Are HBcrAg, HBV RNA an anti-HBc viable Biomarkers?

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What should be expect from an ideal HBV biomarker?

- ability to stratify by disease stages and risk for complications (reactivation, cirrhosis, HCC)
- ability to predict functional cure (HBsAg loss)
- ability to predict definite cure (cccDNA eradication)
- helpful to identify treatment response before or early during treatment

Viability: does it provide benefit over current clinical practice?
Current and novel HBV biomarkers

“HBcrAg” measures simultaneously HBeAg, HBcAg and p22cr


Serum HBV RNA is believed to exist in virus-like particles.
### HBV Transcriptonal Producte Underly Different Regulations

<table>
<thead>
<tr>
<th>Name of the Protein</th>
<th>Symbol</th>
<th>Reported Function in HBV Infection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterogenous Nuclear Ribonucleoproteins</td>
<td>C1/C2=HNRNPC</td>
<td>HBV EnhII transcriptional activator</td>
<td>Tay et al. 1992</td>
</tr>
<tr>
<td>Nucleophosmin</td>
<td>NPM1/B23</td>
<td>Interacts with HBNV core dimers to enhance capsid assembly</td>
<td>Jeong et al. 2014</td>
</tr>
<tr>
<td>Heterogenous Nuclear Ribonucleoprotein K</td>
<td>HNRNPK</td>
<td>HBV EnhII transcriptional activator</td>
<td>Ng et al. 2005</td>
</tr>
<tr>
<td>Poly(ADP-ribose) Polymerase 1</td>
<td>PARP1</td>
<td>HBV core transcriptional activator</td>
<td>Ko and Ren, 2011</td>
</tr>
<tr>
<td>Poly(U)-Binding-Splicing Factor PUF60</td>
<td>PUF60</td>
<td>Transcriptionals and Posttranscriptional regulation of HBV pgRNA</td>
<td>Sun et al, 2017</td>
</tr>
<tr>
<td>Polypyrimidine Tract-Binding Protein 1</td>
<td>PTBP1</td>
<td>HBV mRNA export via posttranscriptional regulatory element (PRE)</td>
<td>Zhang et al, 2001</td>
</tr>
<tr>
<td>Heat Shock Protein 90-Beta</td>
<td>HSP90B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Methods for quantification of serum HBV RNA

<table>
<thead>
<tr>
<th>method</th>
<th>RT primer</th>
<th>Primer sites</th>
<th>LOQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-qPCR#</td>
<td>RNA isolation (including DNase treatment) and subsequent PCR method with specific primers</td>
<td>HBV specific</td>
<td>X, C or S region</td>
</tr>
<tr>
<td>ddPCR#</td>
<td>Droplet digital PCR</td>
<td>HBV specific</td>
<td>X or C region</td>
</tr>
<tr>
<td>3’-RACE-based</td>
<td>oligo(dT) primer plus a unique artificial anchored sequence - to generate cDNA</td>
<td>Oligo(dT) Primer Poly A tail</td>
<td>1.95 log IU/mL</td>
</tr>
<tr>
<td>QuantiGene assays#</td>
<td>hybridization-based and use branched DNA (bDNA) signal amplification technology – measurement via Luminometer</td>
<td>n/a</td>
<td>X region</td>
</tr>
<tr>
<td>Indirect</td>
<td>Serum HBV RNA minus HBV DNA determined by real-time PCR</td>
<td>HBV specific</td>
<td>PreC and C</td>
</tr>
</tbody>
</table>

*In order to avoid DNA contamination during RT-qPCR, DNase I treatment of the nucleic acids extracted from serum is required*

Adapted from Liu S et al. Hepatology 2018; epub
HBcrAg as viable Biomarker – Test Performance

Quantification of HBcrAg via pre-treatment allows measurement of HBc, HBe and p22 (precursor of HBeAg)

HBcrAg as viable marker for
- HCC Incidence?
- Treatment response prediction?

<table>
<thead>
<tr>
<th>Assay</th>
<th>Dynamic range (logU/mL)</th>
<th>Automatic on board dilution</th>
<th>Test principle</th>
<th>Limitations</th>
<th>Sample volume</th>
<th>Repeatability CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fujirebio Lumipulse® G</td>
<td>3.0 - 7.0 (1.0 - 10000.0 kU/mL)</td>
<td>Yes (1:400)</td>
<td>CLEIA / two-step IA</td>
<td>heterophilic antibodies</td>
<td>150µL</td>
<td>≤ 5%</td>
</tr>
</tbody>
</table>

CLIA- Chemiluminescence-immunoassay

Kinetics of HBcrAg, HBV RNA and HBsAg during long term NA treatment
Anti-HBc shows reverse kinetics than viral BM in the natural course

Lin CL, Kao JH. CMH 2016
Serum HBV pgRNA as serum marker for cccDNA activity

Humanized mouse model infected with HBeAg-positive wild-type HBV

Giersch K et al. J Hepatol 2017; 66: 454
Correlation of serum HBcrAg levels with intrahepatic cccDNA

- HBcrAg levels are likely associated with transcription activity from intrahepatic cccDNA.

- Correlation between HBcrAg and HBV cccDNA ($r=0.692$) demonstrated in serum and liver samples from 57 patients ($p<0.001$)

HBcAg correlates with viral load but not with HBsAg levels
Correlations of HBcrAg and HBV RNA with cccDNA may be different by HBeAg status

Correlations of serum viral proteins with intrahepatic cccDNA in HBeAg-positive (A-D) and HBeAg-negative patients (E-H). A, E, Serum HBcrAg; B, F, Serum HBsAg; C, G, Serum HBV RNA; D, H, Serum HBV DNA
Correlation of circulating different HBV BM changes during NUC treatment

Baseline

A

Serum HBV RNA (log Cq/ml)
Intrahepatic cccDNA (log copies/cell)

B

Serum HBV DNA (log Cq/ml)
Intrahepatic cccDNA (log copies/cell)

C

Serum HBsAg (log PEU/ml)
Intrahepatic cccDNA (log copies/cell)

D

Intrahepatic cccDNA (log copies/cell)

Week 96

A

Serum HBV RNA (log Cq/ml)
Intrahepatic cccDNA (log copies/cell)

B

Serum HBV DNA (log Cq/ml)
Intrahepatic cccDNA (log copies/cell)

C

Serum HBsAg (log PEU/ml)
Intrahepatic cccDNA (log copies/cell)

D

Intrahepatic cccDNA (log copies/cell)

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How long remain circulating HBV biomarkers detectable?

Figure 1: Scheme depicting the design of the retrospective study.
Kinetics of HBV biomarkers during TDF or ETV treatment up to 14 years (n=96)

Van Bömmel F,... Lampertico P. AASLD 2019
HBcrAg kinetics are associated with response to NA mono or combination treatment

Serum HBV DNA and HBV RNA before and after start of entecavir in patients (N=11)

Wang J et al. J Hepatol 2018; 65:700
Performance of different HBV biomarkers in predicting HBeAg and HBsAg loss during NA treatment
HBV RNA in serum is an early marker for HBeAg seroconversion during treatment with NUCs

van Bömmel F. et al., Hepatology 2015
Serum HBV RNA in predicting treatment response to Peg-IFNa-2a in HBeAg-positive patients

Early prediction of HBeAg seroconversion

Serum HBV RNA levels

Peg-IFNa-2a monotherapy

Serum HBV RNA levels

Peg-IFNa-2a + lamivudine combination
Association of HBV RNA (pgRNA virion levels) and viral rebound after discontinuation of NUCs

<table>
<thead>
<tr>
<th>HBV RNA</th>
<th>Viral rebound (n)</th>
<th>No viral rebound (n)</th>
<th>Total (n)</th>
<th>*p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>21</td>
<td>0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Below the LoQ</td>
<td>3</td>
<td>9</td>
<td>12</td>
<td>0.001</td>
</tr>
<tr>
<td>Total (n)</td>
<td>24</td>
<td>9</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

*Chi-Square test; n, number of CHB patients.
Early differences in HBcrAg levels in HBeAg neg. patients with or without response to PegIFN

**HBcrAg as clinical biomarker – an overview**

*Virological response - defined as HBeAg clearance and HBV DNA <2000 IU/ml at 24 weeks post treatment

**NPV - negative predictive value

<table>
<thead>
<tr>
<th>Patients</th>
<th>Treatment</th>
<th>Study design</th>
<th>Results</th>
<th>Used specimen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis B sera panels (Boston Biomedica, Inc.)</td>
<td>none</td>
<td>Establishment of a new EIA which detects the precore/core gene products (HBcrAg)</td>
<td>HBcrAg concentrations were well correlated with HBV-DNA levels. On HBeAg/anti-HBe antibody seroconversion panels, the HBcrAg concentration changed in accordance with HBV-DNA levels.</td>
<td>serum</td>
<td>Kimura et al. (2002) Clin Microbiol.</td>
</tr>
<tr>
<td>82 patients with chronic HBV infection</td>
<td>76 patients untreated, 6 patients receiving LMV during the observed time</td>
<td>HBcrAg was measured by CLEIA with monoclonal antibodies to HBeAg and HBCAg</td>
<td>Effectiveness of HBcrAg assay was largely unaffected by HBeAg negative mutants. Changes of HBcrAg and HBV DNA were virtually parallel during the course of natural infection. HBcrAg decreased much slower than HBV DNA after start of LAM treatment.</td>
<td>serum</td>
<td>Rokuhara et al. (2003) J Viral Hepat.</td>
</tr>
<tr>
<td>175 HBeAg positive patients</td>
<td>entecavir (ETV) with or without peginterferon (PEG-IFN) add-on therapy</td>
<td>Analysed serial serum samples to validate the use of HBcrAg in monitoring chronic hepatitis B infection</td>
<td>HBcrAg was associated with combined response (adjusted odds ratio 0.3, 95% confidence interval 0.2-0.5, p &lt;0.001), but was not superior to quantitative HBsAg (qHBsAg).</td>
<td>serum</td>
<td>van Campenhout et al. (2016) Clin Microbiol Infect.</td>
</tr>
<tr>
<td>46 HBeAg positive patients</td>
<td>PEG-IFN for 48 weeks</td>
<td>Analysed for cccDNA from paired liver biopsies and serial serum HBsAg and HBcrAg during therapy</td>
<td>Serum HBcrAg at week 12 was identified as a predictor of VR*. The optimal cut-off value for HBcrAg (log10 8.0 U/ml) provided a NPV** of achieving VR at weeks 12 and 24 of 94.4 and 100%, respectively, while using HBsAg &gt; 20 000 IU/ml provided NPV of 80 and 100% respectively</td>
<td>liver biopsies and patients serial serum</td>
<td>Chuaypen et al. (2016) Liver Int.</td>
</tr>
<tr>
<td>62 HBeAg negative patients</td>
<td>Peg-IFN (n=30) or Peg-IFN + TDF (n=32) for 48 weeks</td>
<td>Baseline prediction of HBsAg loss according to HBsAg and HBcrAg levels</td>
<td>Good diagnostic performance of baseline qHBsAg, qHBcrAg and the combination of qHBsAg and qHBcrAg. The AUC was estimated to be 0.716(95%CI [0.578–0.855]) for HBsAg and 0.668(95%CI [0.524–0.811]) for HBcrAg.</td>
<td>serum</td>
<td>Martinot-PEIGNOUX et al. (2016) J Viral Hepat.</td>
</tr>
</tbody>
</table>
Serum HBV RNA as a predictor of HBsAg seroREversion

The positive and negative predictive value of end of treatment serum HBV RNA levels was 100% and 80%, respectively.
HBV biomarkers for efficacy monitoring of experimental treatments
The main targets & drug discovery efforts

- **Entry inhibitors**
- **Targeting cccDNA**
  - **RNA interference**
  - **Inhibitors of HBsAg release**

- **Immune modulation**
  - Toll-like receptors agonists
  - Anti-PD-1 mAb
  - Vaccine therapy
  - Redirection of T cells

**NUCs**
- "Polymerase inhibitors"

**CpAMs**
- "Capsid inhibitors"

**LHBsAg**

**MHBsAg**

**HBsAg**

**HBV Filament**
- \(~1 \mu g/ml\)

**HBV Sphere**
- \(~100 \mu g/ml\)

**HBV Virion**
- \(~10 \text{ ng/ml}\)

**PD-1**

**Dysfunctional T-cell response**

**Insufficient B-cell response**

**Low**

**High**

**Copy #**

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Treatment with siRNA directed against S-gen HBV mRNA produces changes in HBcrAg levels
Influence of the capsid assembly modulator NVR 3-778 on viral replication

NVR 3-778 inhibited the production of secreted HBV RNA and intracellular HBV RNA encapsidation in HepG2.2.15

In contrast, the nucleotide analog tenofovir (TFV) inhibited HBV DNA production and did not inhibit but actually increased the levels of both intracellular encapsidated HBV RNA and secreted HBV RNA in a dose-dependent manner.
Influence of the capsid assembly modulator NVR 3-778 on viral replication in HBeAg+ patients with chronic HBV infection

NVR 3-778 influences the decrease on serum HBV RNA after the end of treatment

Patients received tenofovir disoproxil fumarate (TDF) on study Day 30, two days after completing study treatment with NVR 3-778 400mg QD or pegIFN

<table>
<thead>
<tr>
<th>Inhibition mechanism</th>
<th>Effect on HBV RNA levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMs</td>
<td>Inhibition of viral replication (rcDNA formation), production of secreted HBVDNA virions and intracellular HBV RNA encapsidation</td>
</tr>
<tr>
<td>RNAi</td>
<td>siRNAs in ARC-520 were designed to target all HBV mRNAs</td>
</tr>
<tr>
<td>3p-siRNA</td>
<td>bi-functional 3p-siRNAs combining RIG-I-mediated immune activation with target gene silencing</td>
</tr>
<tr>
<td>Dihydroquinolizinone (DHQ)</td>
<td>Inhibition via knockdown of PAPD5 and PAPD7</td>
</tr>
<tr>
<td>NAPs</td>
<td>Blocking the release of subviral particles from infected or „integrated“ hepatocytes</td>
</tr>
</tbody>
</table>
Risk of relapse after NAdisc is associated with anti-HBc levels

End-of-treatment anti-HBc level

- <100 IU/mL (65%)
- 100-999 IU/mL (50%)
- ≥1000 IU/mL (21%)

Cumulative rate of clinical relapse (%) vs. Follow-up (years)

No. at risk

<table>
<thead>
<tr>
<th>Range</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100</td>
<td>13</td>
</tr>
<tr>
<td>100-999</td>
<td>48</td>
</tr>
<tr>
<td>≥1000</td>
<td>36</td>
</tr>
</tbody>
</table>

Clinical relapse vs. Follow-up (weeks)

Anti-HBc level (log IU/mL)

- Sustained response
- Clinical relapse

Follow-up (weeks)

<table>
<thead>
<tr>
<th>Week</th>
<th>0</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-value</td>
<td>.96</td>
<td>.002</td>
<td>.20</td>
<td>.14</td>
<td>.002</td>
</tr>
</tbody>
</table>
HBV RNA quantities for the differentiation of disease stages

Phase of CHB

Serum HBV RNA (log_{10} c)

IT  EPIA  ICH  ENH

p=0.000  p=0.000
Quantitative serum HBV RNA levels at different phases of the chronic HBV infection

The upper and lower end of the bar features the 75- and 25-percentile. The mark inside the bar indicates the median. Significant results are given with significance level in the figure. The proportion of positive samples are indicated among the bars.
HBcrAg allows distinction patients with mild vs. minimal liver disease

HBcrAg cut-off value of 4 LogU/ml was able to distinguish between HBeAg- HBcrAg+ patients (n = 21) with mild vs. minimal liver disease (described as fibrosis and/or necroinflammatory activity scores >2 or <1, respectively).
Incidence of HCC based on HBV Biomarkers

Tada et al. (2016) J Hepatol.

- **HBV Genotyp**
  - Number at risk:
    - Genotype C: 621, 596, 319, 195, 83
    - Non-genotype C: 151, 123, 79, 47, 22

- **HBsAg Levels**
  - Number at risk:
    - HBsAg <3 log IU/ml: 369, 271, 162, 90, 37
    - HBsAg ≥3 log IU/ml: 606, 485, 305, 183, 73

- **HBV DNA Levels**
  - Number at risk:
    - HBV DNA <4 log copies/ml: 602, 463, 269, 166, 74
    - HBV DNA ≥4 log copies/ml: 449, 346, 218, 133, 53

- **HBcrAg Levels**
  - Number at risk:
    - HBcrAg <2.9 log IU/ml: 397, 329, 217, 146, 54
    - HBcrAg ≥2.9 log IU/ml: 314, 269, 175, 103, 45
HBsAg proteins for monitoring HBsAg loss

Pfefferkotn M, et al. Submitted 2019
Conclusion – HBcrAg as new viral marker

- HBcrAg might predict HCC Incidence and might distinct patients with mild and severe liver disease
- It reflects the cccDNA transcriptional activity and might be used as surrogate marker

- BUT dynamic range of the test is limited between 3 and 7 log U/mL
  - Not suitable for patients with lower HBcrAg levels
  - Samples of Patients with higher HBcrAg levels should be diluted subsequently
**Conclusion**

**HBV RNA as viable marker?**

- HBV RNA reflects the transcriptional activity of cccDNA during HBV Infection
  - Useful as clinical marker for treatment decision
  - No validated assay

- Strong predictor for HBeAg Seroconversion during antiviral treatment

- BUT: Currently no diagnostical assay available