mice, with good correlations between serum and intrahepatic pgRNA levels, but not with cccDNA levels, since such pgRNA reduction mostly reflected the suppression of cccDNA activity\textsuperscript{150}. In HBeAg-positive patients, low HBV RNA levels may also help predict response, HBeAg loss and sustained virological control off-treatment after IFN-α and a combined IFN-α/NA therapy\textsuperscript{126,146,151}. Although the relevance and correlation between viral RNA levels and liver damage still needs clarification, serum HBV RNA could be helpful to define treatment endpoints\textsuperscript{127,152}.

Shortcomings of current and novel circulating biomarkers

While these markers can in some cases reflect transcriptionally-active cccDNA, several shortcomings remain to be addressed.

Firstly, standardization and optimization of novel markers (HBcrAg and HBV RNA) have not been fully established; protocols, standards, and reagents vary among labs and different studies. Moreover, these assays appear to suffer from poor sensitivity. In a recent study of untreated North American HBV patients, more than a third of the whole cohort fell below
the limit of quantification for HBcrAg and 40% of HBeAg-negative patients had unquantifiable serum HBV RNA.

An additional limitation for HBcrAg quantification is its reliance on the simultaneous quantification of three targets. Each of these viral proteins may be independently influenced by immune response against HBV infections or by HBV treatments, and contribution of each component is not known. Thus, HBeAg is the predominant form of HBcrAg, and any factor affecting HBeAg quantities will affect HBcrAg quantities. On the other hand, HBcAg is a good marker for cccDNA expression because it is not affected by factors affecting HBeAg expression, thus its inclusion into HBcrAg may compensate for the variability of HBeAg. The relatively narrow dynamic range of HBcrAg (quantification limit of $3 \log_{10} \text{U/mL}$) also affects its use, though assays of greater sensitivity have been developed and could hold promise for future studies.

Even with more classical biomarkers where the assay is well-established, the correlation to cccDNA levels remains difficult to measure. This is largely due to the numerous approaches and the lack of harmonization of cccDNA quantification assays. As efforts to standardize these assays across the field are being finalized, this may soon play a lesser role.

Relatedly, the measurement of active vs. inactive cccDNA is a contentious issue. It is currently unclear to what extent inactive cccDNA is converted to active cccDNA (or vice-versa). By measuring only the former with circulating biomarkers (Table 2), researchers could be missing the potential for viral reactivation.

To address this, researchers will need to measure total cccDNA levels. Direct measurement of cccDNA is difficult because it requires a liver biopsy. It needs to be shown if approaches like fine needle biopsy represent a possibility to get easier access to liver tissue and whether
this allows a more precise characterisation of cccDNA status, and potentially future end points as sterilising cure from HBV infections. With any form of liver biopsy, there is the possibility of sampling error where intrahepatic heterogeneity may lead to inaccurate readings of cccDNA levels, but this may be lessened by the “fanning” technique that samples FNAs over a broad area used by some investigators.

Finally, there appears to be no perfect surrogate marker for active cccDNA levels. However, combinations of these candidates could be used to achieve a more robust and flexible measure. Substantial efforts will need to be made to design this combination panel with large, multi-centre studies and populations of different ethnicities to avoid over-fitting of data, a significant problem when multiple variables are introduced. Like the individual markers themselves, these combination panels will need to show superiority over current markers, be measurable with standardised assays, have a linear range of quantification for most patients, and perform equally across genotypes.

Other implications of cccDNA quantification and future directions

Measuring activity of cccDNA is not only necessary for determining if patients will progress to one of these cure states, but also could facilitate translational research, inform clinical management, directly affect the experiences of those living with chronic hepatitis B.

Measuring the efficacy and mechanism of action of new therapeutics

To achieve a therapeutic cure that targets the cccDNA levels, researchers need to measure the effect on active cccDNA levels in patients. However, with the wide range of approaches being used to target virus-infected cells, not every marker is an appropriate measure of
transcriptionally active cccDNA (Table 3). For example, capsid assembly modulators will reduce the cellular secretion of HBc and therefore reduce HBcrAg levels. However, this result is a post-translational effect and does not reflect transcriptional activity or amount of established cccDNA, which are not efficiently reduced by these agents. Similarly, the target engagement of HBV-specific siRNAs can be measured by most of these markers, but do not necessarily reflect the amount of active cccDNA remaining after a finite course of treatment. More accurate markers for cccDNA activity would therefore be helpful in the accurate assessment of therapeutic efficacy.

**Prediction of disease progression**

Greater levels of cccDNA (or associated biomarkers) have generally been linked to increased risk of liver disease progression or HCC (previously reviewed by us). Several recent studies have reported that HBV DNA levels (in NA-naive patients), HBsAg, and HBcrAg levels are associated with greater risk of HCC occurrence, particularly in HBeAg-negative patients. Conversely, HBeAg-seroconversion (associated with a dramatic decrease in cccDNA levels) is generally associated with reduced progression of liver disease and lower risk of liver cancer. Together, these data suggest a likely role of cccDNA persistence in driving HCC. However, these markers are still far from perfect, and their predictive power is not absolute. Whether novel assays or combinations of existing markers can improve upon these figures remains to be seen, but improvements here could be highly valuable for the clinical management of chronic hepatitis B patients.
Prediction of functional cure after treatment cessation

One of the most exciting potentials for markers of cccDNA level and/or transcriptional activity is prediction of which patients will undergo HBsAg-seroconversion (or maintain virological response) after NA cessation. In transforming NA therapy into a limited term treatment, this is essentially realizing a functional cure, at least for a subset of patients. Multiple studies have reported that lower HBsAg and HBCrAg levels can predict (to some extent) those who will undergo HBsAg-loss after NA cessation. However, these predictions have unfortunately not been validated in controlled studies. It is possible that appropriate subsets of patients need to be selected or novel markers need to be developed, so this remains a topic of ongoing research.

Implications of active cccDNA quantification for people living with Hepatitis B

If novel markers of active cccDNA could be established and used ubiquitously, this could directly affect the lives of people living with Hepatitis B. Currently, due to the collective community fear of HBV transmission, people who test positive for HBsAg unfairly face stigma and discrimination, often limiting patients’ access to close relationships, education, employment, visas, affordable insurance, and appropriate health care. Loss of HBsAg (functional cure) reduces this discrimination and has been used as one of the rationales for pursuing this endpoint. However, this societal response is not only unjust, but also not based on scientific evidence. Low or undetectable serum HBV DNA already signify low transmission risk and reduced disease progression. Greater promotion by the scientific and
clinical communities as an intermediate end-point (similar to the HIV undetectable = untransmissible paradigm) could stem discriminatory practices in the broader community.

A concern regarding using low/undetectable HBV DNA as an end-point is the potential for breakthrough replication and viral relapse. Establishing new markers of active cccDNA could address this worry by facilitating the understanding of this risk. For example, these markers could quantify the risk by measuring the load of active cccDNA in the liver while under NA therapy or measuring the rate at which inactive cccDNA is converted into active cccDNA.

A widely-accepted measure of cccDNA could even change the definition of a functional cure (independent of HBsAg loss) to one that more accurately describes the underlying biology of the liver. As mentioned previously, even if cccDNA is eliminated from the liver (and therefore no longer a risk for transmission or significant disease progression), some people may remain HBsAg-positive due to transcriptionally-active integrated HBV DNA. A specific measure of intrahepatic cccDNA could provide a scientific basis for denoting a lack of transmission risk. However, to convey this concept and liberate a broad sector of HBV patients from discrimination would require an appropriate community education program aimed at health care staff, affected community, and general society.

Conclusions
Given what the HBV research and clinical fields stand to gain with a highly accurate circulating marker for transcriptionally-active cccDNA levels, we expect even greater efforts
to be made to develop them. We show here that the progress in the field has expanded significantly in the last few years and appears to be settling on 2 major candidates (HBcrAg and HBV RNA) to add to the arsenal of output measurements for upcoming trials of antivirals targeting HBV, though these have clear shortcomings in particular scenarios. We foresee continual development of standardized assays of a panel of surrogate markers from which the most appropriate marker(s) can be selected to measure drug efficacy, or to categorize patients into different sub-populations (e.g. by risk of viral relapse, levels of virus replication, or stages of liver disease).

Conflicts of Interest

Dr. Tu consults for and advises for the Gilead, Excision Biosciences, and GlaxoSmithKline.

Prof Dr Berg consults for, advises, is on the speakers’ bureau for, and received grants from Gilead Biosciences, Janssen, MSD/Merck and AbbVie. Prof Dr van Bömmel consults for, advises, is on the speakers’ bureau for, and received grants from Gilead, GSK, Janssen, MSD/Merck, Roche, Eisai.

References

4. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL 2017 Clinical Practice Guidelines


46. Wong DK, Yuen MF, Ngai VW, Fung J, Lai CL. One-year entecavir or lamivudine therapy results in reduction of hepatitis B virus intrahepatic covalently closed circular DNA levels. *Antivir Ther*. 2006;11(7):909-16.


98. Yuen MF, Schiefke I, Yoon JH, et al. RNA Interference Therapy With ARC-520 Results in Prolonged Hepatitis B Surface Antigen Response in Patients With


124. Butler EK, Gersch J, McNamara A, et al. Hepatitis B Virus Serum DNA andRNA Levels in Nucleos(t)ide Analog-Treated or Untreated Patients During


Table 1. The state of viral serum markers and intrahepatic HBV DNA for clinical end-points, based on

<table>
<thead>
<tr>
<th>Presence of HBV markers</th>
<th>Cure type</th>
<th>Serum HBV DNA</th>
<th>Serum HBsAg</th>
<th>Intrahepatic integrated HBV DNA</th>
<th>Intrahepatic cccDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterilising cure</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cure Type</td>
<td>HBeAg-positive patients</td>
<td>HBeAg-negative patients</td>
<td>Current limitations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Integrated HBV DNA</td>
<td>Active cccDNA</td>
<td>Inactive cccDNA or HBV mutant s</td>
<td>Requires invasive liver biopsy to measure</td>
<td></td>
</tr>
<tr>
<td>Complete cure</td>
<td>-</td>
<td>-</td>
<td>++/++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Functional cure</td>
<td>-</td>
<td>-</td>
<td>++/++</td>
<td>+*</td>
<td></td>
</tr>
<tr>
<td>Partial cure</td>
<td>-</td>
<td>+ to +++</td>
<td>++/++</td>
<td>+/++*</td>
<td></td>
</tr>
<tr>
<td>HBeAg-negative chronic infection phase</td>
<td>+</td>
<td>+ to +++</td>
<td>++/++</td>
<td>+/++</td>
<td></td>
</tr>
</tbody>
</table>

*Present, but majority is transcriptionally suppressed or replication-deficient.

Table 2. Biomarkers and how well they correlate with different forms of intrahepatic HBV DNA.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>HBeAg-positive patients</th>
<th>HBeAg-negative patients</th>
<th>Current limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct PCR for cccDNA</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Serum HBV DNA</td>
<td>-</td>
<td>+++*</td>
<td>-</td>
</tr>
<tr>
<td>Serum HBsAg</td>
<td>+</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Serum HBcAg</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 3. Effect of direct-acting antiviral therapeutic target engagement on serum biomarker levels (only monotherapy studies included)

<table>
<thead>
<tr>
<th></th>
<th>Nucleot(s)ide analogues</th>
<th>Entry inhibitors</th>
<th>CpAMs</th>
<th>Post-transcriptional inhibitors (e.g. RNAi/siRNA/ASOs)</th>
<th>HBsAg secretion inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HBV DNA</strong></td>
<td>+++</td>
<td>-</td>
<td>+++66,67</td>
<td>+++70,71</td>
<td>+++72</td>
</tr>
<tr>
<td><strong>HBsAg</strong></td>
<td>-</td>
<td>-</td>
<td>+*25,74,75</td>
<td>++++69</td>
<td>++++70</td>
</tr>
<tr>
<td><strong>HBeAg</strong></td>
<td>+*76</td>
<td>NR</td>
<td>NR</td>
<td>++425,75</td>
<td>+72</td>
</tr>
<tr>
<td><strong>HBcrAg</strong></td>
<td>+*76,78</td>
<td>+*79</td>
<td>++77</td>
<td>+++69</td>
<td>+++70</td>
</tr>
<tr>
<td><strong>HBV RNA</strong></td>
<td>+*80</td>
<td>NR</td>
<td>+++66,67</td>
<td>++++69</td>
<td>++++70</td>
</tr>
</tbody>
</table>

+++ = strongly affected by target engagement; - = not known to be affected by target engagement; NR = Not reported

CpAMs = core protein assembly modulators; RNAi = RNA interference; siRNA = short interfering RNAs; ASOs = anti-sense oligonucleotides

+++/+++ = high correlation between marker and DNA template; - = poor correlation between marker and DNA template; *Except when treated with NAs; ? = unknown.
* Likely no direct effect, but reduction in serum biomarker levels observed in clinical studies;
‡ predominantly in HBV genotypes A or D; ¥ in vitro/murine models

The implementation of circulating surrogate markers for the transcriptional activity of cccDNA provides new insights into the biology of this molecule and thus also into HBV infection. Firstly, patients are likely more willing to participate in trials that do not involve regular invasive collection of liver biopsy, thereby facilitating recruitment to clinical studies and serial sample analysis. Moreover, many of these candidates are relatively stable proteins that are not affected by freezing, thereby facilitating the analysis of samples in established serum biobanks. Finally, circulating biomarkers provide a way to circumvent the given marked heterogeneity in the liver (as some regions of the liver show marked viral replication, while others are virus-free\textsuperscript{81,82}) and avoid sample bias associated with liver biopsies.