Antiviral Research 169 (2019) 104553

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Non-invasive biomarkers for chronic hepatitis B virus infection management

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ARTICLE INFO

Keywords:
HBV
Antiviral therapy
cccDNA
Chronic hepatitis B
Serum marker

ABSTRACT

Chronic hepatitis B virus (HBV) infection remains a major health burden with over 250 million cases worldwide. This complex infection can lead to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Complete recovery is seldom achieved due to the persistence in infected hepatocytes of covalently closed circular (ccc) DNA, which is not targeted by current antiviral therapies. Routine circulating biomarkers used for clinical monitoring of patients do not accurately reflect the cccDNA pool and transcriptional activity. New biomarkers, such as serum HB core-related Ag and circulating HBV RNAs, are under development. In this review, we discuss surrogate non-invasive biomarkers for evaluating intrahepatic cccDNA abundance and transcriptional activity. We also present their relevance for improving the classification of patients with regards to their natural history and for evaluating novel compounds to assess target engagement and to define new virological endpoints.

1. Introduction

More than 250 million people worldwide are chronically infected with hepatitis B virus (HBV) *i.e.* they harbor hepatitis B surface antigens (HBsAg) in their serum ("WHO | Global hepatitis report, 2017," n.d.). Chronic HBV infection (CHB) is a complex chronic disease leading to chronic liver inflammation, cirrhosis and hepatocellular carcinoma (HCC). Different phases of the disease (detailed in paragraph 2.2) have been characterized depending on the status of viral replication, as well as host immune and inflammatory responses as summarized by Lampertico et al. and Terrault et al. (Lampertico et al., 2017; Terrault et al., 2018).

The optimal management of chronic HBV infection relies on a population-wide-screening, and the accurate identification of patients requiring antiviral therapy. Routine monitoring is mainly performed on blood samples and currently relies on HBsAg, hepatitis B e antigen (HBeAg) and HBV DNA quantification.

Hepatitis B viral load, *i.e.* the levels of serum HBV DNA, is currently the most relevant marker of HBV replication and is a strong predictor of liver disease progression towards liver cirrhosis and HCC. Treatment decision is mainly based on HBV DNA cut-offs according to major international guidelines (Lampertico et al., 2017; Sarin et al., 2016; Terrault et al., 2018). HBV DNA is a highly efficient marker to monitor current treatments based on pegylated interferon alpha (IFN) or nucleos(t)ide analogs (NUCs). Most of the patients under NUCs treatment reach undetectable viral loads, with improved outcomes (lower risk of developing cirrhosis and HCC). The lower limit of detection (LOD) of HBV DNA by current assays is around 5 IU/mL corresponding to approximately 15–25 copies/mL.

However, there is currently no cure for HBV, owing to the persistence of the so-called covalently closed circular (ccc)DNA, the viral minichromosome, in the liver of infected patients. Occult HBV infection (OBI) is defined as the presence of replication competent HBV DNA (*i.e.* cccDNA) in the liver and/or HBV DNA in blood of persons testing negative for HBsAg by currently available assays. OBI may result in transmission of HBV infection to blood or organ transplant recipients, and reactivation of HBV replication in patients receiving cancer chemotherapy or other immunosuppressive therapies (Raimondo et al., 2019). Assessment of the viral reservoir in the liver requires a liver biopsy, an invasive procedure which is currently seldom performed outside clinical studies/trials. Furthermore, the very small amounts of tissues obtained with liver biopsies might not faithfully reflect the infection status throughout the liver. A better appraisal of the risks of viral reactivation in patients who discontinue antiviral treatment or in those undergoing immunosuppressive therapy is much needed. Hence, the development of novel serum biomarkers that better reflect the pool of transcriptionally active cccDNA in the liver than those routinely used for patient monitoring is warranted.

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https://doi.org/10.1016/j.antiviral.2019.104553
Received 26 March 2019; Received in revised form 5 July 2019; Accepted 5 July 2019
Available online 06 July 2019

Abbreviations

Ag	antigen
ALT	alanine aminotransferase
HBcrAg	serum hepatitis B core related antigen
HBsAg	hepatitis B surface antigen
CAGE	cap analysis of gene expression
CAM	capsid assembly modulators
CARD	caspase activation and recruitment domain
cccDNA	covalently closed circular DNA
CHB	chronic hepatitis B virus infection
CTD	C-terminal arginine-rich domain
DNA	desoxyribonucleic acid
dslDNA	double stranded linear DNA
ESCRT	endosomal sorting complex required for transport
HBcAb	hepatitis B core antibody
HBcAg	hepatitis B core antigen
HBeAb	hepatitis B e antibody
HBeAg	hepatitis B e antigen
HBsAb	hepatitis B surface antibody
HBsAg	hepatitis B surface antigen
HBxAg	hepatitis B x antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HR	hazard ratio
ICT-CLEIA	immune complex transfer chemiluminescence enzyme immunoassay
IFN	pegylated interferon alpha
L-HBs	large hepatitis B surface antigen
LOD	lower limit of detection
M-HBs	middle hepatitis B surface antigen
NUCs	nucleos(t)ide analogs
NAP	nucleic acid polymer
OBI	occult HBV infection
ORF	open reading frame
p22cr	22 kilo-Dalton truncated core-related protein
pgRNA	pregenomic RNA
preC mRNA	pre-core mRNA
PRR	pathogen recognition receptor
qHBcAb	quantitative anti-HBc antibody
qHBsAg	quantitative HBsAg
RACE	rapid amplification of complementary DNA cDNA-ends
rcDNA	relaxed circular DNA genome
RNA	ribonucleic acid
RIG-I	regulatory domain of retinoic acid inducible gene
S-HBs	small hepatitis B surface antigen
siRNA	small interfering RNA
SVP	subviral particle
TE	target engagement
trRNA	truncated RNA
VP	viral particle

Novel compounds, aiming at silencing the cccDNA or reducing the size of the cccDNA pool to achieve a functional cure with finite treatment duration, are under development. Persistent suppression of serum HBV DNA and loss of HBsAg are considered to be the most important primary efficacy endpoints for novel compounds in phase 2/3 clinical development. However, undetectable viral load is not an appropriate endpoint for trials that enroll patients who are already treated with NUCs, who are virally suppressed, and viral suppression is not a good predictor of HBsAg loss. Moreover, it was shown that despite undetectability of serum HBV DNA during prolonged NUCs therapy, a low level of viral DNA synthesis persists in the liver leading to the replenishment of the cccDNA pool (Boyd et al., 2016). Furthermore, the kinetics of HBsAg decline is slow during NUCs therapy (Zoulim et al., 2015). Thus, biomarkers predicting early stage HBsAg decline are warranted to assist the development of novel compounds.

Due to the cost and side effects of treatments, these biomarkers could also be helpful to predict response to IFN at an early stage and only extend treatment to patients who display sensitivity, or to identify patients who could discontinue NUCs without risk of relapse.

Several of the classic diagnostic tools, such as HBsAg, HBeAg and HBcAb detection, were revisited to provide a quantitative evaluation, since their variation was suggested to predict treatment response and

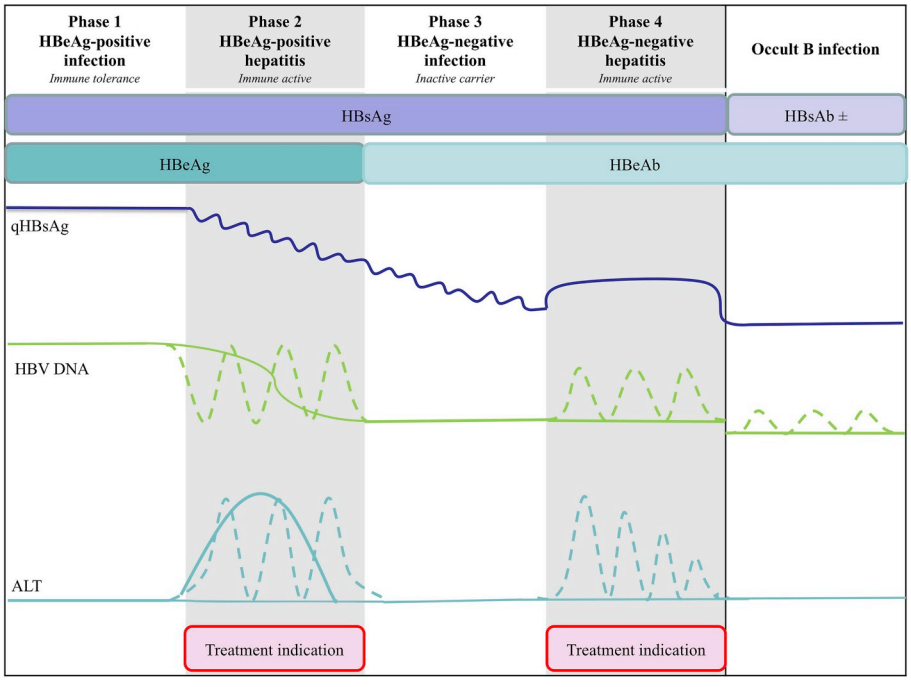

Fig. 1. Chronic hepatitis B phases.
Hepatitis B virus (HBV) DNA, alanine aminotransferase (ALT), and quantitative hepatitis B surface antigen (qHBsAg) according to the different phases of chronic HBV infection (former terminologies are indicated in italic). Of note, intermittent flares represented in dashed lines can be less frequent in some patients. Evolution of the disease is not necessarily longitudinal through the phases. Treatment is indicated in phase 2 and 4 "hepatitis" phases. HBeAg, hepatitis B e antigen; HBsAg, hepatitis B s antigen; HBeAb, antibodies against HBeAg, HBsAb, antibodies against HBsAg.

outcome. New biomarkers aiming at better evaluating the pool and transcriptional activity of cccDNA are also under development.

Herein, we review current knowledge on circulating surrogate biomarkers used to assess HBV infection in the liver with a specific focus on serum HB core-related Ag (HBcrAg) and circulating viral RNAs. These non-invasive biomarkers are being developed to assess the size of the pool of intrahepatic cccDNA and its epigenetic status, and to predict functional cure, *i.e.* HBsAg loss, using current antiviral therapies or novel ones under clinical development. We will also discuss their relevance for better classifying patients according to their natural history.

2. Chronic hepatitis B

2.1. Markers for hepatitis B diagnosis

Diagnosis of HBV infection is based on the detection of three viral antigens and/or their related antibodies (Ab) by Enzyme-Linked ImmunoSorbent Assay (ELISA):

(i) hepatitis B surface antigen/hepatitis B surface antibody (HBsAg/HBsAb),
(ii) hepatitis B e antigen/hepatitis B e antibody (HBeAg/HBeAb) and
(iii) hepatitis B core Ag/hepatitis B core antibody (HBcAg/HBcAb).

The diagnosis of chronic hepatitis B virus (HBV) infection is based on the presence of HBsAg in serum over six months, with negative IgM anti-HBc. Of note, HBcAg is currently not detectable in serum and can only be visualized in hepatocytes by immunostaining of liver sections. HBcAg is highly immunogenic and it can induce anti-HBc responses without the help of T cells (Lee et al., 2009; Milich and McLachlan, 1986). Thus, anti-HBc is usually present, even in HBV carriers who develop no other immune responses to HBV, and is considered to be the marker of a past-HBV contact.

2.2. Classification of patients with HBV chronic infection

The natural history of chronic HBV infection has been classified into four major phases according to 2017 EASL clinical practice guidelines on the management of hepatitis B virus infection (Lampertico et al., 2017) (Fig. 1):

- (phase 1) the HBeAg-positive *chronic HBV infection* phase formerly called immune-tolerant phase,
- (phase 2) the HBeAg-positive *chronic hepatitis* B phase formerly called the HBeAg-positive immune-active phase,
- (phase 3) the HBeAg-negative *chronic HBV infection* phase formerly called the inactive carrier phase and finally
- (phase 4) the HBeAg-negative *chronic hepatitis* B phase formerly called HBeAg-negative immune-active phase.

This nomenclature is based on HBeAg serostatus, serum HBV DNA levels, alanine aminotransferase (ALT), liver inflammation and fibrosis and defines indications for treatment of the patients in the "*chronic hepatitis*" phases. Patients in phases 1 and 2 are HBeAg-positive and those in phases 3 and 4 are HBeAg-negative. Furthermore, those with an inactive disease (phases 1 and 3) are considered to have chronic infection, while those with an active disease (phases 2 and 4) are considered to have chronic hepatitis.

However, it remains sometimes difficult to precisely classify the patients who have present criteria belonging to two phases, in particular for patients with fluctuating viral loads (below and above 2000 IU/mL) but normal ALT levels that can neither be classified in phase 3 nor in phase 4.

2.3. Definitions of HBV cure

In the majority of cases, current therapies provide a "*partial cure*", *i.e.* undetectable HBV DNA in serum, with the persistence of HBsAg in serum and cccDNA in the liver (Fig. 2). Indeed, they do not target the cccDNA, from which the transcription of different RNAs pursues, leading to the production of HBV proteins, in particular HBsAg. Only a limited number of patients lose serum HBsAg (defined as the "*functional cure*"). The "*parafunctional cure*" is an intermediate phase proposed by Jie Wang et al. characterized by a sustained loss of circulating HBV RNA with HBSAg still positive (Jie Wang et al., 2017a) "*Complete cure*" would be achieved by eliminating cccDNA, while a "*sterilizing cure*" refers to the removal of all integrated HBV forms (Lok et al., 2017).

2.4. The HBV life cycle

HBV has a complex life cycle (Fig. 3) that has previously been extensively reviewed (Seeger and Mason, 2015). We will focus herein on

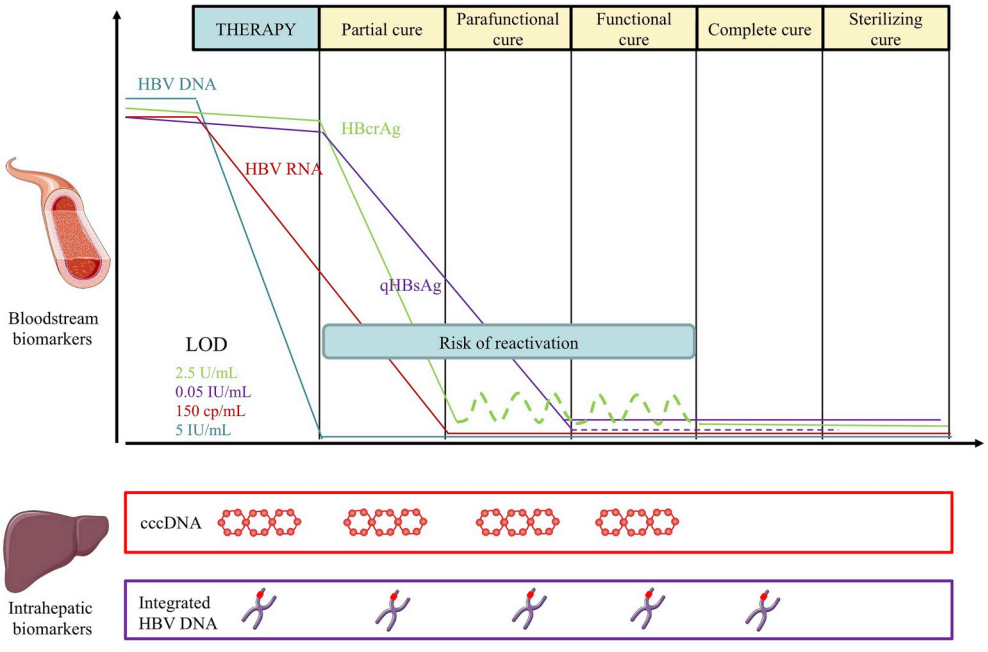

Fig. 2. Classification of HBV-cure with new biomarkers. Partial cure: detectable serum hepatitis B surface antigen (HBsAg), hepatitis B core-related antigen (HBcrAg) and circulating RNAs (cirRNAs) but persistently undetectable serum HBV-DNA after a finite course of treatment. Parafunctional cure: detectable serum HBsAg but sustained loss of circulating HBV RNA and probably HBcrAg reflecting the transcriptional silencing of covalently-closed circular DNA (cccDNA) and predicting a limited risk of relapse after NUCs therapy cessation. Functional cure: sustained, undetectable HBsAg and serum HBV-DNA with or without anti-HBs seroconversion; at this stage the persistence of HBcrAg after treatment is associated with a risk of reactivation. Complete cure: elimination of cccDNA, undetectable HBsAg; HBsAg might still be produced from integrated sequences probably below the limit of detection (LOD) of current assays (dashed line). Sterilizing cure: undetectable HBsAg in serum, elimination of both cccDNA and integrated viral DNAs.

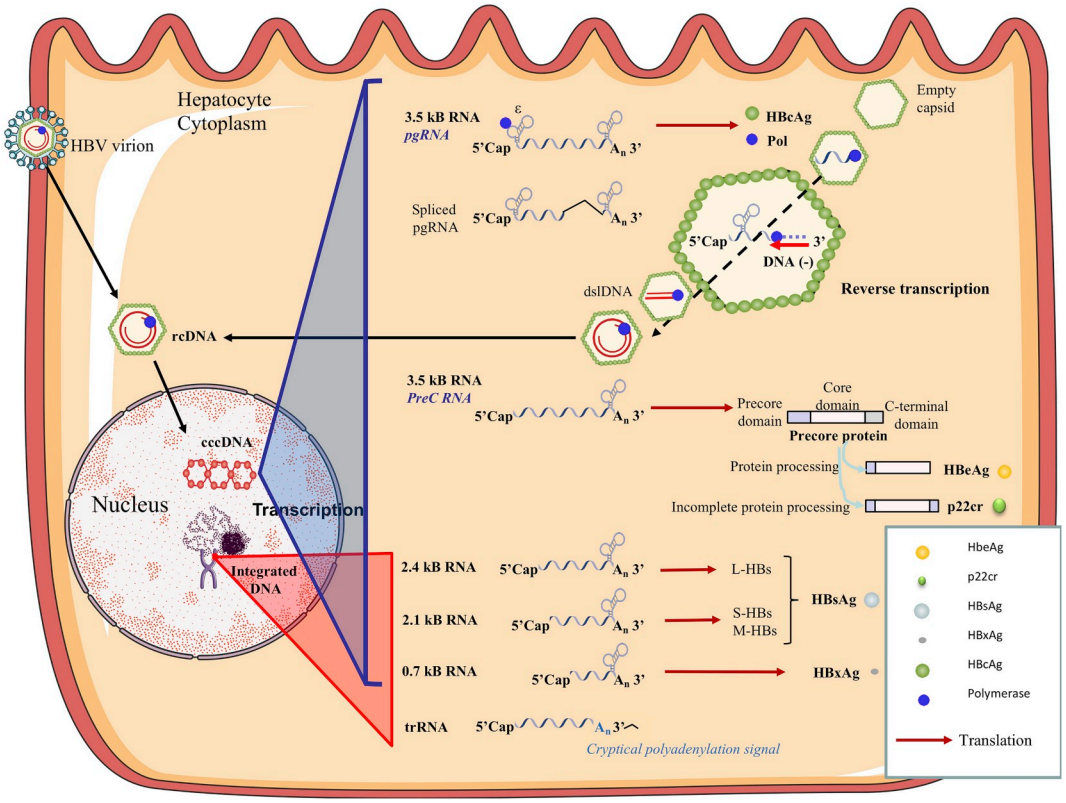

Fig. 3. The HBV replication cycle: genesis of biomarkers.
Covalently closed circular DNA (cccDNA) serves as a template for transcription of all viral RNAs, a greater-than-genome pregenomic RNA (pgRNA) and subgenomic RNAs that have different 5′ ends, but share a common 3′ poly-A end. There are two 3.5-kb transcripts, one subgenomic RNA called the pre-core RNA (preC RNA) and the pgRNA. The preC RNA is translated into the precore protein precursor that leads to the hepatitis B e antigen (HBeAg) and the 22 kDa precore related protein (p22cr) after differential protein processing. The pgRNA serves as template for the translation of the hepatitis B core antigen (HbcAg) and the polymerase. The 2.4-kb and 2.1 kb transcripts are translated into the hepatitis B surface antigens (HBsAg). The 0.7-kb transcript is translated into the hepatitis B x antigen (HBxAg). The 2.4 kb, 2.1 kb and 0.7 kb transcripts can also be transcribed from integrated HBV sequences. The pgRNA is reverse-transcribed within nucleocapsids: after a step of priming at the 5′ end ε, polymerase-DNA primer is transferred to the 3′ DR1 region to initiate minus-strand DNA synthesis. The 3′ end ε is cleaved by RNase H domain of polymerase. During the extension of minus-strand DNA, pgRNA is continuously cleaved from 3′ end leading to pgRNA fragments with receding 3′ ends. Polymerase than synthesizes the second DNA strand forming a double stranded linear intermediate (dslDNA) that can be integrated randomly within the human genome or recircularizes to form relaxed circular DNA (rcDNA) that can replenish the cccDNA pool or undergo further packaging to be released from the hepatocyte.

viral determinants that are important for the understanding of the novel biomarkers, their related assays, and the clinical relevance of their testing.

Viral particles contain a 3.2 kb relaxed circular partially double-stranded DNA genome (rcDNA) that harbors four overlapping open reading frames (ORFs) encoding at least seven different proteins:

- a capsid protein (the HB core antigen or HBcAg) and a secreted dimeric protein (HBeAg),
- a polymerase, exhibiting several activities (priming, reverse transcriptase, RNaseH and DNA-polymerase),
- three envelope proteins (large, medium and small) harboring the HBsAg,
- and a transcriptional activator (hepatitis B x antigen or HBxAg).

An important intermediate in HBV replication is the cccDNA, an HBV minichromosome that acts as a template for the transcription of all viral RNAs and represents the genomic form of viral persistence (Nassal, 2015). The expression of viral transcripts from the cccDNA is regulated by four promoters and two enhancers, which guide the transcription of at least five RNAs: a greater-than-genome pregenomic RNA and subgenomic RNAs that have different 5′ ends, but share a common 3' poly-A end. There are two 3.5 kb transcripts, one subgenomic RNA called the pre-core mRNA (preC mRNA) translated into HBeAg and sometimes into an aberrantly processed protein called p22cr, and one pregenomic RNA (pgRNA) that serves as template for the translation of the core protein and the polymerase (Blondot et al., 2016), as well as for reverse transcription into rcDNA within the newly formed nucleocapsids. A 2.4 kb transcript is translated into the large hepatitis B surface protein (L-HBs). The L-HBs encompasses the PreS1-domain, the PreS2-domain, and the S-domain. Two 2.1 kb transcripts are translated into the middle (M-HBs) that consists of the PreS2- and the S-domain and the small surface proteins (S-HBs) which contains the S-domain, respectively. Finally, a 0.7 kb transcript is translated into the HBxAg.

A stem-loop structure epsilon (ε) in the 5′ terminal region of pgRNA is recognized by the polymerase to form a covalently-bound polymerase-ε complex, followed by recruitment of the core protein to mediate pgRNA selective encapsidation. A second copy of ε present at the 3′-end of the RNAs does not play any role in packaging (Jeong et al., 2000). However a recent study found HBx RNA in cell culture supernatant and serum sample of patients and suggests that it may be present in viral particles, subviral particles (SVP) or microvesicles questioning the selectivity of the packaging (Niu et al., 2017). pgRNA is reverse-transcribed within the nucleocapsid to synthesize rcDNA (Hu and Seeger, 2015): after a step of priming at the 5′ end ε, polymerase-DNA primer is transferred to the 3′ DR1 region to initiate minus-strand DNA synthesis. The 3′ end ε is cleaved by RNase H domain of polymerase. During the extension of minus-strand DNA, pgRNA is continuously cleaved from 3′ end leading to pgRNA fragments with receding 3′ ends. HBV polymerase then synthesizes the second DNA strand finally

forming the double stranded rcDNA. Mature nucleocapsids containing rcDNA are then enveloped and secreted through multivesicular bodies or re-enter the nucleus to replenish the cccDNA pool. The latter route can be maintained even under clinically efficient NUCs treatment (Boyd et al., 2016), suggesting an incomplete viral suppression.

It was long thought that only mature nucleocapsids could be enveloped and secreted (Bruss, 2004; Gerelsaikhan et al., 1996). However, throughout the replication process, many replicative intermediates including non-enveloped HBV DNA-containing nucleocapsids and enveloped pgRNA-containing forms are produced, and in some cases, secreted (Hu and Liu, 2017; Ning et al., 2011; Wang et al., 2016). Non enveloped but encapsidated pgRNA-containing particles within immune complexes have also been observed (Bai et al., 2018).

Several spliced RNA variant transcripts originating from the same promoter as pgRNA and containing ε, but lacking several introns removed have been described (Abraham et al., 2008; Günther et al., 1997). The presence of ε at the 5' end in the spliced RNA transcripts allows the packaging of these RNAs into capsids, their reverse transcription and secretion as spliced DNA-containing particles (Terré et al., 1991).

In around 10% of cases, RNA primer translocation does not occur during the second strand synthesis and plus-strand DNA synthesis is primed from DR1, resulting in the formation of a double-stranded linear DNA (dslDNA) instead of rcDNA (Haines and Loeb, 2007). dslDNA is also generated after hepatocyte infection, during the formation process of cccDNA from rcDNA (Gómez-Moreno and Garaigorta, 2017). dslDNA can be randomly integrated into the host genome (Bill and Summers, 2004). During this process, the core promoter is cleaved from its ORF and linearization prevents the synthesis of greater-than-genome pgRNA (Tu et al., 2017). Thus, functional pgRNA for viral DNA replication and preC RNA cannot be generated from integrated sequences. However, some HCCs express transcripts containing the HBe/HBcAg ORFs, presumably due to active cellular promoters upstream of the integration site (Lee, 2000). The S ORF remains under the control of its native promoter (Tu and Jilbert, 2017), subgenomic RNAs leading to the formation of HBsAg can thus be generated also from integrated sequences and are indeed the major form produced in HBe-negative patients (Wooddell et al., 2017). Finally, enhancer 1 is known to be active in the integrated form, and so can produce transcripts of the HBx ORF (Shamay et al., 2001), but this ORF is truncated at its 3' end and stop codon is missing, resulting in the possible synthesis of truncated HBx transcripts or chimeric HBV-human fusion transcripts such as HBx-LINE1 (Lau et al., 2014).

HBsAg comprises three proteins, S-HBs, M-HBs and L-HBs which form the viral envelope. All 3 proteins share the same carboxy-terminal, the S-domain, but differ in their amino-terminal sequences and glycosylation status. In serum, they can be part of the complete infectious virions known as Dane particles also called viral particles (VP) or are present in large excess (1:1000 to 1:100000 ratio) (Désiré et al., 2015) as non-infectious SVP (Blumberg, 1977; Chai et al., 2008). One ng of HBsAg corresponds to ca. 2×10^8 22 nm SVP (also called spheres) and to ca. 5×10^7 HBV virions (Gerlich et al., 2007). S-HBs is the predominant protein present in both virions and SVP and L-HBs is relatively enriched in virions and filaments and barely detectable in spheres (Heermann et al., 1984). Pre-S1 domain of L-HBsAg plays an important role in viral entry into hepatocytes (Yan et al., 2012).

3. Description of new biomarkers for HBV management

3.1. Improved HBsAg assays

Current solid phase immunoassay with labelled antibodies against HBsAg should detect at minimum 0.05 IU/mL (= 0.2 ng/mL) HBsAg which corresponds to around 4×10^7 HBsAg particles/mL of serum (Deguchi et al., 2004). This analytical sensitivity corresponds to a mean 14-day window period compared to HBV-DNA in seroconversion panels (Paul Ehrlich Institute data). Abbott Diagnostics recently developed an ultra-sensitive qualitative HBsAg assay with an improved analytical sensitivity at 5.2 mIU/mL (Lou et al., 2018), but is not commercially available yet.

Antibodies used in the immunoassays mainly recognize the "a" determinant contained in the S-domain, which is common to the 3 HBsAg isoforms. Thus S-HBs, M-HBs and L-HBs forms of HBsAg of all HBV genotypes can be detected, but not distinguished. Furthermore, assays cannot discriminate between the different forms of HBsAg produced, either virion-associated, SVP forms or produced from integrated sequences.

3.1.1. Quantitative HBsAg assays

HBsAg seroclearance is now considered to be the standard definition of a "*functional cure*" for chronic HBV infection and the goal for novel antiviral strategies. Accordingly, quantifying serum HBsAg (qHBsAg) has become a routine test in clinical studies of new HBV therapies.

Three assays (Table 1), namely the Architect HBsAg assay (Abbott Diagnostics, Abbott Park, IL, USA), the Elecsys HBsAg II quant assay (Roche Diagnostics, Indianapolis, IN, USA) and the DiaSorin Liaison XL

Table 1
Analytical performances of current and improved HBsAg assays.

HBsAg assays			Analytical performances		
			Lower limit of Detection (mIU/mL) (HBsAg subtype adw2, genotype A; NIBSC code number: 00/588)	Lower limit of Quantification (mIU/mL)	Specificity
Qualitative	Classic	Abbott HBsAg Qual II	21 (datasheet) 21 (Lou et al., 2018)	–	99.9% datasheet
		Siemens HBsAg II	40 (datasheet) 29 (Lou et al., 2018)	–	–
		Roche HBsAg II	33 (datasheet) 32 (Lou et al., 2018)	–	99.98% datasheet
		Beckman Access HBsAg	75 (Lou et al., 2018)	–	99.96%
	Ultra-sensitive	Abbott HBsAg prototype	5.2 (Lou et al., 2018)	–	100%
Quantitative	Classic	Abbott HBsAg	50 (datasheet) 26 (Thibault et al., 2017)	50	99.87%
		DiaSorin HBsAg	50 (datasheet) 59 (Thibault et al., 2017)	50	> 99,5% datasheet
		Roche Elecsys HBsAg II Quant II	50 (datasheet) 39 (Thibault et al., 2017)	50	100%
	Ultra-sensitive	Fujirebio, Lumipulse HBsAg-HQ (Yang et al., 2016)	4	4	99.84%
		ICT-CLEIA (Takeda et al., 2013)	0.2	0.5	99.5–99.95%

(DiaSorin, Saluggia, Italy) are commonly used for HBsAg quantification with a sensitivity of 0.05 IU/mL (50 mIU/mL) and a good inter-assay correlation. However, slight differences have been highlighted between systems and absolute values for different genotypes, thus a 0.1 log IU/mL difference should not be considered as clinically relevant (Thibault et al., 2017).

Updated knowledge on qHBsAg use has been nicely reviewed in (Cornberg et al., 2017).

The degree of correlation between qHBsAg and intrahepatic viral markers, in particular cccDNA levels, varies greatly between studies and is still debated, particularly in HBeAg-negative carriers, in whom transcription from HBV integrants may significantly contribute to HBsAg production, in addition to its transcription from the cccDNA template (Wooddell et al., 2017).

3.1.2. Ultrasensitive HBsAg assays

The analytical sensitivity of 0.05 IU/mL is not sufficient to detect HBV in all phases of infection especially OBI, and to detect HBV reactivation at an early stage under immunosuppressive therapy. To circumvent these issues, several assays with a higher sensitivity have been developed.

Lumipulse HBsAg-HQ (Fujirebio) is a highly sensitive HBsAg chemiluminescent enzyme quantitative immunoassay with a 10-fold greater sensitivity (0.005 IU/mL) than current commercial qHBsAg assays (Matsubara et al., 2009; Yang et al., 2016).

A semiautomated immune complex transfer chemiluminescence enzyme immunoassay (ICT-CLEIA) claims a 100-fold improvement of the sensitivity (0.5 mIU/mL) with a specificity of over 99%. ICT-CLEIA requires sample pretreatment and has a turn-around assay time of around 3 h (Shinkai et al., 2017; Takeda et al., 2013). A fully automated assay would thus be required for commercial use.

With the 10-fold improved sensitivity, an additional 1% of patients were classified as positive for HBsAg in a screening assay in a highly prevalent country. Six patients with HBV DNA between 32 IU/mL and 600 IU/mL that were previously identified as negative with the Architect qHBsAg assay, tested positive with Lumipulse (Yang et al., 2016). The mean closure of the window period in HBs seroconversion panels of the ICT-CLEIA assay was 23.6 days shorter than the Architect qHBsAg assay. In a retrospective study (Shinkai et al., 2017), the sensitivity of ICT-CLEIA with minimal sample (120 µL) was shown to be similar to that of HBV DNA quantification with a threshold of 20 IU/mL to detect HBV reactivation in patients undergoing immunosuppressive therapy.

Few studies have evaluated the association between extremely low levels of HBsAg (below the classical thresholds) and the clinical outcome. Re-examination of sera from Japanese patients that had cleared their HBsAg using highly sensitive HBsAg assays revealed that HBsAg was positive in approximately 50% of cases, and that the majority of the patients having discordant results seroreverted during follow-up (Ozeki et al., 2018).

Although the usefulness of ultra-sensitive tests to confirm “functional cure” and predict the risk of reactivation have to be further evaluated, they undoubtedly improve the characterization of OBI and the early identification of HBV reactivation-related hepatitis in patients with resolved HBV infection during the course of chemotherapy and/or immunotherapy (especially in rituximab-based therapy).

Analytical performances of all aforementioned HBsAg assays are summarized in Table 1.

3.1.3. Quantification of the different isoforms of HBsAg

Differential assessment of HBsAg components has been proposed as a potential biomarker in HBV infection (Heermann et al., 1984; Petit

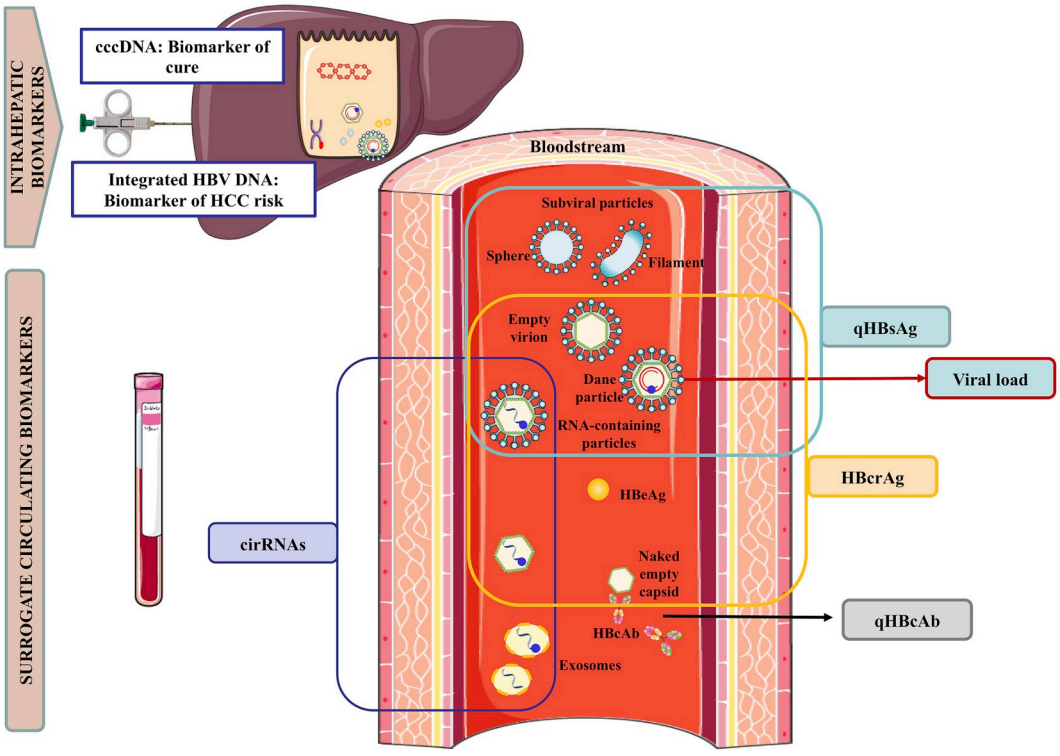

Fig. 4. Novel biomarkers for intrahepatic hepatitis B virus activity.
The direct assessment of cccDNA quantity and integrated HBV require an invasive liver biopsie. Infected hepatocytes release HBV particles containing rcDNA (virions also called Dane particles), RNA-containing particles (virions-like, in naked capsids or exosome-associated), empty virions and non-infectious subviral particles (filaments and spheres). Standard surrogate biomarkers are the viral load (Dane particles), the quantitative HBsAg (particles highlighted in a light blue box), the HBe antigen and antibodies (Ab) such as HBc, HBs and HBe antibodies. New biomarkers include quantitative HBcAb, circulating RNAs (cirRNAs, in purple box) and hepatitis B core-related antigen (HBcrAg, in orange box).

et al., 1990). The three HBs proteins can be measured using an in-house quantitative ELISA. A first immune-reaction is based on 3 monoclonal antibodies that bind specifically to the S-domain (total HBsAg), the preS1-domain (L-HBs) and the N-glycosylated preS2-domain (M-HBs) of the HBs protein. A biotin-conjugated antibody and peroxidase-conjugated streptavidin then enable detection. S-HBs can be indirectly quantified by subtracting L-HBs and M-HBs from total HBsAg (Rinker et al., 2015). This differential HBsAg assay seems to be a promising biomarker to accurately identify phase 3 patients (Pfefferkorn et al., 2017).

3.2. Quantitative HBc antibody

Quantitative anti-HBc antibodies (qHBcAb) levels can be measured using a commercially available immunoassay (Wantai, Beijing, China) and have been found to be a sensitive and specific indicator of the host immune response against HBV. HBcAb production is significantly higher during the "hepatitis" phases (2 and 4) than during the "infection" phases (1 and 3) (Song et al., 2015). Unlike qHBsAg, qHBcAb testing is not routinely used in management and still requires international standardization. As for ultrasensitive HBsAg assays, qHBcAb might improve the characterization of OBI (Caviglia et al., 2018; Yang and Kao, 2018) and the early identification of HBV reactivation-related hepatitis in patients with resolved HBV infection during the course of chemotherapy and/or immunotherapy (especially in rituximab-based therapy (Yang et al., 2018)).

3.3. Hepatitis B core-related antigen (HBcrAg)

Hepatitis B virus core-related antigen (HBcrAg) is a composite biomarker that comprises HBcAg, HBeAg and a 22 kDa truncated core-related protein (p22cr) (Fig. 4). p22cr is an aberrantly processed Pre-Core protein containing the entire PreC region including the uncleaved signal peptide (*i.e.*, with a 29 residue N-terminal extension relative to HBc or 19 residue extension relative to HBe) but similarly to HBe, lacks the C-terminal arginine-rich domain (CTD), which mediates RNA and DNA binding by HBcAg (Kimura et al., 2005).

This assay was first described in 2002 (Kimura et al., 2002) and is available since 2014 as a fully automated quantitative assay using the Lumipulse G1200 CLEIA (ChemiLuminescent Enzyme Immunoassay) analyzer (Fujirebio, Tokyo, Japan). However, this commercial assay has currently no FDA or CE-certification. The 3 proteins share a common sequence of 149 amino acids forming a linear epitope that can be recognized by a monoclonal antibody after denaturation. A pretreatment is required to release all core-related antigens from immune complexes or enveloped particles. This assay can thus detect all capsid-containing particles (HBcAg), either virions or free capsids, p22cr-related particles and secreted HBeAg. Unlike qHBsAg, HBcrAg quantification should not be influenced by translation from integrated viral sequences and could be a more accurate surrogate marker to evaluate the transcriptional activity of cccDNA. Indeed, Testoni et al. showed that patients with HBcrAg level under 3 log U/mL had a lower intrahepatic amount of cccDNA, a reduced cccDNA transcriptional activity and lower fibrosis and necro-inflammatory activity scores (Testoni et al., 2019).

Quantification is estimated by comparing luminescence signals to a Pro-HBeAg recombinant standard curve. The range of measurement varies from 3 to 7 log U/mL and the LOD was determined at 2.4 logU/mL (Kimura et al., 2002). Many studies (Maasoumy et al., 2015; Oliveri et al., 2017; Riveiro-Barciela et al., 2017; Seto et al., 2016; L. Wang et al., 2018; Zhang et al., 2017) using a LOD of 2 log U/mL may have misinterpreted non-specific signals, since unpublished data from Fujirebio revealed a specificity of 99% in blood donors above 2.8 log U/mL but only 68% with a threshold set at 2 log U/mL.

Improvement of the sensitivity of this test will be required prior to its use in evaluating novel antiviral compounds under clinical development, since only 30–50% of HBeAg-negative patients are currently identified as positive for HBcrAg (Testoni et al., 2019).

3.4. Serum HBV RNA

HBV RNAs can be detected in the serum of patients at levels ranging between 0.1 and 1% of HBV DNA levels (Fig. 4), in the absence of antiviral treatment (Butler et al., 2018; Huang et al., 2018, 2015; 2010; Jansen et al., 2016; Rokuhara et al., 2006; van Bömmel et al., 2015; Wang et al., 2016). Circulating HBV RNA was initially observed by Miller et al. in 1984 in a form of HBV DNA-RNA hybrid molecules (Miller et al., 1984). In 1996 Kock et al., confirmed the presence of circulating polyadenylated HBV RNAs in the sera of HBV-infected patients by using rapid amplification of complementary DNA (cDNA)-ends (RACE) (Köck et al., 1996). However, the precise species of circulating

RNA species		Source	Circulating species	References
5' Cap A_n 3'	3.5 kb pgRNA	cccDNA	++	Wang *et al.*, 2016 Jansen *et al.* 2016 Butler *et al.* 2018
5' Cap	3.5 kb pgRNA with receding 3'end	cccDNA	+	Bai *et al.*, 2018
5' Cap A_n 3'	3.5 kb PreC RNA	cccDNA	+ Minority compared to pgRNA	Wang *et al.*, 2016 Jansen *et al.*, 2016 Prakash *et al.*, 2018 Actinel *et al.*, 2016
5' Cap A_n 3'	Spliced pgRNA	cccDNA	+	Lam *et al.*, 2017
5' Cap A_n 3'	2.4 kb S RNA	cccDNA / [integrated]	-	
5' Cap A_n 3'	2.1 kb S RNA	cccDNA / [integrated]	-	
5' Cap A_n 3'	0.7 kb X RNA	Incoming virions / cccDNA [integrated] ?	+	Niu *et al.*, 2017
5' Cap A_n 3' *Cryptical polyadenylation signal*	trRNAs	cccDNA /Integrated ?	+	van Bömmel *et al.*, 2015

Fig. 5. Circulating RNA species.
All HBV RNA species with template from which they are transcribed are represented. References are indicated when RNA species have been reported in serum. pgRNA: pregenomic RNA; preC RNA: pre-core RNA; cccDNA: covalently-closed circular DNA; trRNAs: truncated RNA.

RNAs and the molecular entities they are associated with (either free RNA, exosome containing RNA or viral particle containing RNA) remain a matter of debate.

3.4.1. Circulating RNA species (Fig. 5)

Recently, several studies using multi-PCR identification or cap analysis of gene expression (CAGE) (Altinel et al., 2016) highlighted that circulating HBV RNAs were mainly pgRNAs either polyadenylated or poly-A free. Full-length transcripts ending at the canonical polyadenylation signal "TATAAA" motif downstream of the HBx ORF are the major form described (Butler et al., 2018; Jansen et al., 2016; van Bömmel et al., 2015; Wang et al., 2016). Van Bömmel et al. also found RNAs truncated at the 3′-end in serum (van Bömmel et al., 2015). Truncated RNAs were discovered in HCCs by Hilger et al., who described a cryptic polyadenylation signal CATAAA located within the HBx ORF (Hilger et al., 1991). This cryptic polyadenylation site is usually used upon transcription from viral integrated sequences (Breitkreutz et al., 2001). Circulating truncated RNAs (trRNAs) may thus reflect the reservoir of integrated viral sequences. Accumulation of circulating 3′-truncated polyA-free pgRNA has also been observed in sera of CHB patients under NUCs treatment (Jie Wang et al., 2017b).

As NUCs hamper reverse transcription of encapsidated pgRNA, increased levels of circulating pgRNA are generally observed after instauration of NUCs treatment. Circulating HBV RNAs may also include replicative intermediates of pgRNA, formed as a result of abortive RNaseH degradation by viral polymerase, *i.e.* residual pgRNA sequences present in minus strand DNA-pgRNA hybrids. Those hybrids mimicking double-stranded DNA may then be released from hepatocytes according to state of advancement of reverse transcription and DNA maturation (Hu and Liu, 2017). In addition, a distribution of pgRNA fragments with receding 3' ends was observed in treatment-naïve HBV infected patients and in the supernatant of HBV-replicating stably transduced cell lines (HepAD38) (Bai et al., 2018). These results suggest that circulating HBV RNAs may also include replicative intermediates of pgRNA according to reverse transcription advancement.

Several splice variants of pgRNA with internal deletions preserving both the 5′ and the 3' ends have also been described as circulating forms of HBV RNAs (Lam et al., 2017). *In vitro*, over a dozen splice-derived variants have been recognized (Günther et al., 1997), SP1-RNA representing over 30% of the total pgRNAs (Wu et al., 1991). Spliced cDNA variants resulting from reverse transcription of the spliced RNAs were found in the blood of CHB patients (Chen et al., 2015).

Although pgRNA or viral RNA derived from pgRNA species are the major species currently described, other viral RNA species may circulate in the bloodstream. For example, HBx mRNA was recently detected in sera of patients with CHB (Niu et al., 2017).

Chimeric viral-human transcripts resulting from the transcription of integrated sequences have so far been detected only intracellularly (*i.e.,* not circulating freely in the bloodstream) (Lau et al., 2014).

3.4.2. Circulating RNA containing particles (Fig. 6)

As for the exact composition of circulating RNA species, the nature of circulating RNA-containing particles (associated with exosomes, naked capsids or fully enveloped) still remains controversial. *In vivo*, the association between hepatic cytolysis and the level of circulating RNAs (Bai et al., 2018; van Campenhout et al., 2018) led to the hypothesis that RNA-release might result, at least partly from lysis of infected cells. Nevertheless, by inhibiting exosome secretion, Wang et al. observed no decrease in the total 3.5 kb RNA level (Wang et al., 2016). Secretion by exosomes might thus be restricted to some subgenomic RNAs. Secretion of the truncated RNAs was observed without other HBV markers (Su et al., 2001; van Bömmel et al., 2015), which makes the association with particle secretion in this case highly improbable and is probably linked to the release of vesicles during the lysis of the infected hepatocytes.

In accordance with *in vitro* experiments showing the presence of extracellular viral RNA-containing naked capsids (Ning et al., 2011), Bai et al. recently found viral RNA-containing naked capsids, in an immune complex form, in the plasma from 4 patients with CHB (Bai et al., 2018). *In vitro,* it has been shown that pgRNA-containing naked capsids can be secreted via the natural endosomal sorting complex required for transport (ESCRT) pathway (Chou et al., 2015). In normal conditions, the CTD domain of HBcAg is highly phosphorylated, impairing the packaging of non-specific RNA. However, some conditions might lead to a hypophosphorylated state of the CTD (Zhao et al., 2018), leading to unspecific packaging of RNAs in naked capsids (Ludgate et al., 2016). Genome-free secreted HBV particles were shown to contain p22cr instead of HBcAg (Kimura et al., 2005).

Other experiments imply that viral HBV RNA may circulate in viral particles: 1) serum RNAs were co-purified with HBV DNA and core

Circulating RNAs forms			Literature	References
RNA containing viral particles	pgRNA		+	Jansen et al., 2016, Rokuhara et al., 2006; Wang et al., 2016
	Subgenomic RNAs		-	
RNA containing capsids	pgRNA		+	Bai et al., 2018
	Subgenomic RNAs		-	
Exosomes	pgRNA		-	
	Subgenomic RNAs			
Free RNAs	pgRNA		-	
	Subgenomic RNAs			

Fig. 6. Compartimentalization of circulating RNAs.
Hypothetic forms of circulating RNAs (cirRNAs) are represented. References are indicated when the presence of cirRNA form are clearly be demonstrated. pgRNA, pregenomic RNA.

protein in a sucrose gradient (Rokuhara et al., 2006; Wang et al., 2016), suggesting a similar composition; 2) viral RNA was found in the supernatant of a hepatoma cell line stably transfected with HBV genome (HepG2.2.15) after precipitation with anti-HBs antibodies suggesting the presence of an HBsAg envelope (Jansen et al., 2016); 3) pgRNA was also detected after anti-HBc immunoprecipitation in the plasma of 3 treatment naïve HBeAg-positive patients with CHB demonstrating an association between pgRNA and the capsid. The detection was enhanced using a detergent treatment to remove the HBV envelope, suggesting that pgRNA may be both encapsidated and enveloped (Jansen et al., 2016). This observation is consistent with the protection of serum viral RNA from exogenous RNAses (Jansen et al., 2016; Prakash et al., 2018). Altogether these observations suggest a wide heterogeneity of circulating HBV RNA species that may vary depending on different stages of chronic HBV infection.

3.4.3. *Measurements of circulating viral RNAs (Fig. 7)*

Methods of quantification of serum HBV-RNA differ according to studies. There are two main strategies to detect and quantify HBV transcripts. One is based on a 3′ end amplification that amplifies the majority of HBV transcripts (except truncated RNAs) as they share the same 3' end (Gao et al., 2017; van Bömmel et al., 2018, 2015; van Campenhout et al., 2018; Wang et al., 2016). Quantification of circulating truncated RNAs by assays using a primer in the cryptic polyA signal site could reflect the transcriptional activity of viral integration (van Bömmel et al., 2015).

The second is based on a 5' amplification specifically targeting 3.5 kb transcripts (pgRNA and preC mRNA) (Butler et al., 2018; Jansen et al., 2016; Jie Wang et al., 2017a, 2017b; Jing Wang et al., 2017; J. Wang et al., 2018; Wang et al., 2016). Assays based on the aforementioned techniques, generally use a low input volume, ranging from 100 to 300 µL of plasma or supernatant. However, their sensitivity remains to be improved: currently the LOD ranges from 1.85 log copies/mL (Jansen et al., 2016) to 3.4 log copies/mL (van Bömmel et al., 2018). A high throughput HBV RNA test developed recently (Abbott m2000 RNA) claimed a LOD of 1.65 logU/mL, where 1U HBV RNA = 1 IU HBV DNA (Butler et al., 2018) corresponding to around 150 copies/mL. Of note, there is currently no direct standardization of HBV RNA quantification. In most assays, viral RNA is indirectly estimated after the reverse transcription step. cDNA is then quantified by comparing signals to an HBV DNA standard curve (usually plasmid) (Butler et al., 2018; Jansen et al., 2016; Jie Wang et al., 2017a, 2017b; Wang et al., 2016).

Careful interpretation of the results should be made depending on the assay and especially primers used. Furthermore, quantification of circulating RNA seems to depend on the HBV genotype but also on the presence of basal core promoter mutation and pre-core mutation (Prakash et al., 2018; van Campenhout et al., 2018). Standardization of quantification is thus needed to rigorously compare results from different studies with the aim of using circulating RNA as a new biomarker to assess target engagement (TE) of new compounds or to be used as a new treatment endpoint. DNase digestion conducted to remove viral DNA before RNA detection, might not be sufficient to completely remove all viral DNA. Indeed, in some cases, residual HBV DNA may be measured instead of or in addition to viral RNA, leading to conflicting results between studies.

4. Relevance of these new biomarkers in the classification and monitoring of HBV infection

The management of CHB is challenged by its varying natural course. Indeed, not all HBV-infected patients will develop complications of infection. However, it is of utmost importance to identify patients who are at risk and require antiviral treatment and/or close surveillance.

4.1. *Novel biomarkers to follow the natural history of HBV*

4.1.1. *Classification of the patients*

Currently the distinction between phase 3 and phase 4 patients is based on ALT and serum HBV DNA statuses. However, as these biomarkers may fluctuate over time, periodical measurements are recommended before rigorously assessing the stage (Lampertico et al., 2017; Terrault et al., 2018). Combinations of novel biomarkers to the classical ones may improve the assignment of patients to specific disease phases using a single timepoint measurement.

In HBeAg-positive patients (Chan et al., 2007), the level of HBsAg mainly reflects the transcriptional activity of cccDNA. However, in HBeAg-negative patients, HBsAg may still be highly expressed despite low cccDNA transcriptional activity, since integrated HBV DNA can maintain HBsAg production (Wooddell et al., 2017). HBsAg levels in the serum are thus poorly correlated with intrahepatic cccDNA levels in

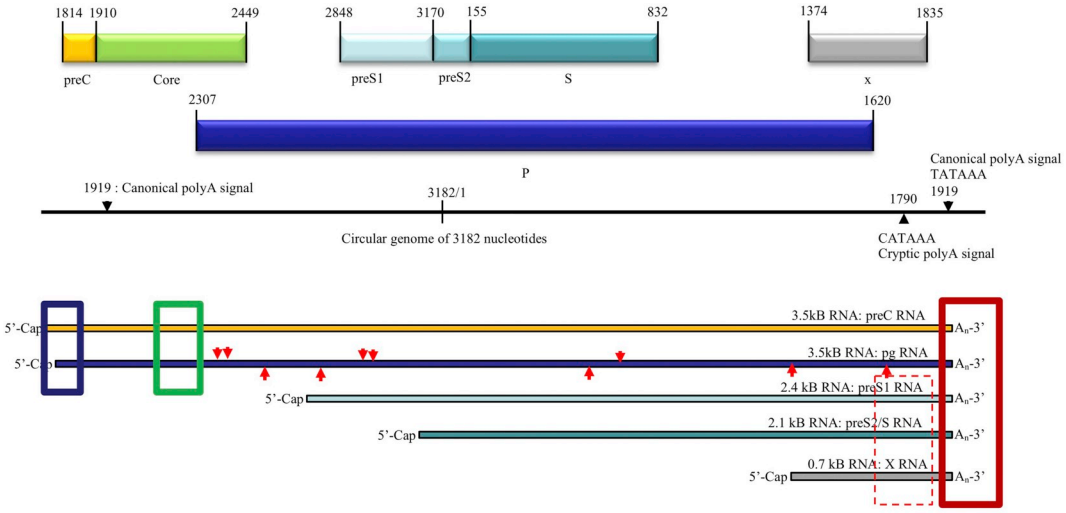

Fig. 7. Different strategies described for measuring circulating RNAs.
HBV ORFs and their end nucleotide positions are represented with colored boxes. Full length RNAs are represented with colored solid lines. There are two main strategies to detect and quantify HBV transcripts. One is based on a 3′ end amplification (red box) that amplifies the majority of full-length HBV transcripts as they share the same 3′ end. The other one is based on 5′ amplification that permit to amplify 3.5 kB transcripts (both pgRNA and preC mRNA; green box). A preC RNA specific PCR (blue box) showed that preC RNA is insignificant in the serum and that 3.5 kB transcripts are mainly pgRNA. Specific primers were also designed to specificaly amplify truncated RNAs that end at the cryptic polyA site (dashed box). Splicing site on pgRNA are indicated by red arrows.

HBeAg-negative patients. Yet, qHBsAg is currently proposed as a useful marker to identify phase 3 patients with different cut-offs (but usually 1000 IU/mL) depending on the HBV genotype and the origin of the patient (Cornberg et al., 2017). In Asian cohorts of patients mostly infected with HBV genotype B and C, it has been shown that in a context of low viral load (HBV DNA < 2000 IU/mL), a qHBsAg level > 1000 IU/mL is associated with a higher risk of hepatitis flare, cirrhosis and HCC (Tseng et al., 2012, 2013).

It has been shown in a European cohort of untreated genotype A and D HBV infected patients that the composition of HBsAg differed according to the phases of HBV infection. Moreover, percentages of L-HBs and M-HBs were better predictors of phase 3 carriers than viral load or total HBsAg. In HBeAg-negative patients with a total HBsAg level > 1000 IU/mL, proportions of L-HBs and M-HBs were significantly lower in HBV infection carriers than in patients with chronic hepatitis regardless of their HBeAg status (Petit et al., 1990; Pfefferkorn et al., 2017). Therefore, differential HBsAg seems to be a promising biomarker to accurately identify phase 3 patients.

Serum HBcrAg levels differ significantly according to the aforementioned CHB phases and is reported to classify patients even better than qHBsAg (Riveiro-Barciela et al., 2017; Testoni et al., 2019). In both Asian (Chen et al., 2017; Seto et al., 2014; L. Wang et al., 2018) and European (Maasoumy et al., 2015; Testoni et al., 2019) cohorts of treatment-naïve CHB patients, serum HBcrAg has been shown to be higher in HBeAg-positive patients than in HBeAg-negative patients. Among HBeAg-positive patients, the serum HBcrAg level was significantly lower in a context of chronic hepatitis than in a context of chronic infection (Seto et al., 2014). Conversely, among HBeAg-negative patients, serum HBcrAg level was significantly higher in a context of chronic hepatitis than in a context of chronic infection. In accordance with this finding, Zhang et al. highlighted the potential of serum HBcrAg to significantly predict necro-inflammation and significant fibrosis in HBe-negative patients (Zhang et al., 2016). Another study reported that HBcrAg levels below 3 log U/mL combined with a viral load below to 2000 IU/mL, at a given timepoint, accurately identifies phase 3 patients, irrespective of HBV genotypes (Riveiro-Barciela et al., 2017). In this context, combination of HBcrAg with the classical markers has been proposed to accurately discriminate patients following a single timepoint assessment (Brunetto et al., 2018).

Levels of circulating HBV RNA were also reported to differ between phases of infection, with highest levels observed during HBeAg-positive infection phase. A decrease in circulating HBV RNA levels was noticed in the order of the following mentioned phases: HBeAg-positive hepatitis, HBeAg-negative hepatitis, HBeAg-negative infection phase (Wang. JVH. 2018). Given that circulating HBV RNA both correlate with HBV DNA and hepatic cytolysis (Campenhout. Hepatology. 2018) among treatment-naïve patients, this distribution among phases of disease is not surprising. However, the clinical relevance of serum HBV RNA levels in the classification of the patients remains unclear.

4.1.2. Prediction of spontaneous HBeAg and HBsAg seroconversion

Serum HBcrAg has also been shown to be a useful biomarker to predict spontaneous HBeAg seroconversion (Bae et al., 2012; Song et al., 2017) and HBsAg seroclearance (Seto et al., 2014, 2012).

A decrease in circulating HBV RNA is also a good predictor of HBe-loss in HBe-positive patients (van Bömmel et al., 2018).

4.1.3. Prediction of HBV reactivation during immunosuppression

Owing to the fact that serum HBcrAg can be detected when serum HBV DNA or HBsAg remain undetectable (Seto et al., 2012; Suzuki et al., 2009), it has been measured in HBsAg-negative HBcAb-positive patients undergoing high-risk immunosuppressive therapy (*e.g.,* rituximab administration) and its positivity was reported to be a risk factor for HBV reactivation (Seto et al., 2016). Nevertheless, these results should be interpreted with caution as in this latter study, the LOD was set at 2 logU/mL.

qHBcAb might be an even more valuable tool to identify patients at risk of reactivation. Indeed, in a cohort of liver transplant donors, Caviglia et al. demonstrated that an HBcAb value > 4.4 (cut-off index) was independently associated with the detection of intrahepatic HBV cccDNA while most individuals were negative for serum HBcrAg (Caviglia et al., 2018). In a longitudinal cohort of patients with lymphoma and resolved hepatitis B, defined by the absence of HBsAg and presence of anti-HBc, a higher total HBcAb level (≥6.41 IU/mL) at baseline was associated with increased risk of HBV reactivation (HR of 4.52). Furthermore, a combination of high total HBcAb level and low anti-HBs Ab level (< 56 mIU/mL) at baseline could identify patients with a very high risk of HBV reactivation (HR of 17.29) (Yang et al., 2018).

4.1.4. Prediction of HCC development

HBcrAg has been reported to be a predictor of HCC development among CHB patients, not receiving NUCs therapy (Pan To et al., 2018; Suzuki et al., 2019; Tada et al., 2016), and HBcrAg detection after a long term NUCs therapy is also associated with a risk of HCC development (Honda et al., 2016; Suzuki et al., 2019).

Increased ratio of blood HBV DNA splice variants compared to the wild-type virus is correlated with liver fibrosis and HCC (Bayliss et al., 2013; Soussan et al., 2008).

Some deletions in preS1 and preS2-domains that might decrease L-HBs secretion following its accumulation in the endoplasmic reticulum, also appear to be related to a higher risk of HCC development (Chen et al., 2006, 2007). Thus, a low level of L-HBs should be considered with caution and further studies, both in European and in Asian cohorts, are needed to evaluate its predicting role in disease progression especially among inactive carriers across viral genotypes, and HBV mutants (Lin et al., 2018).

Studies assessing the presence of trRNAs or chimeric transcripts in blood are still missing to evaluate their potential use in the prediction of HCC development.

4.2. Evaluation of the efficacy of current treatment

Current virologic and serologic endpoints consist in HBV viral load suppression, HBe seroconversion and finally HBsAg clearance followed by HBsAg seroconversion that define functional cure. Persistent suppression of serum HBV DNA and loss of HBsAg are considered to be the most important primary efficacy endpoints for novel compounds in phase 2/3 clinical development. Nevertheless, these biomarkers are not sufficient to assess cccDNA loss or complete cure. Moreover, the use of HBsAg loss may be an overly stringent criterion for a cure if the remaining serum HBsAg is produced exclusively from integrated HBV DNA following the loss of hepatic cccDNA without major pathological consequence. New off-therapy endpoints before HBsAg clearance are needed. Indeed HBsAg clearance might be obtained only after sterilizing cure in some cases where cccDNA has been efficiently cleared.

4.2.1. Use of novel biomarkers during IFN treatment

Due to the side effects of IFN treatments, the use of novel biomarkers could also be helpful to predict response to IFN at an early stage and only extend treatment to patients who display sensitivity. IFN has been described to induce (i) a decrease in HBV transcription as a result of cccDNA epigenetic modification (Belloni et al., 2012), (ii) the degradation of pgRNA (Liu et al., 2017) and (iii) the inhibition of pgRNA encapsidation (Liu et al., 2017; Wieland et al., 2005). Consistent with these mechanisms of action, circulating HBV RNAs decrease upon IFN treatment (Farag et al., 2018; Jansen et al., 2016; van Bömmel et al., 2018). Circulating HBV RNAs may help predict the IFN-response. A decrease in circulating HBV RNA is more pronounced in responders to a combination of IFN/NUCs therapy than in non-responders, irrespective of the analysis time-point and of the HBeAg serostatus (Jansen et al., 2016), and is a good predictor of HBe-loss in HBe-positive patients (van Bömmel et al., 2018). In HBeAg-negative patients, a low

HBV RNA level at baseline is predictive of response to an IFN/adefovir combination therapy (Jansen et al., 2016). Conversely, HBV RNA values above 3.2 log copies/mL at week 12 are associated with no response to IFN treatment (Farag et al., 2018).

A decline in serum HBcrAg may reflect that of cccDNA during IFN treatment (Ma et al., 2016). Moreover, the level of serum HBcrAg and the extent of its decline may predict HBeAg seroconversion in a context of IFN monotherapy (Chuaypen et al., 2016) or IFN combined with NUCs therapy (Matsumoto et al., 2015; van Campenhout et al., 2016). In addition, the predictive performance of HBcrAg appears to be better than that of quantitative HBsAg (M.-L. Wang et al., 2018). Of note, the study by *Caviglia* et al. did not reveal any significant decline in serum HBcrAg during IFN treatment (Caviglia et al., 2017). HBcrAg at baseline has also been shown to predict HBsAg loss in HBe-negative patients treated with IFN combined or not with NUCs (Martinot-Peignoux et al., 2016). Nevertheless, the performance of HBcrAg or HBcrAg combined with qHBsAg was not better than that of qHBsAg alone.

qHBcAb levels at baseline were considered to be the best independent predictor of HBeAg seroconversion following either IFN or telbivudine/adefovir treatments compared to the baseline predictive value of ALT or HBV DNA levels, with a baseline predictive threshold value of qHBcAb ($\geq$4.4–4.5 log U/mL) (Fan et al., 2016; Hou et al., 2015). Using qHBcAb as a surrogate marker of the overall immune response may improve the identification of responders to therapy and has been shown to be independent of HBV genotype.

4.2.2. Use of novel biomarkers during NUCs therapy

Inhibition of the viral polymerase activity by NUCs leads to a rapid decline in viral load, whereas cccDNA remains unaffected. However, after receiving a long-term NUCs therapy, a decline in cccDNA may occur due to the decrease in cccDNA replenishment. Indeed, during adefovir treatment of HBeAg-positive patients, the decline in qHBsAg was correlated with the decline in cccDNA (Werle–Lapostolle et al., 2004). In NUCs-treated patients, a decrease in HBcrAg levels was reported to occur slower than that of serum DNA (Rokuhara et al., 2003; Tanaka et al., 2006) and was significantly correlated with that of cccDNA (Rokuhara et al., 2003; Wong et al., 2017, 2007). A combination of serum HBcrAg and qHBsAg appears to be a highly accurate predictor of HBeAg seroconversion (B. Wang et al., 2018). Moreover, a stronger decline in HBcrAg was also reported in HBe-positive patients who had lost HBsAg following NUCs treatment alone or in combination with IFN (van Campenhout et al., 2016).

NUCs therapy leads *in vitro* and *in vivo* to a relative increase in circulating HBV pgRNA-containing particles compared to DNA-containing particles (Jansen et al., 2016; Wang et al., 2016). Consistently, serum HBV RNA might be a potential surrogate biomarker to monitor the persistence of intrahepatic cccDNA among patients receiving NUCs therapy, especially when viral load is undetectable. Both a decline in serum 3.5 kb RNA (Jansen et al., 2016) and an early stage decrease in total serum RNA (measured by 3′RACE) and its level at baseline (van Bömmel et al., 2015) have been shown to predict HBeAg seroconversion during NUCs treatment. Low serum HBV RNA levels also appear to predict initial virological response (around week 12) in the context of NUCs therapy (Huang et al., 2015).

Finally, among virally-suppressed patients, circulating HBV RNA has been shown to reflect liver disease activity and predict its progression (Jing Wang et al., 2017).

4.2.3. Use of novel biomarkers for NUCs treatment discontinuation

The sustained transcription from cccDNA and integrated viral genomes may explain the relatively minor decrease in serum HBsAg levels during NUCs therapy despite undetectable serum HBV DNA (Seto et al., 2013). In virally-suppressed patients, persistence of the viral reservoir (cccDNA), can be responsible for relapse or viral reactivation. As mentioned before, current assays for circulating HBsAg cannot distinguish between transcription of HBsAg from cccDNA than transcription from integrated HBV DNA. Current recommendations are thus to maintain life-long NUCs treatment, while HBsAg remains detectable. If sensitivity of this biomarker is improved, HBcrAg that cannot be produced from viral integrated sequences, might be a better surrogate marker of cccDNA loss or silencing than HBsAg.

Serum HBcrAg was shown to be measurable in some virally-suppressed patients (Chuaypen et al., 2016; Hosaka et al., 2010; Seto et al., 2014; Wong et al., 2017). Thus, serum HBcrAg may constitute a suitable biomarker for monitoring residual infection under NUCs treatment, and has also been put forward as a biomarker to predict viral relapse after NUCs discontinuation. Consistently, it was shown that a serum HBcrAg levels above 3.7 logU/mL at the end of treatment predict relapse within 1 year after NUCs withdrawal (Jung et al., 2016). Interestingly, the combination of serum HBsAg and HBcrAg is recommended as a predictor for viral relapse after discontinuation of NUCs therapy in Japanese CHB guidelines for the management of hepatitis B virus infection (Drafting Committee for Hepatitis Management Guidelines and the Japan Society of Hepatology, 2014).

Another potential biomarker for predicting rebound after NUCs treatment cessation is circulating HBV RNA, since it has been associated with the residual transcriptional activity of cccDNA in the context of NUCs therapy (Jie Wang et al., 2017a; Jing Wang et al., 2017). Thus, contrary to circulating HBV DNA, disappearance of circulating HBV pgRNA may reflect the elimination of cccDNA or its transcriptional silencing, and predict NUCs therapy discontinuation with a limited risk of relapse (Tsuge and Chayama, 2013; Wang et al., 2016). Consequently, serum HBV pgRNA could be a new interesting end-point to guide cessation of NUCs therapy. In this regard, a new classification of HBV-cure characterized by a sustained loss of circulating HBV pgRNA has been proposed: the "*parafunctional cure*" (Jie Wang et al., 2017a).

5. The importance of new biomarkers in the development and evaluation of novel compounds

Novel compounds, aiming at silencing the cccDNA or reducing the size of the cccDNA pool to achieve a functional cure with finite treatment duration, are under development. New biomarkers aiming at better evaluating the pool and transcriptional activity of cccDNA are needed to assess their efficacy.

These new direct-acting antivirals, targeting different steps of the viral cycle, have been developed and their target engagement (TE) has to be accurately evaluated. TE is defined as the interaction between ligands and their target biomolecules. Understanding these interactions should enable us to accurately assess target validation including issues such as mechanism of action (or pharmacodynamics), efficacy, selectivity, pharmacokinetics and relationships between biological targets and disease.

Based on the mechanism of action of the new drugs being developed, target-specific biomarkers are needed to assess the efficacy of each therapeutic class and to define new endpoints. In this context new surrogate noninvasive biomarkers such as circulating RNAs and HBcrAg have to be evaluated in clinical trials.

Loss of HBsAg might be a too stringent endpoint for early phase clinical trials because of its slow decline kinetics. Furthermore, the impact of the expression of integrated viral sequences on the level of HBsAg, in contexts in which cccDNA would have been cleared or silenced, remains unknown. In both situations, biomarkers such as circulating pgRNA and HBcrAg could be useful to assess antiviral activity of novel compounds and improve the decision-making process to continue or stop the clinical development of a given compound.

However, substudies in selected patients who will undergo liver biopsy to analyze viral and immunological markers are warranted in proof of concept studies to demonstrate target engagement following investigational drug administration. Combination trials will require a careful design to ensure accurate evaluation of the antiviral activity of the investigational drugs and their combination. For instance, in add-on

trials in NUC suppressed patients, the pretreatment period under NUCs will be critical to evaluate the baseline values of each biomarker and their variation during administration of the investigational drugs.

5.1. Capsid assembly modulators (CAMs)

CAMs are a group of small molecules that bind to HBcAg leading either to the formation of core aggregates or to defective capsids unable to encapsidate polymerase-pgRNA complexes (Zhao et al., 2018). Accordingly, rcDNA neosynthesis, as well as virion secretion, are inhibited (Bourne et al., 2008; Wang et al., 2012). Conversely to NUCs therapy, circulating pgRNA were shown *in vitro* to decrease proportionally to HBV DNA upon CAM treatment (Lahlali et al., 2018; Lam et al., 2017; Wang et al., 2016). This observation has been confirmed in a humanized mouse model of persistent HBV infection (Klumpp et al., 2018) and in phase 1b studies (ABI-H0731, NVR 3–778) among HBe-positive patients (Yuen et al., 2019; M.-F. Yuen et al., 2018). Moreover, another CAM (JNJ-56136379) tested in a phase 1 trial decreased HBV RNA regardless of the HBe status (Zoulim et al., 2018). It has also been shown that some CAMs can block *de novo* formation of cccDNA and regulate it's transcriptionnal activity (Berke, 2017). Thus, a decrease in blood HBV pgRNA in CAM-treated patients appears to be the reflexion of the mechanisms of action of these molecules. Nevertheless, it will be difficult to distinguish the effect due to the primary mechanism of action from the secondary that lead to a depletion of cccDNA and in the end also to a decrease in circulating pgRNA. Kinetics studies performed with robust and validated assays in clinical trials will be mandatory to address this question.

In these phase 1b studies, no decline in HBsAg nor HBeAg were observed during the 28-day treatment time-course, which was expected (Yuen et al., 2019; M.-F. Yuen et al., 2018; Zoulim et al., 2018). Indeed, a sharp decline in the cccDNA pool after only 4 weeks of administration was not likely to occur. HBcrAg may also be used to measure TE by CAMs with a decreased release of HBc-containing viral particles, but this would be only measurable in HBeAg-negative patients (Lenz et al., 2019).

Detection of circulating RNA by a 5' specific amplification combined with HBV viral load would be a very promising way of following TE of such treatments. However, besides misdirecting HBcAg assembly, some CAMs have been described *in vitro* to interfere with cccDNA synthesis but also directly impair HBeAg secretion at an early stage without affecting cccDNA amounts (Lahlali et al., 2018). This latter observation suggests that some CAMs can also act at a transcriptional or a post-transcriptional level. Combination of several biomarkers will be crucial to accurately assess the complex mechanism of action of CAMs in patients.

5.2. Small interfering RNAs (siRNAs)

Some siRNAs have been designed to specifically target HBV mRNA. One of the first strategies, in which siRNA were designed to target the 3′ end of all transcripts, was unsuccessful in decreasing serum HBsAg levels in HBeAg-negative patients, due likely to the frequent presence of integrated viral sequences leading to truncation of viral RNAs at their 3' end thus losing the target sequence in this group of patients (Wooddell et al., 2017). Recently, siRNAs have been redesigned to target mRNA transcribed from both cccDNA and integrated viral sequences. As expected, a significant decline in HBsAg, serum DNA, RNA and HBcrAg was observed during a phase 1 clinical trial using a combo of 2 siRNAs (ARO-HBV). No difference was noticed according to the HBeAg status (HBeAg-positive = 11, HBeAg-negative = 13) (Gane et al., 2018). In this context, circulating HBV RNA appears to be an interesting surrogate tool to assess TE, consisting here in intrahepatic HBV mRNA degradation. The downstream pharmacological effect of mRNA degradation, *i.e.* downregulation of HBV DNA and proteins synthesis and secretion, could also be evaluated using other viral parameters such as HBV DNA, HBsAg and HBcrAg. It is important to note that in this case, circulating biomarkers would not reflect the cccDNA pool.

5.3. RIG-1 activator or inarigivir

Inarigivir is a dinucleotide prodrug delivered as an oral antiviral agent for the treatment of chronic hepatitis infections in humans. It binds to both the caspase activation and recruitment domain (CARD) and regulatory domain of retinoic acid inducible gene (RIG-I) protein, a key pathogen recognition receptor (PRR), resulting in a novel dual mechanism of action involving: (i) the activation of the host cytosolic protein RIG-I implicated in recognition of viral nucleic acids, which results in the stimulation of type I and type III IFN production and induction of an antiviral state in cells, and (ii) direct inhibition of viral replication upstream of the viral polymerase by inhibiting the interaction of RIG-I with the 5' ε region of the pgRNA (Colledge et al., 2018).

In the majority of patients receiving RIG-1 activator for 12 weeks, inarigivir therapy has resulted in a significant decline in HBV DNA, HBV RNA and HBcrAg levels with only a moderate decline in HBsAg (Walsh et al., 2018). However, the level of HBsAg at baseline was reported to be a predictive parameter of response to inarigivir therapy (M. F. Yuen et al., 2018).

5.4. Nucleic acid polymer (NAPs)

Nucleic acid polymers (NAPs) are phosphorothioated oligonucleotides with broad spectrum antiviral activity (Vaillant, 2016). They block the HBV life cycle both during and after viral entry. They may block secretion of HBsAg from infected hepatocytes and further may selectively block the assembly and secretion of SVPs. Indeed, a high rate of durable HBsAg clearance associated in most of cases with ALT flares, likely due to an immune reactivation was reported in clinical trials with NAPs. However, new biomarkers such as RNA and HBcrAg have not yet been evaluated during NAPs treatments (Bazinet et al., 2018).

6. Conclusions

In the near future, the most promising use of measurement of serum HBV RNAs and HBcrAg is the identification of patients that may safely discontinue NUCs therapy and those at risk of reactivation upon immunosuppressive therapy. HBcrAg appears to be an interesting tool to differentiate the different phases of CHB.

The molecular characteristics of serum HBV RNA still remains to be better defined and the techniques for its measurement to be standardized, in order to more reliably and accurately interpret the meaning of serum HBV RNA levels.

While the novel circulating biomarkers appear to be correlated to the cccDNA level and transcriptional activity during the natural course of HBV infection, their measurement might be misleading during treatment with novel compounds. Indeed, therapeutics designed to block pgRNA packaging into nucleocapsids such as CAMs or to degrade it such as siRNAs, would lead to a loss of serum HBV RNA-containing virions despite cccDNA persistence in the liver. In these cases, circulating RNA would rather reflect their on-target efficacy. Understanding the mechanism of action of the novel compounds on specific targets and their downstream effect is crucial to adequately use the circulating biomarkers. As the development of these novel direct antiviral therapies requires in parallel the development and evaluation of new viral biomarkers reviewed herein, the efforts to develop immunomodulatory therapies for HBV will have to be accompanied by the evaluation of specific clinical assays to monitor anti-HBV immune responses.

Acknowledgements

This work was supported by public grants overseen by the French National Research Agency as part of the second « Investissements d'Avenir » program (reference: "CirB-RNA" project – ANR-17-RHUS-0003) and by « Agence Nationale pour la Recherche sur le SIDA et les hépatites virales» (ANRS) to FZ (nN16003CR) and to FZ and ML

(nECTZ8323).

We are grateful to Brigitte Manship for critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104553.

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