Novel markers on HBV and HDV infection

Valentina Svicher

University of Rome Tor Vergata
cccDNA: the key molecule allowing HBV persistence
• cccDNA is the driver of HBV replication: it is used as template for the synthesis of viral mRNAs
• Among viral mRNAs, pre-genomic RNA is converted into DNA by HBV Reverse Transcriptase
- The burden and transcriptional activity of cccDNA is modulated by immune responses against the virus:
  - the interplay between the virus and immune system plays a crucial role in disease progression.
Quantification of cccDNA is hampered by the need of a liver biopsy

Need of serum biomarkers to retrieve information on intrahepatic cccDNA
Classical biomarkers used in clinical practise:

Markers of viral replication:
- Serum HBV-DNA quantification
- HBsAg quantification
- HBeAg detection

Serological Markers:
- Anti-HBe detection
- Anti-HBs quantification
- Anti-HBc detection

Classical biomarkers used in clinical practice:

Markers of viral replication:
- Serum HBV-DNA quantification
- HBsAg quantification
- HBeAg detection

Serological Markers:
- Anti-HBc detection
- Anti-HBs quantification
- Anti-HBe detection

HBsAg quantification

Stopping rules to to interferon-alpha

Differential diagnosis between HBeAg-negative infection and hepatitis

Prediction of vertical transmission

HBsAg>4.1 log10IU/mL identified mothers who had transmitted the virus to their newborn with 100% sensitivity and 71% specificity


Brunetto et al., Hepatology 2010
• HBsAg correlates with intrahepatic cccDNA in HBeAg-positive patients. Conversely, this correlation tends to be lost in patients with HBeAg-negative chronic hepatitis.

Thompson et al., Hepatol 2010

Manesin et al., J Hepatol 2011
• The lack of correlation between HBsAg and cccDNA in HBeAg negative hepatitis can be explained by HBV-DNA integration in human genome

• In HBeAg-negative hepatitis, integrated HBV DNA may be important source of HBsAg (even in presence of completely silenced cccDNA)

**HBsAg amount can derive from**

cccDNA

Integrated HBV DNA

---

*Wooddell et al., Sci Trans Med 2017; Revill et al., Nat Rev Gastroenterol Hepatol 2016; Cornberg et al., J Hepatol 2016; Freitas et al., J Virol 2014*
Overall findings highlight the need to integrate classical and novel virological markers to better measure the burden and transcriptional activity of cccDNA.
Optimization of classical HBV biomarkers

Quantification of:

- HBeAg
- Anti-HBc
qHBeAg decline can predict HBeAg seroconversion during interferon treatment better than serum HBV-DNA and can allow to distinguish between late responder and non-responder.

>1log decrease in qHBeAg levels at week 12 was predictive of HBeAg seroconversion and undetectable serum HBV-DNA during entecavir treatment:

- OR for HBeAg seroconversion: 23.9 (1.8-316.0), P=0.016
- OR for undetectable serum HBV-DNA: 9.6 (2.1-42.7), P=0.033

Fried et al., Hepatology 2008

Kwong et al., 2012
Optimization of classical HBV biomarkers

Quantification of:
- HBeAg
- Anti-HBc
Which is the biological meaning of anti-HBc?

- Anti-HBc titer has been proposed to measure the strength of immune responses against the virus.

- According to recent studies, anti-HBc titer can mediate antibody-dependent cytotoxicity, playing a role in the setting of acute liver failure.

  Farci et al., PNAS 2010; Chen et al, PNAS 2018
Which can be the clinical positioning of anti-HBc titer according its the biological function?

Anti-HBc titer correlates with ALT and hepatitis activity in eAg-positive and eAg-negative infection (Song et al., CMI 2015; Li et al., Hep Res 2018)

Baseline anti-HBc (>4.5logIU/mL) is the best predictor of HBeAg seroconversion after interferon or NA treatment compared to level of ALT or HBV DNA (Fan et al., Gut 2016)

Baseline anti-HBc titer is associated with spontaneous HBeAg seroclearance in drug-naive patients (Liu et al., Clin Gastroenterol Hepatol 2018).
Anti-HBC titer can contribute to the diagnosis of occult HBV infection (defined as the presence of HBV-DNA in the liver in an HBsAg-negative individual)
In anti-HBc+/HBsAg- patients, anti-HBc titer > 4COI can predict intrahepatic presence of cccDNA
UPDATE OF THE STATEMENTS ON BIOLOGY AND CLINICAL IMPACT OF OCCULT HEPATITIS B VIRUS INFECTION

**DIAGNOSIS**

- Diagnosis of OBI is based on the detection of HBV DNA in blood or liver of HBsAg negative persons.
  - Detection of HBV DNA in the liver is the gold standard.
  - Detection of HBV DNA in the blood is commonly used.
  - Detection of anti-HBc in the blood is often used as a surrogate.

The diagnosis of OBI is based on the sensitivity of assays used in the detection of HBV DNA and HBsAg. HBsAg assays with inadequate sensitivity or inability to detect HBV S variants may lead to false negative HBsAg result and misdiagnosis of OBI in persons who have overt HBV infection. On the other hand, HBV DNA assays with inadequate sensitivity resulting in false negative HBV DNA result may lead to missed diagnosis of OBI.
Clinical Case

- Female, born in 2000
- At 2 months of age: diagnosis of Alagille Syndrome
- 2002: Liver Transplantation from anti-HBc+ liver donor despite successful vaccination. Induction therapy with Basiliximab and immunosuppression with steroids and tacrolimus
Liver biopsy for the evaluation of HBV intrahepatic markers and serum HBV-DNA by highly sensitive assay of ddPCR

Total HBV-DNA = 8.0 copies/ml
cccDNA = 5.6 copies/ml
pgRNA = 1.1 copies/ml

High-sensitive quantification of serum HBV-DNA = 1 copy/ml

Ultra-deep sequencing HBV RT/S region
HBV genotype: D3
HBsAg immune-escape mutations: P120T (88.2%), T126I (99.4%), P142S (87.1%), G145R (99.6%): contribute to HBV transmission despite vaccination
Innovative HBV biomarkers

Quantification of:
- HBcrAg
- Serum HBV-RNA
Which is the virological meaning of these biomarkers?
The quantification of HBcrAg reflects the capsid protein HBcAg, HBeAg and a precore protein (p22) coded with the precore/core region

*Testoni et al., J Hepatol 2018*
• HBcrAg is strongly correlated with tHBV-DNA, cccDNA and pgRNA levels both in HBeAg+ and HBeAg patients.

• Notably, qHBsAg and serum HBV-DNA correlations with the same intra-hepatic markers are much weaker.

| Table 2. Correlations between HBcrAg, qHBsAg, serum HBV-DNA and intrahepatic viral markers. |
|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
|                                                  | Liver markers                                   | tHBV-DNA                                         | cccDNA                                         | pgRNA                                          | cccDNA transcriptional activity (pgRNA/ccDNA)    |
|                                                  |                                                  |                                                  |                                                |                                                |                                                |
| ALL                                              |                                                  |                                                  |                                                |                                                |                                                |
| HBcrAg                                           |                                                  | R = 0.85; p < 0.0001                             | R = 0.74; p < 0.0001                           | R = 0.75; p < 0.0001                           | R = 0.52; p < 0.0001                           |
| qHBsAg                                           |                                                  | R = 0.38; p = 0.003                             | R = 0.26; p = 0.044                           | R = 0.35; p = 0.006                           | R = 0.29; p = 0.023                           |
| Serum HBV DNA                                    |                                                  | R = 0.78; p < 0.0001                             | R = 0.57; p < 0.0001                           | R = 0.41; p < 0.0001                           | R = 0.25; p = 0.015                           |
| HBeAg+ chronic hepatitis (n = 32)                |                                                  |                                                  |                                                |                                                |                                                |
| HBcrAg                                           |                                                  | R = 0.79; p < 0.0001                             | R = 0.80; p < 0.0001                           | R = 0.68; p = 0.004                           | R = −0.02; p = n.s.                           |
| qHBsAg                                           |                                                  | R = 0.49; p = n.s.                              | R = 0.33; p = 0.01                           | R = 0.32; p = n.s.                           | R = 0.26; p = n.s.                           |
| Serum HBV DNA                                    |                                                  | R = 0.50; p = 0.003                             | R = 0.29; p = n.s.                           | R = 0.41; p = 0.07                           | R = 0.18; p = n.s.                           |
| HBeAg− chronic hepatitis (n = 43)                |                                                  |                                                  |                                                |                                                |                                                |
| HBcrAg                                           |                                                  | R = 0.61; p < 0.0001                             | R = 0.25; p = n.s.                           | R = 0.81; p < 0.0001                           | R = 0.70; p < 0.0001                           |
| qHBsAg                                           |                                                  | R = −0.15; p = n.s.                             | R = −0.4; p = 0.01                           | R = −0.02; p = n.s.                           | R = 0.15; p = n.s.                           |
| Serum HBV DNA                                    |                                                  | R = 0.71; p < 0.0001                             | R = 0.19; p = n.s.                           | R = 0.79; p < 0.0001                           | R = 0.66; p = 0.0002                           |
| HBeAg− chronic infection (n = 18)                |                                                  |                                                  |                                                |                                                |                                                |
| HBcrAg                                           |                                                  | R = 0.34; p = n.s.                              | R = 0.47; p = 0.05                           | R = 0.29; p = 0.09                           | R = 0.11; p = n.s.                           |
| qHBsAg                                           |                                                  | R = 0.24; p = n.s.                              | R = −0.03; p = n.s.                           | R = −0.12; p = n.s.                           | R = 0.08; p = n.s.                           |
| Serum HBV DNA                                    |                                                  | R = −0.02; p = n.s.                             | R = 0.27; p = n.s.                           | R = 0.39; p = n.s.                           | R = 0.28; p = n.s.                           |

HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; pgRNA, pregenomic RNA; qHBsAg, quantitative hepatitis B surface antigen. The correlation coefficient was calculated using Spearman’s correlation test. Twotailed p value was calculated for a risk threshold α = 0.05.

1 Only patients with positive HBcrAg quantification (i.e. >3 LogU/ml) were included in the analysis.

2 HBeAg+ chronic infection category was composed by only 4 patients (see Table S1), therefore it was not included in the analysis.
HBV-infected hepatocytes can release:
- viral particles containing HBV-DNA produced by RT
- viral particles containing pre-genomic RNA (not retrotranscribed)

- Thus, serum HBV-RNA measures the release of viral particles containing pre-genomic RNA
• Levels of serum HBV-RNA correlate with intrahepatic pgRNA and cccDNA ...........

Girsch et al., J Hepatol 2017

Wang et al., Hepatol 2018
and are more genetically homogenous to intrahepatic pgRNA than cccDNA:

- This suggests that serum HBV-RNA can serve as a biomarker for cccDNA transcriptional activity

*Wang et al., Hepatol 2018*
According to their virological meaning which can be the role of HBcrAg and serum HBV-RNA?

• Differential diagnosis between HBeAg-negative infection and hepatitis
  - low/undetectable levels of HBcrAg and serum HBV-RNA can predict HBeAg-negative infection (Buti et al., CMI 2015; Brunetto et al., AASLD 2018; Wang et al., JVH 2018)

Serum HBV-RNA

HBcrAg

Brunetto et al., AASLD 2018

Wang et al., JVH 2018
According to their virological meaning which can be the role of HBcrAg and serum HBV-RNA?

**Differential diagnosis between HBeAg-negative infection and hepatitis:**
- Low/undetectable levels of HBcrAg and serum HBV-RNA can predict HBeAg-negative infection (Buti et al., CMI 2015; Brunetto et al., AASLD 2018; Wang et al., JVH 2018)

**Predict sustained virological response to peg-interferon alpha treatment:**
- Serum HBcrAg declines in all patients, but more significantly in responders (Chuaypen et al., Liver Int 2016 and CMI 2018)
- IFN-treatment induces a stronger decrease in serum HBV-RNA than NUC. Responders show a stronger and earlier decline in HBV-RNA during IFN-treatment (Janssen et al., JID 2016)
• NUCs cannot affect the production of HBcrAg and serum HBV-RNA
• The biomarkers provide the advantage to measure intrahepatic HBV reservoir in virologically suppressed patients
NUC treatment can favour an increase in the release of serum HBV-RNA:
- The release of RNA-containing virions may become the prominent type of virions during NUC treatment

Girsch et al., J Hepatol 2017
Wang et al., J Hepatol 2017
• In HBeAg-negative patients, HBcrAg and serum HBV-RNA progressively decrease during long term NUC treatment.
• At 5 year of treatment, 14% and 35% of patients are still positive to serum HBV-RNA and HBcrAg.
  - potential weakening of cccDNA transcriptional activity during NUC treatment?

Carey et al., EASL 2019
Undetectable serum HBV-RNA at NUC suspension correlates with maintenance of virological suppression after NUC interruption

<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.

Wang et al., J Hepatol 2017
Table 2. NA treatment cessation recommendations in the current hepatitis B virus guidelines

<table>
<thead>
<tr>
<th>Society</th>
<th>HBeAg(+)</th>
<th>HBeAg(-)</th>
<th>Cirrhosis</th>
</tr>
</thead>
</table>
| EASL (2017)[9] | HBsAg clearance (safest)  
HBsAg seroconversion and HBV DNA undetectability with 6-12 mo of ensuing consolidation therapy | HBsAg clearance  
Selected patients with ≥ 3 yr virological suppression if guaranteed close postNA monitoring for at least 1 yr  
HBsAg clearance | Not recommended |
| AASLD (2016)[20] | HBsAg clearance  
HBsAg seroconversion with at least 12 mo of persistently normal ALT levels and undetectable serum HBV DNA levels (close monitoring for at least 1 yr) | HBsAg clearance with antiHBs seroconversion  
HBsAg loss with at least 12 mo of consolidation period  
After treatment for at least 2 yr with undetectable HBV DNA documented on 3 separate occasions, 6 mo apart | Not recommended |
| APASL (2016)[22] | HBsAg seroconversion with undetectable HBV DNA and persistently normal ALT levels with 1-3 yr of consolidation therapy |  | Could be considered in compensated cirrhosis with careful monitoring |

AASLD: American Association for the Study of Liver Diseases; ALT: Alanine aminotransferase; APASL: Asian Pacific Association for the Study of the Liver; EASL: European Association for the study of the Liver; HBeAg: Hepatitis B e antigen; HBs: Hepatitis B surface protein; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; NA: Nucleos(t)ide analogue treatment.

Moreno-Cubero et al., 2018
What happens after NUC suspension?

Median FU post-treatment = 69 months (range 67-72)

- Sustained biochemical and virologic response: 55% (18/33)
- HBsAg loss: 39% (13/33)

Hadziyannis et al., Gastroenterology 2012
A major unmet need is the identification of patients who can safely and successfully suspend NUC treatment.
A lower HBsAg (reflecting a limited HBV reservoir) at NUC suspension correlates with higher probability to achieve HBsAg loss

Jeng et al., J Hepatology 2017
Combining hepatitis B core-related and surface antigens at end of nucleos(t)ide analogue treatment to predict off-therapy relapse risk

- the combination of HBsAg together with HBcrAg quantification help to predict safe discontinuation after NA treatment cessation

- In all patients who later achieved HBsAg loss, HBsAg and HBcrAg levels at NUC suspension were lower than 100IU/mL and 1000U/mL respectively

*Hsu et al., APT 2018*
Detectable HBcrAg and serum HBV-RNA are observed only in patients with clinical relapse

Carey et al., EASL 2019
The patients who did not relapse after NUC suspension are characterized by an increased frequency of functional HBV-specific T cells

Patients with functional HBV-specific CD8 T cells may no longer need NUC treatment and should be considered for treatment cessation
Higher anti-HBc titer correlates with lower risk of clinical relapse

Chi et al., Clin Gastroenterol Hepatol 2019
The integration of virological and immunological markers could help in individualize therapeutic approach to each patient.

- **High viral reservoir and/or low immunological control**:
  - Continuing therapy *(candidate to new approaches?)*

- **Low viral reservoir and/or high immunological control**:
  - Suspending NUC

Further studies are necessary to investigate this issue!
The issue of immune-suppression driven HBV reactivation
Immunosuppressed patients with occult HBV infection at screening

Monitoring?  Antiviral Prophylaxis?
### Lower baseline anti-HBs levels associated with subsequent HBV reactivation

<table>
<thead>
<tr>
<th>Reference</th>
<th>Nº of patients</th>
<th>Clinical setting</th>
<th>% of patients with HBV-R</th>
<th>Association of biomarker with risk of HBV-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seto et al., 2014</td>
<td>260</td>
<td>Oncohematological patients receiving rituximab</td>
<td>30.2%</td>
<td>Anti-HBs negativity at BL: HR (95%CI): 3.51(1.37-8.98), P=0.009 Anti-HBs&lt;100 IU/ml</td>
</tr>
<tr>
<td>Cho Y et al. 2016</td>
<td>108</td>
<td>B-cell lymphoma receiving rituximab not antiviral prophylaxis</td>
<td>11.6%</td>
<td>HBV reactivation rates at 6, 12, 36, and 48 months after chemotherapy as high as 8.3, 17.3, 21.1, and 25.7%, Anti-HBs&lt;100 IU/ml</td>
</tr>
<tr>
<td>Fukuda et al. 2016</td>
<td>1042</td>
<td>Rheumatic diseases</td>
<td>3.3%</td>
<td>Anti-HBs&lt;100 IU/ml OR (95% CI): 2.8 (1.3 to 6.8)</td>
</tr>
<tr>
<td>Lee et al., 2018</td>
<td>366</td>
<td>Kidney transplanted patients using rituximab for desensitization</td>
<td>2.5%</td>
<td>Anti-HBs&lt;100IU/l at transplantation: HR (95%CI): 9.06 (1.11-74.3), P=0.04 standard-dose rituximab at transplantation: HR (95%CI): 10.60 (2.52–44.60), P=0.001</td>
</tr>
<tr>
<td>Kotake et al., 2018</td>
<td>243</td>
<td>Patients with solid tumors</td>
<td>2.1%</td>
<td>Anti-HBs negativity at BL: OR(95% CI): 5.94 (1.15-30.6), P=0.03 dexamethasone &gt;1.0 mg/day at BL: OR (95% CI): 8.69 (1.27-58.8), P=0.02</td>
</tr>
<tr>
<td>Tien et al. 2018</td>
<td>380</td>
<td>Rheumatic diseases treated with biologic therapy</td>
<td>4.4%</td>
<td>Anti-HBs&gt;100 IU/ml at BL Rate of HBV reactivation person year 0/100 Anti-HBs 10-100 IU/ml at BL Rate of HBV reactivation person year 2.5/100 Anti-HBs &lt;10 IU/ml at BL Rate of HBV reactivation person year 4.7/100</td>
</tr>
<tr>
<td>Seto et al., 2016</td>
<td>62</td>
<td>Oncohematological patients receiving rituximab</td>
<td>29%</td>
<td>HBcrAg positivity at BL: HR(95%CI):3.65(1.35–9.86), P=0.011 anti-HBs negativity at BL: HR (95%CI): 2.84 (1.10–7.37), P=0.032</td>
</tr>
</tbody>
</table>
Quantification of HBV core antibodies may help predict HBV reactivation in patients with lymphoma and resolved HBV infection

Baseline anti-HBc/anti-HBs levels may predict HBV reactivation in patients with lymphoma and help optimize prophylactic antiviral therapy for high-risk patients

Yang et al., J Hepatol 2018
High-sensitive assays & HBV reactivation

The case of ultra-sensitive HBsAg:
- Limit of detection of the classical assays: 50mIU/ml
- Limit of detection of the highly sensitive assays: 5mIU/ml (1log higher sensitivity)
Clinical Case from oncohematological setting

Anti-HBc + HBsAg – at screening

2 years LMV prophylaxis

HBV-DNA < 20 IU/ml & ALT < 40 U/L

High-sensitivity HBsAg
Clinical Case from oncohematological setting

Anti-HBc + HBsAg – at screening

2 years LMV prophylaxis

HBV-DNA < 20 IU/ml & ALT < 40 U/L

High-sensitivity HBsAg
HDV: the smallest RNA virus, causing the most severe forms of hepatitis

Virological markers of HDV replication:

- Serum HDV RNA
- HDAg
Comprehensive analysis revealed a very high heterogeneity of assay characteristics, including their technical steps and technologies. Thirteen labs (46.3%) properly quantified all 18 positive samples; 16 (57.1%) failed to detect one to up to 10 samples, and several others underestimated (>3 log IU/mL) HDVL of African genotype strains (1 and 5-8). Discrepancies were mainly attributed to either primers or probe mismatches related to the high genetic variability of HDV and, possibly, to the complex secondary structure of the target genomic RNA.

**Conclusion**: The results of this international quality-control study underline the urgent need to improve methods used to monitor HDV viremia and will be instrumental in achieving that goal.
Clinical Case

- 42 years old male from Ukraine, positive to HBsAg since 5 years of age

<table>
<thead>
<tr>
<th>Serological profile at March 2019</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>+</td>
</tr>
<tr>
<td>HBeAg</td>
<td>-</td>
</tr>
<tr>
<td>Serum HBV-DNA</td>
<td>&lt;10IU/ml</td>
</tr>
<tr>
<td>HBsAg</td>
<td>9860 IU/ml</td>
</tr>
<tr>
<td>Anti-HDV IgG</td>
<td>+</td>
</tr>
<tr>
<td>HDAg</td>
<td>-</td>
</tr>
</tbody>
</table>
Clinical Case

- 42 year old male from Ukraine, positive to HBsAg since 5 years of age

<table>
<thead>
<tr>
<th>Serological profile</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>+</td>
</tr>
<tr>
<td>HBeAg</td>
<td>-</td>
</tr>
<tr>
<td>Serum HBV-DNA</td>
<td>&lt;10IU/ml</td>
</tr>
<tr>
<td>HBsAg quantitativo</td>
<td>9860 IU/ml</td>
</tr>
<tr>
<td>Anti-HDV IgG</td>
<td>+</td>
</tr>
<tr>
<td>HDAg</td>
<td>-</td>
</tr>
<tr>
<td>Serum HDV-RNA</td>
<td>2,696,930 copies/ml</td>
</tr>
</tbody>
</table>

HDAg failed to detect high levels of viral replication
Conclusions

• The spectrum of HBV biomarkers has increased during the last period

• Need of standardization, understanding factors affecting the level of biomarkers, their proper positioning in clinical practise

• Collaborative studies are necessary to better understand how to integrate these biomarkers in order to allow a precision medicine approach of patients with chronic HBV infection