La diagnostica dell'epatite da HBV e HDV tra vecchi e nuovi biomarkers

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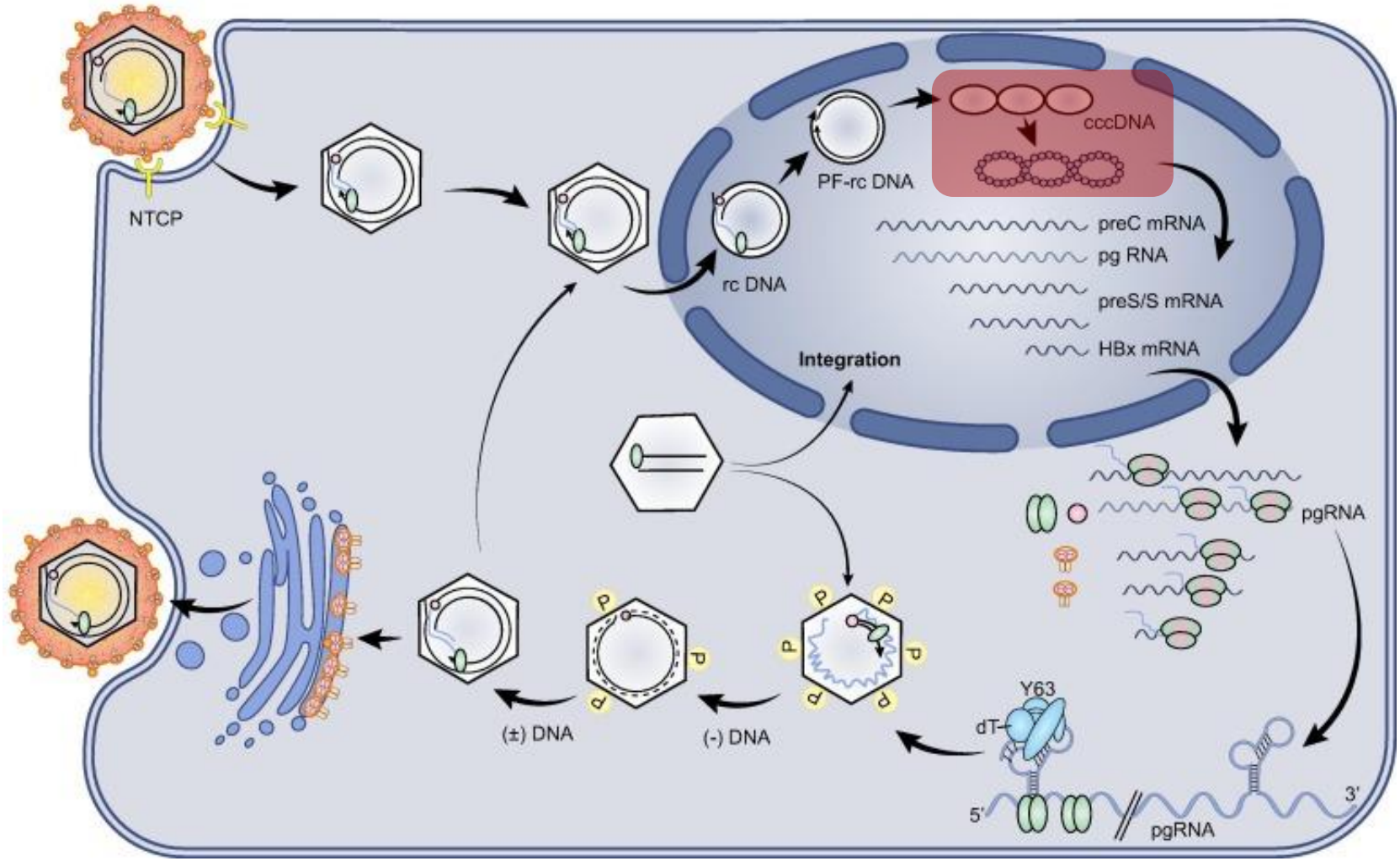
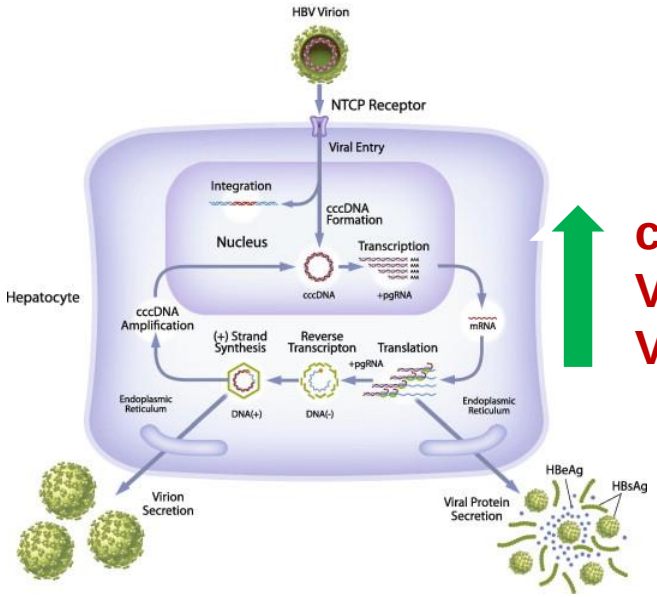
cccDNA: the key molecule allowing HBV persistence



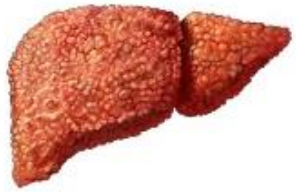
- The burden and the metabolic activity of cccDNA modulate HBV replication and in turn anti-HBV immune response and disease progression

- Several studies have shown that **a larger and transcriptionally active** intrahepatic HBV reservoir **increases the risk of liver inflammation and disease progression**, including HCC



cccDNA
Viral mRNA
Viral proteins

Inflamed liver

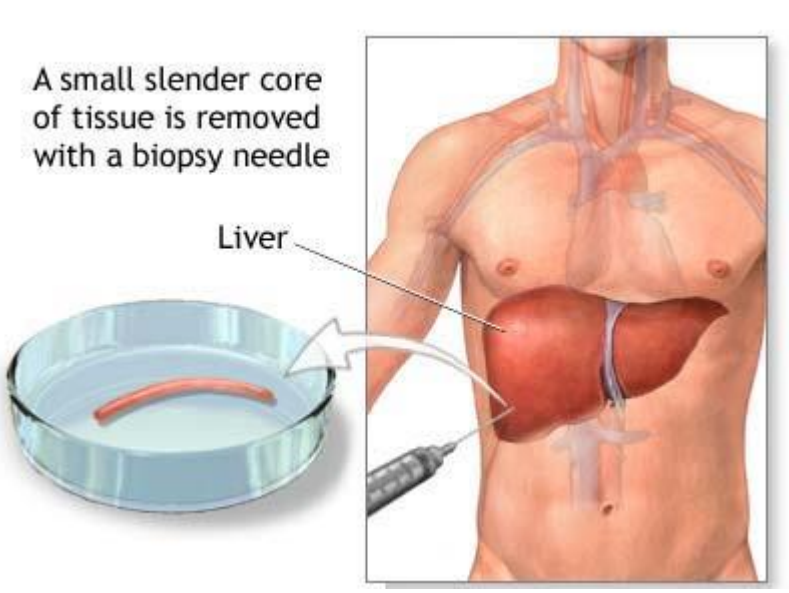


Cirrhosis of liver

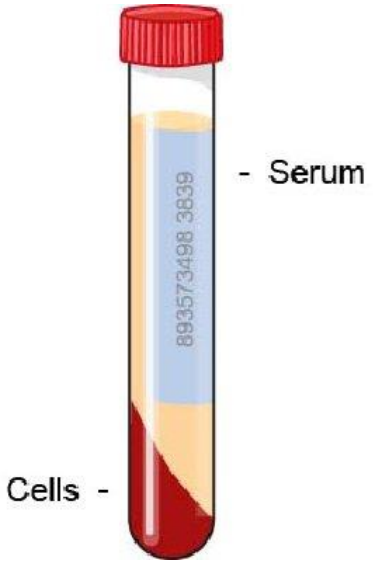
Liver disease progression

Meng et al., Transplant Proced 2019
Liang LB, International J of Infectious Disease, 2016
Larsson SB wt al., Liv Int, 2014
Cheng PN et al, J Med Virology, 2011

Quantification of cccDNA is hampered by the need of a liver biopsy



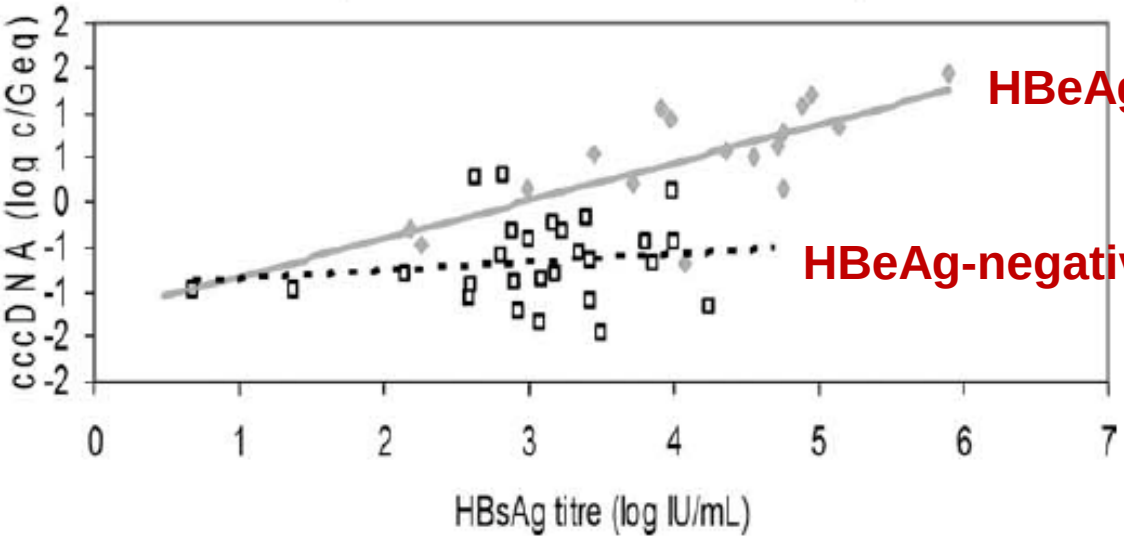
Need of serum biomarkers to retrieve information on intrahepatic cccDNA



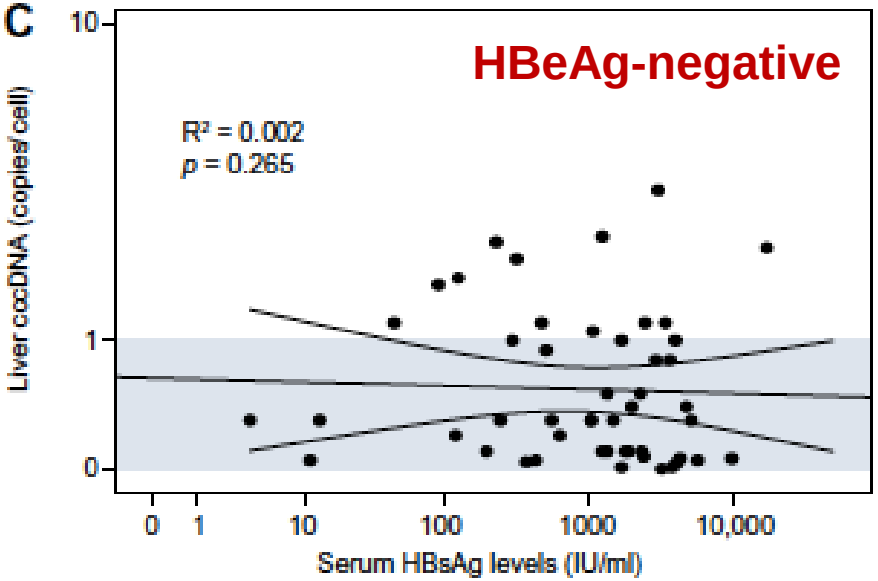
HBV biomarkers: classical and novel

Classical biomarkers	Classical biomarkers optimized	Novel biomarkers
HBV-DNA	Three forms of HBsAg	HBcrAg
Quantitative HBsAg	Ultra-sensitive HBsAg	Serum HBV-RNA
Qualitative HBeAg/anti-HBe	Quantitative HBeAg	
Qualitative anti-HBc 	anti-HBc titer 	
Anti-HBs titer 		

- **HBsAg correlates with intrahepatic cccDNA in HBeAg-positive patients. Conversely, this correlation tends to be lost in patients with HBeAg-negative chronic hepatitis…....**

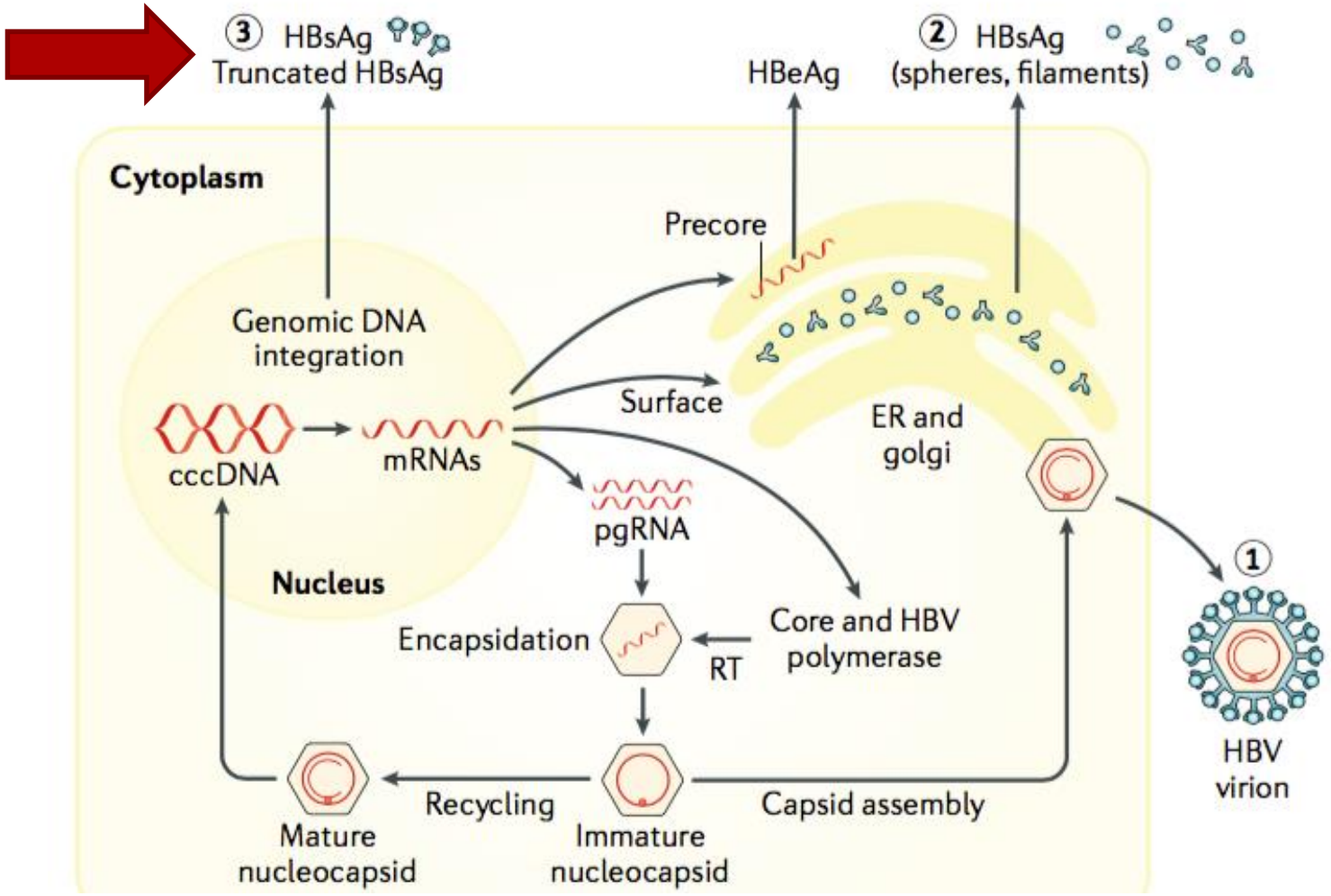


Thompson et al., Hepatol 2010



Manesin et al., J Hepatol 2011

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



Liaw Nature Reviews 2019

HBsAg quantification

- **Stopping rules to to interferon-alpha**

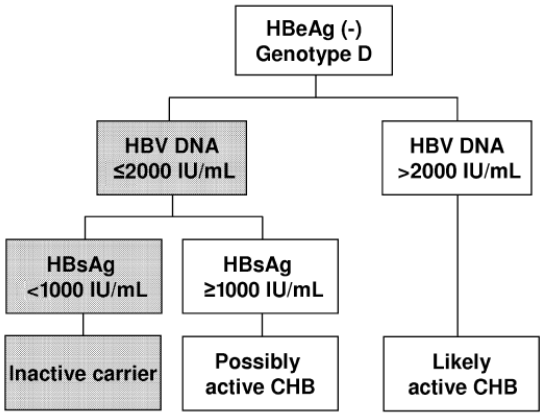
Week 12 Versus the Baseline			
HBsAg Decline	HBV DNA Decline ≥2 Log Copies/mL	Chance of SR	Recommendation to Continue
no	no	Absent	stop
no	yes	Intermediate	continue
yes	no	Intermediate	continue
yes	yes	High	strong recommendation for continuation

Rijckborst et al. Hepatology 2010, Rijckborst et al. J Hepatol 2012, Lampertico P, et al. EASL 2012

- **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

- **Differential diagnosis between HBeAg-negative infection and hepatitis**



Brunetto et al., Hepatology 2010

Hepatitis B Surface Antigen Serum Levels Help to Distinguish Active From Inactive Hepatitis B Virus Genotype D Carriers

Brunetto et al., Gastroenterology 2010

Normal ALT levels combined with HBV DNA levels ≤2,000 IU/ml and qHBsAg levels <1,000 IU/ml at a single time point had a positive predictive value (PPV) of 88% and a negative predictive value (NPV) of 97% for the iden- tification of inactive carriers in those with genotype D chronic HBV infection

Serum levels of hepatitis B surface antigen and DNA can predict inactive carriers with low risk of disease progression

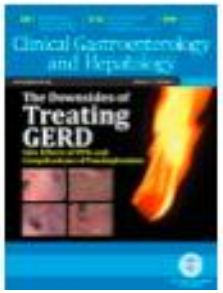
Liu et al., Hepatology 2016

In HBeAg-negative patients with genotype B or C HBV infection, these three combined criteria had a PPV of 83% and a NPV of 74% for identifying inactive carriers

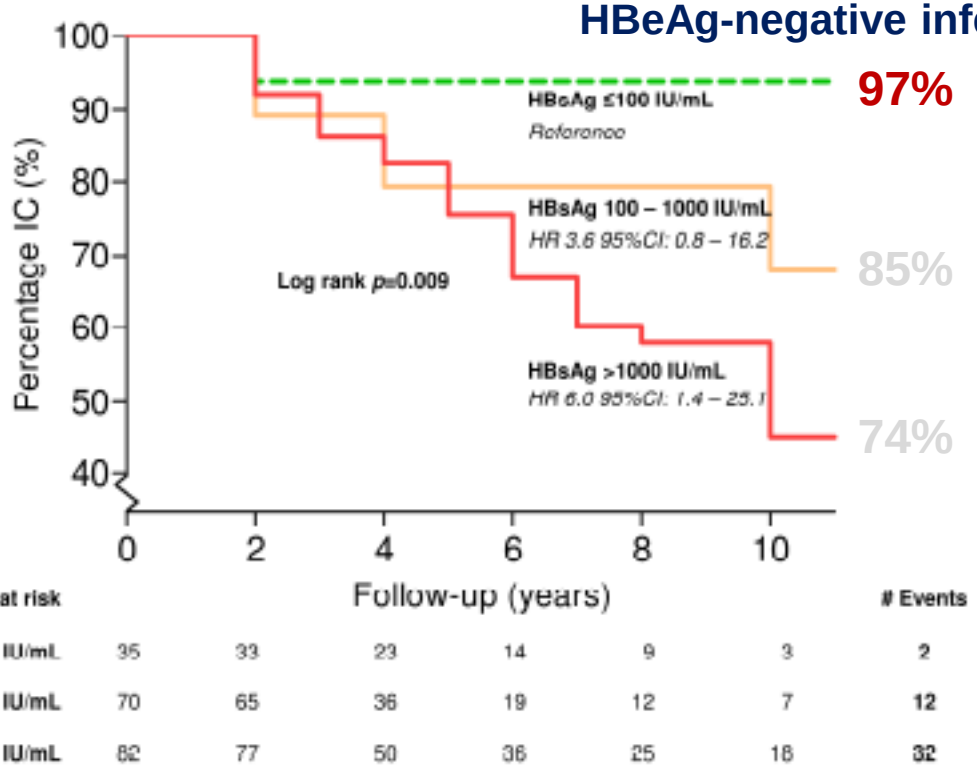
Another step forward….

Repeated Measurements of Hepatitis B Surface Antigen Identify Carriers of Inactive HBV During Long-Term Follow-up

Brouwer et al., 2016



Probalibity to remain in HBeAg-negative infection phase

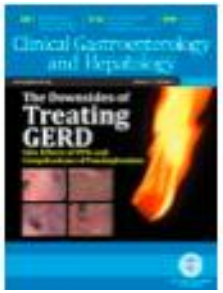


# Patients at risk							# Events
≤100 IU/mL	35	33	23	14	9	3	2
100 – 1000 IU/mL	70	65	36	19	12	7	12
>1000 IU/mL	82	77	50	36	25	18	32

Patients with HBsAg <100IU/ml have higher probability to achieve HBsAg loss (PPV of 36% and 50% after 1 and 3 years

Repeated Measurements of Hepatitis B Surface Antigen Identify Carriers of Inactive HBV During Long-Term Follow-up

Brouwer et al., 2016



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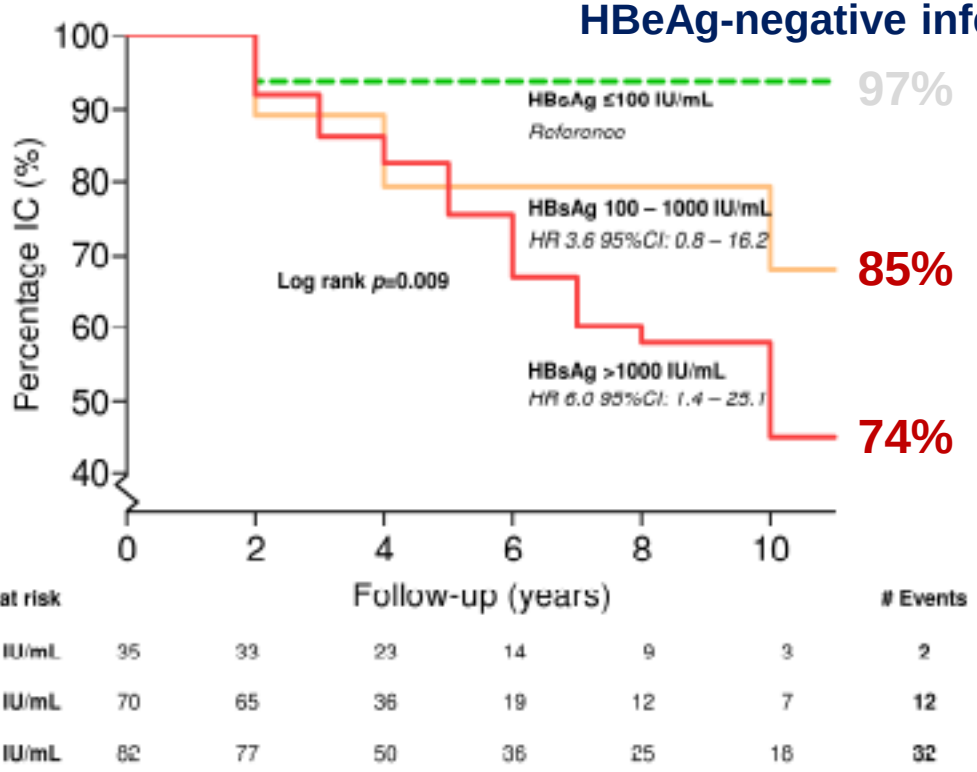

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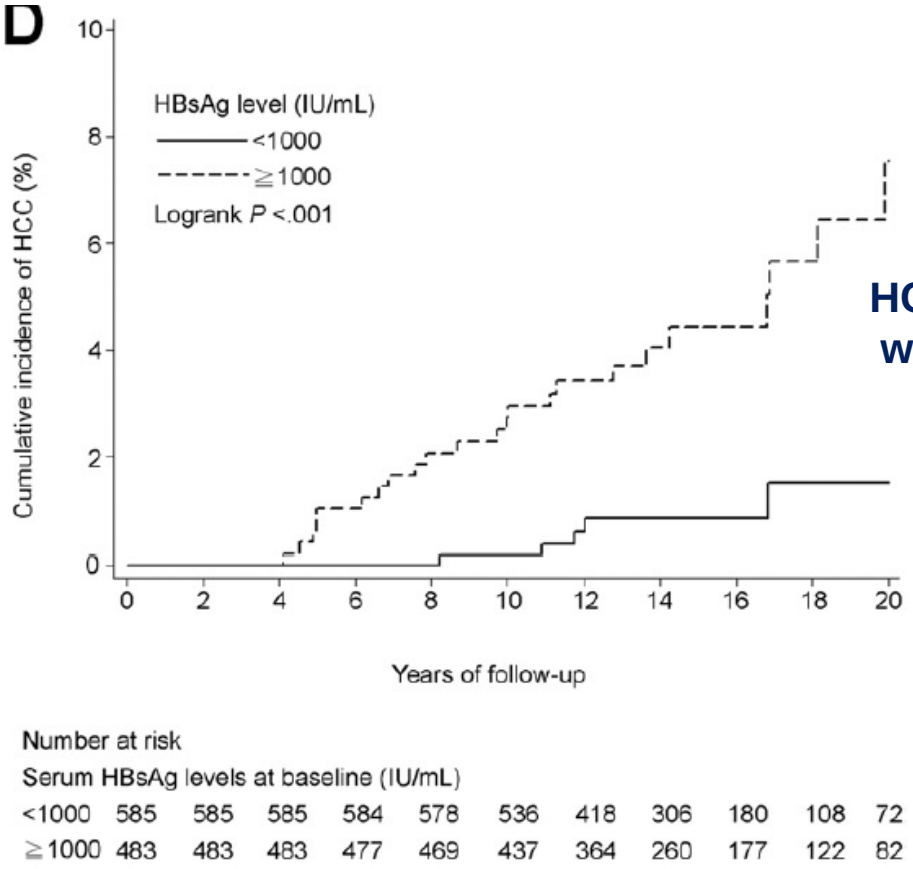
By increasing levels of HBsAg, there is an increase in the probability to lose the status of HBeAg-negative infection

High Levels of Hepatitis B Surface Antigen Increase Risk of Hepatocellular Carcinoma in Patients With Low HBV Load

TAI–CHUNG TSENG,[*,§,**] CHUN–JEN LIU,[‡,§] HUNG–CHIH YANG,[‡,#] TUNG–HUNG SU,[‡,§] CHIA–CHI WANG,[*,**] CHI–LING CHEN,[§] STEPHANIE FANG–TZU KUO,[‡‡] CHEN–HUA LIU,[‡,§] PEI–JER CHEN,[‡,§] DING–SHINN CHEN,[‡,§] and JIA–HORNG KAO[‡,§,‖,¶]

GASTROENTEROLOGY 2012;142:1140–1149



HCC risk:13.7 fold higher with HBsAg >1,000IU/ml

When we evaluated HBeAg-negative patients with levels of HBV DNA 2,000IU/mL, factors that determined HCC risk included sex, age, and levels of alanine aminotransferase and HBsAg (1000 IU/mL), but not level of HBV DNA. Multivariate analysis showed that the **adjusted hazard ratio for HCC in patients with levels of HBsAg 1000 IU/mL** versus 1000 IU/mL was **13.7** (95% confidence interval: 4.839.3)

The integration of Hepatitis B virus in relevant regions of human genome occurs frequently in HBeAg negative chronic infection despite limited liver disease and low-viraemia: implications for an altered cell metabolism

Romina Salpini[1], Luca Carioti[1], Arianna Battisti[1,2], Luna Colagrossi[1,3], Lorenzo Piermatteo[1], Matteo Surdo[4], Valeria Cacciafesta[4], Andrea Nuccitelli[4], Navjyot Hansi[2], Francesca Ceccherini-Silberstein[1], Carlo Federico Perno[5], Upkar S. Gill[2], Patrick T.F. Kennedy[2], Valentina Svicher[1]

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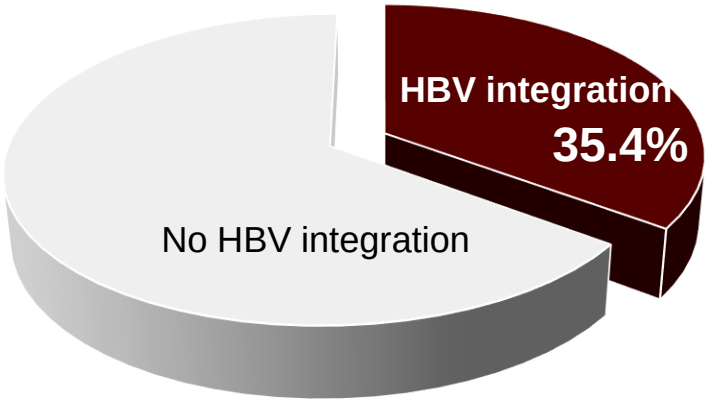
4. GENOMA, Molecular Genetics Laboratory, Rome, Italy.
5. Department of Oncology and Haematooncology, University of Milan, Milan, Italy.

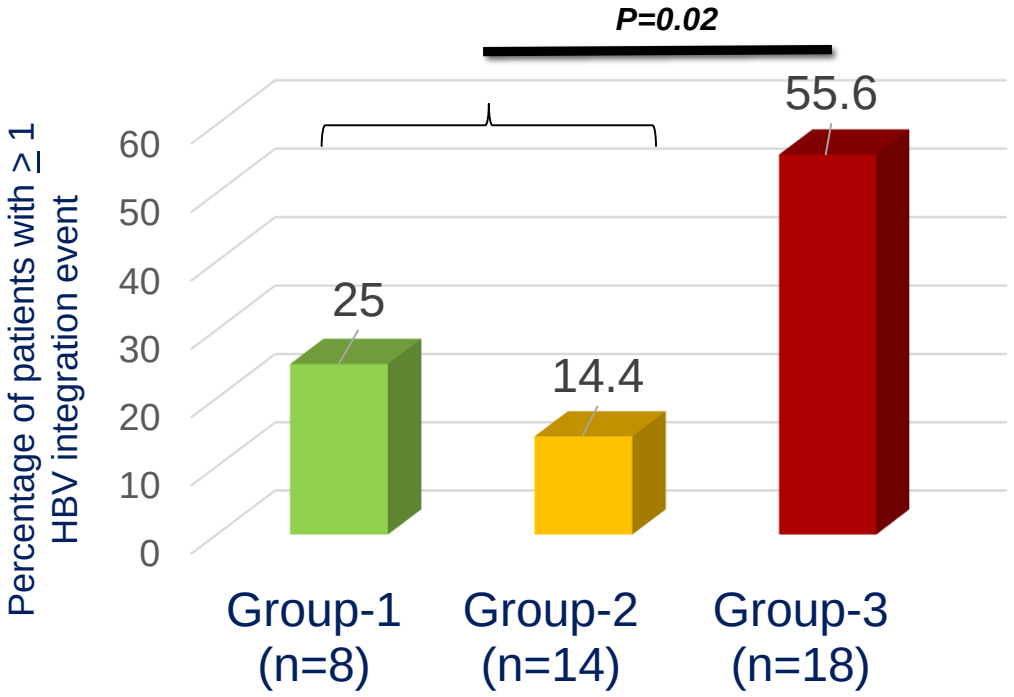
Best Oral Presentation Basic Science, International Liver Congress 2019, Wien
Best Oral Presentation Clinical Hepatitis, SIMIT Special Awards, ICAR 2019, Milano

Integration of HBV-DNA into human genome

- Overall, HBV integration in genomic regions relevant for gene expression is detected in 35.4% (14/40) of patients

  → Notably, **HBV integration is evident in a significant number of patients in groups 1 & 2, despite low viraemia and a limited intrahepatic reservoir**





Group 1= serum HBV-DNA <2,000IU/ml

Group 2= seruma HBV-DNA between 2,000 and 20,000IU/ml

HBV Integration: the downstream effects

HBV integration involves genes regulating :

Cell proliferation

NUP85
Nuclear pore complex
ANKRD52
Ankyrin Repeat Domain
AGBL-5
Metallocarbossipeptidase
COL-18-A1
Collagene XVIII
ELAC-2
Ribonuclease Z

Lipid and drug metabolism

CYP2UI
Cytochrome P450
LMF-1
Lipoprotein Maturation Factor 1

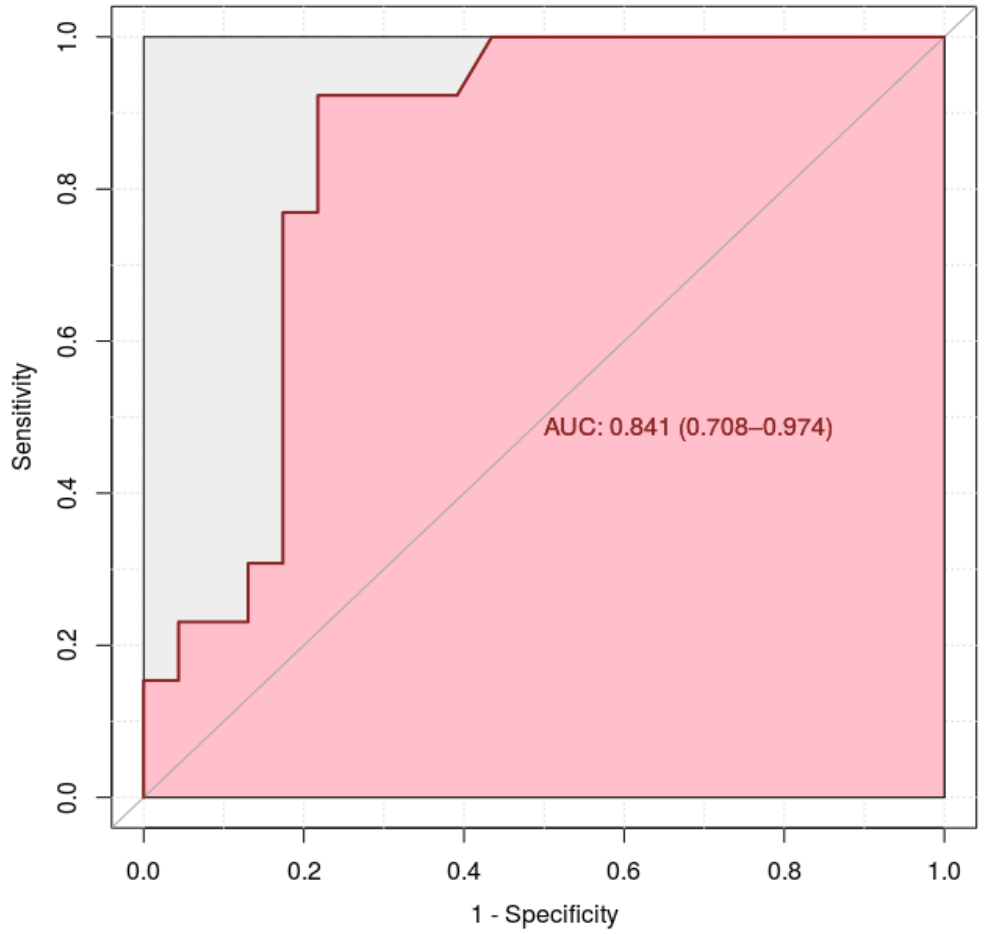
Antiviral and inflammatory response

NR3C1
Glucocorticoid receptor
IFITM-1
IFN-induced antiviral protein

Unfavourable prognosis in liver cancer

Retrieved from: The Human Protein Atlas, Gene Cards, KEGGS

By AUROC, HBsAg >5,000 IU/ml identifies HBV integration with the best diagnostic-accuracy (83.5%)



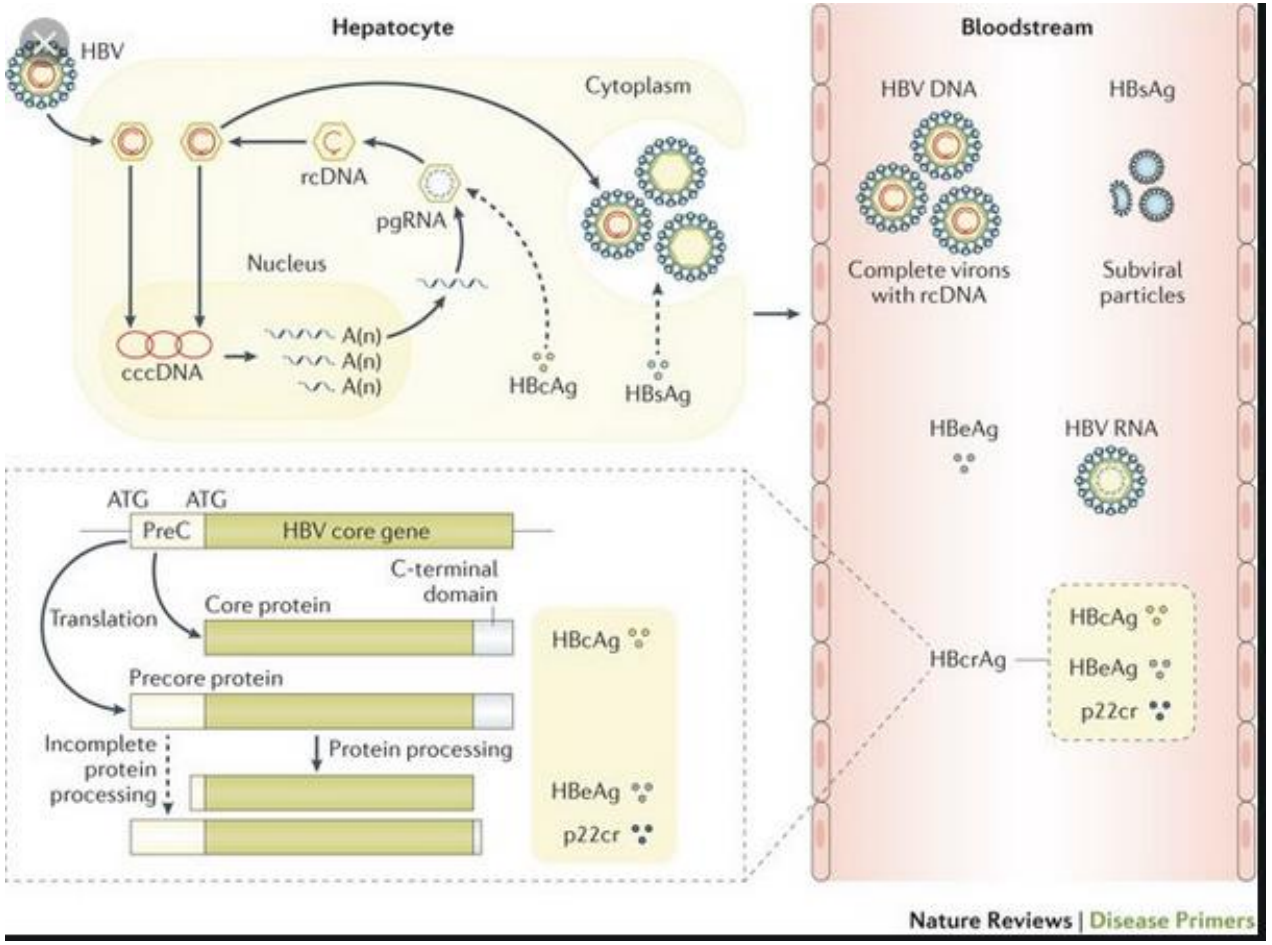
Area under receiver operating characteristics (AUROC) curve of HBsAg levels and occurrence of HBV integration (area under curve [AUC] = 0.841; threshold: 5,000 IU/ml).

Negative Predictive Value	94.7%
Positive Predictive Value	70.6%

Sensitivity	**92.3%**
Specificity	**78.3%**

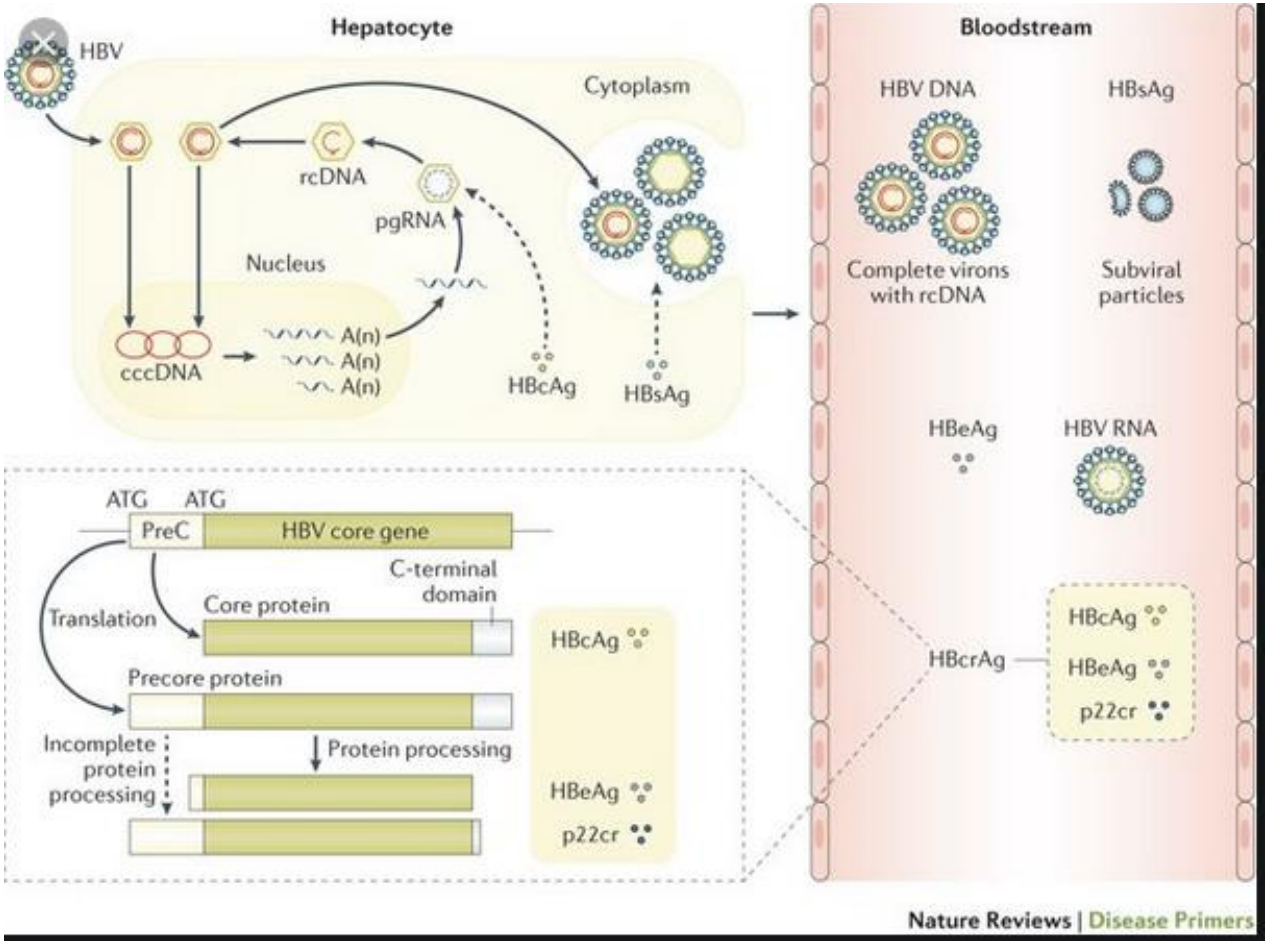
Beyond HBsAg…..

- **The quantification of HBcrAg reflects the capsid protein HBcAg, HBeAg and a precore protein (p22) coded with the precore/core region**



Yuen et al., Nature Reviews 2018

- **Serum HBV-RNA mainly reflects the amount of pgRNA not retrotranscribed and present in virions or naked particles**

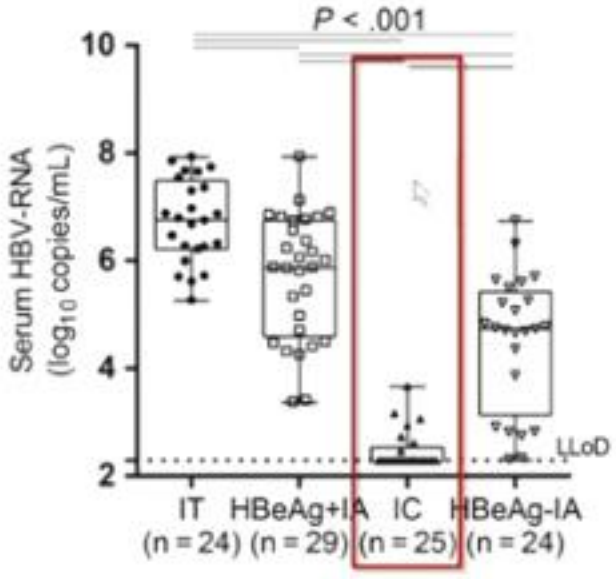


Yuen et al., Nature Reviews 2018

- Serum HBV RNA is likely a mixture of intact, pregenomic (pg) and subgenomic, spliced, truncated, and polyA-free species:
  - this highlights the importance to use adequate assays for the detection of pgRNA in serum

- **Differential diagnosis between HBeAg-negative infection and hepatitis**
  - low/undetectable levels of HBcrAg or 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



Wang et al., JVH 2018

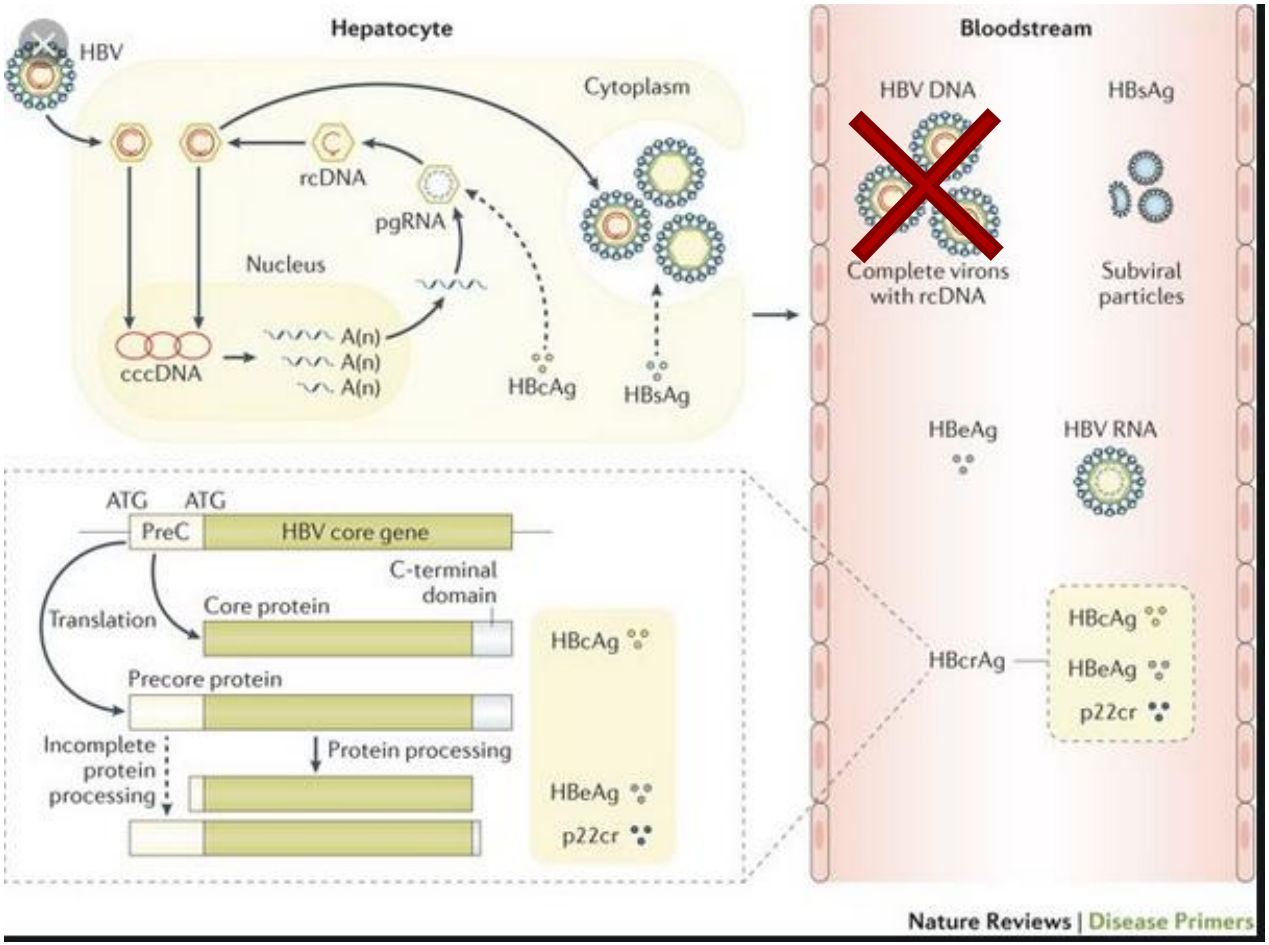
HBcrAg

ROC to discriminate CHB form HBeAg negative infection (CI-1)

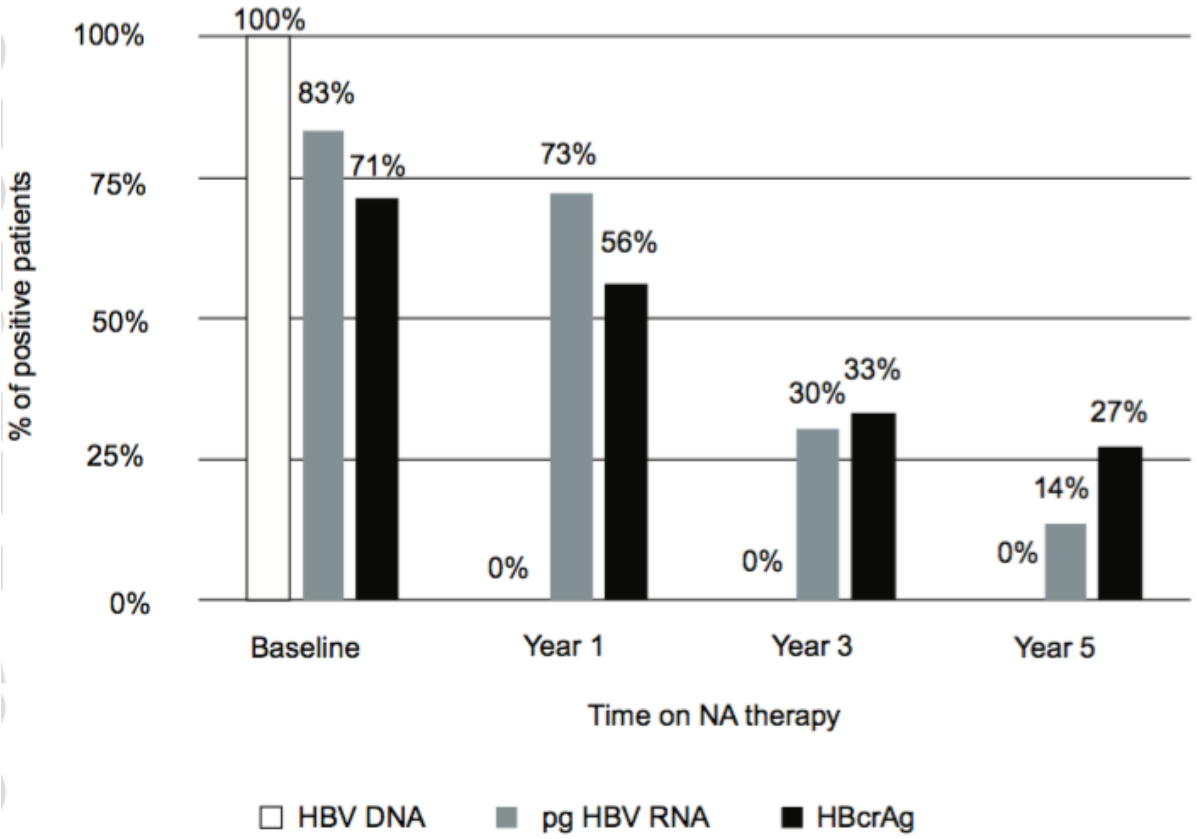
Viral markers	AUC	Cutoff	Sensitivity	Specificity
HBV-DNA	0.996	3.329	0.998	0.992
HBsAg	0.754	2.962	0.569	0.880
HBcrAg	0.963	3.138	0.914	0.930

Brunetto et al., AASLD 2018

- **Differently from serum HBV-DNA, the production of serum HBV-RNA and also HBcrAg cannot be affected by NUCs**
- **These biomarkers provide the advantage to measure intrahepatic HBV reservoir in virologically suppressed 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? Or reduced pool of infected hepatocytes?**



Carey et al., Hepatology 2019

- **identification of patients who can safely and successfully suspend NUC treatment.**

Diagnostic objective

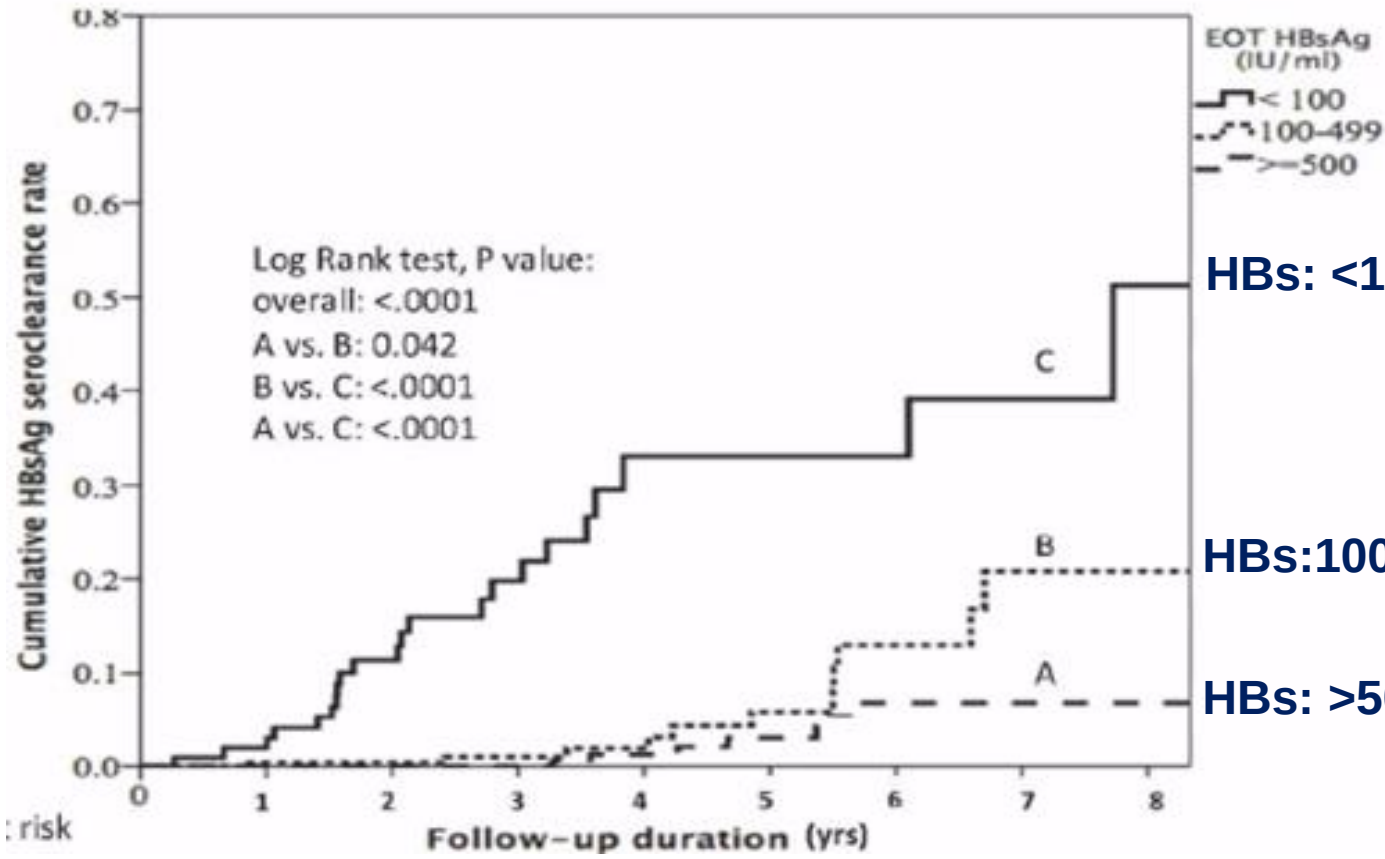
Patients under NUC treatment

Continuing treatment?

Suspending treatment?

Need to identify patients who can safely and successfully suspend antiviral treatment.

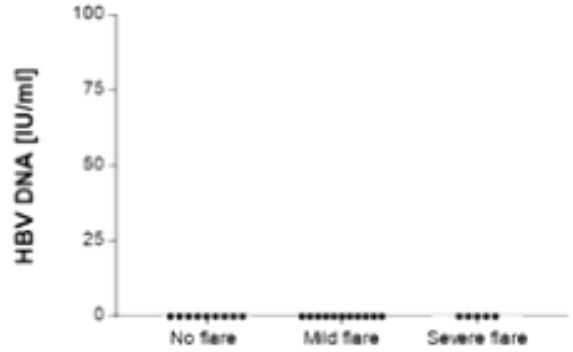
A lower HBsAg (reflecting a limited HBV reservoir) at NUC suspension correlates with higher probability to achieve HBsAg loss

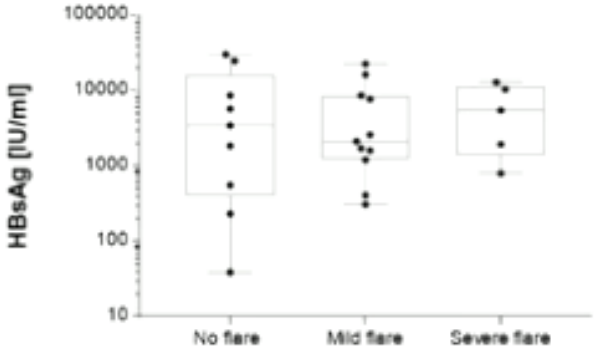


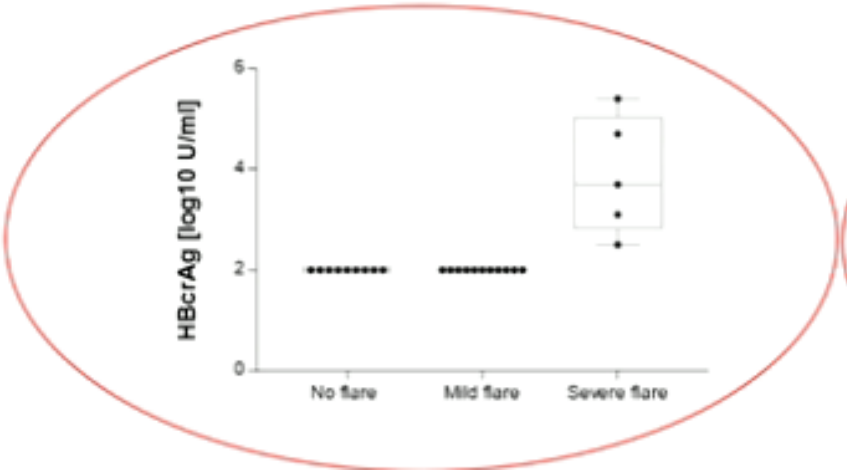
- Stopping NUC therapy in sustained responders and those who achieve HBsAg <100 IU/ml by the end of therapy might increase the 5-year HBsAg loss by up to >30%.

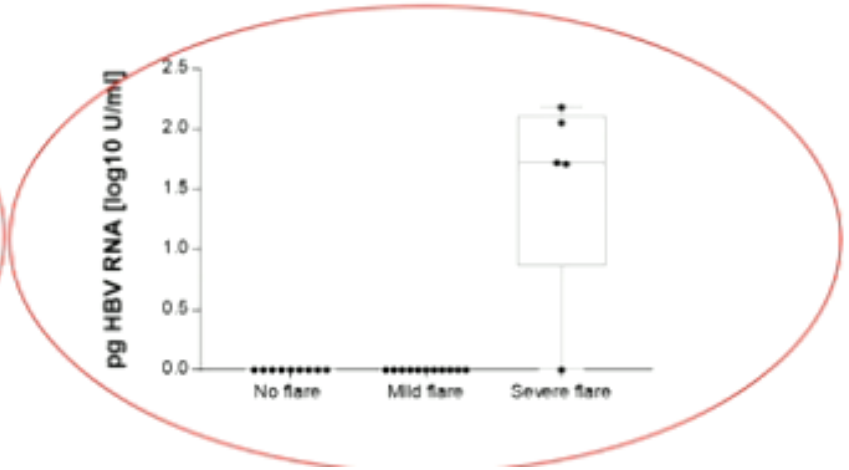
Jeng et al., J Hepatology 2017

Detectable HBcrAg and serum HBV-RNA at NUC suspension are observed only in patients with severe clinical relapse









Carey et al., Hepatology 2019

Stop & watch strategy

qHBsAg decline during hepatic flare

No need to re-start treatment

Stable or increasing qHBsAg during hepatic flare

Need to re-start treatment

Liaw et al., Gastroenterology 2018

- **identification of patients at risk of immune-suppression driven HBV reactivation**

Diagnostic objective

Immunosuppressed patients with occult HBV infection at screening

Monitoring?

Antiviral Prophylaxis?

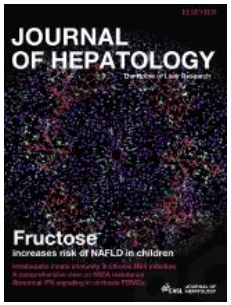
Lower baseline anti-HBs levels associated with subsequent HBV reactivation

Reference	N° of patients	Clinical setting	% of patients with HBV-R	Association of biomarker with risk of HBV-R
Seto et al., 2014	260	Oncohematological patients receiving rituximab	30.2%	**Anti-HBs** negativity at BL: HR (95%CI): 3.51(1.37-8.98), P=0.009
Cho Y et al. 2016	108	B-cell lymphoma receiving rituximab not antiviral prophylaxis	11,6%	**Anti-HBs**<100 mIU/ml HBV reactivation rates at 6, 12, 36, and 48 months after chemotherapyas high as 8.3, 17.3, 21.1, and 25.7%,
Fukuda et al. 2016	1042	Rheumatic diseases	3,3%	**Anti-HBs**<100 mIU/ml OR (95% CI): 2.8 (1.3 to 6.8)
Lee et al., 2018	366	Kidney transplanted patients using rituximab for desensitization	2.5%	**Anti-HBs** <100mIU/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
Kotake et al., 2018	243	Patients with solid tumors	2.1%	**Anti-HBs** negativity at BL: OR(95% CI):5.94 (1.15-30.6), P=0.03 **dexamethasone** >1.0 mg/day at BL: OR (95% CI): 8.69 (1.27-58.8), P=0.02
Tien et al. 2018	380	Rheumatic diseases treated with biologic therapy	4,4%	**Anti-HBs**>100 IU/ml at BL Rate of HBV reactivation person year 0/100 **Anti-HBs 10-100 mIU/ml at BL** Rate of HBV reactivation person year 2.5/100 **Anti-HBs <10 IU/ml at BL** Rate of HBV reactivation person year 4.7/100
Seto et al., 2016	62	Oncohematological patients receiving rituximab	29%	**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

Svicher et al., 2019

- **An added value can be provided by anti-HBc titer……**

J Hepatol. 2018 Apr 2. pii: S0168-8278(18)31963-9. doi: 10.1016/j.jhep.2018.03.021. [Epub ahead of print]



Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: a new tool to detect occult infection.

Caviglia GP[1], Abate ML[2], Tandoi F[3], Ciancio A[2], Amoroso A[4], Salizzoni M[3], Saracco GM[2], Rizzetto M[2], Romagnoli R[3], Smedile A[2].

Author information

Abstract

BACKGROUND & AIMS: The accurate diagnosis of occult HBV infection (OBI) requires the demonstration of HBV DNA in liver biopsies of HBsAg-negative subjects. However, in clinical practice a latent OBI is deduced by the finding of the antibody to the HB-core antigen (anti-HBc). We investigated the true prevalence of OBI and the molecular features of intrahepatic HBV in anti-HBc-positive subjects.

METHODS: The livers of 100 transplant donors (median age 68.2 years; 64 males, 36 females) positive for anti-HBc at standard serologic testing, were examined for total HBV DNA by nested-PCR and for the HBV covalently closed circular DNA (HBV cccDNA) with an in-house droplet digital PCR assay (ddPCR) (Linearity: $R^2 = 0.9998$; lower limit of quantitation and detection of 2.4 and 0.8 copies/10^5 cells, respectively).

RESULTS: A true OBI status was found in 52% (52/100) of the subjects and cccDNA was found in 52% (27/52) of the OBI-positive, with a median 13 copies/10^5 cells (95% confidence interval 5-25). Using an assay specific for anti-HBc of IgG class, the median antibody level was significantly higher in HBV cccDNA-positive than negative donors (5.7 [3.6-9.7] vs. 17.0 [7.0-39.2] COI, $p = 0.007$). By multivariate analysis, an anti-HBc IgG value above a 4.4 cut-off index (COI) was associated with the finding of intrahepatic HBV cccDNA (OR = 8.516, $p = 0.009$); a lower value ruled out its presence with a negative predictive value of 94.6%.

CONCLUSIONS: With a new in-house ddPCR-based method, intrahepatic HBV cccDNA was detectable in quantifiable levels in about half of the OBI cases examined. The titer of anti-HBc IgG may be a useful surrogate to predict the risk of OBI reactivation in immunosuppressed patients.

LAY SUMMARY: The covalently closed circular DNA (cccDNA) form of the Hepatitis B virus (HBV) sustains the persistence of the virus even after decades of resolution of the florid infection (Occult HBV infection=OBI). In the present study we developed an highly sensitive method based on droplet digital PCR technology for the detection and quantitation of HBV cccDNA in the liver of subjects with OBI .We observed that the amount of HBV cccDNA may be inferred from the titer in serum of the IgG class antibody to the hepatitis B core antigen (anti-HBc IgG). The quantitation of anti-HBc IgG may represent a surrogate to discriminate the patients at the highest risk of HBV reactivation following immunosuppressive therapies.

Anti-HBc titers can represent a valid surrogate marker for estimating HBV intrahepatic reservoir

The use of accurate virological and immunological hepatitis B markers can allow an early diagnosis of HBV reactivation in HBsAg-negative/anti-HBc-positive patients with oncohematological diseases

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EASL 2019; ICAR 2019

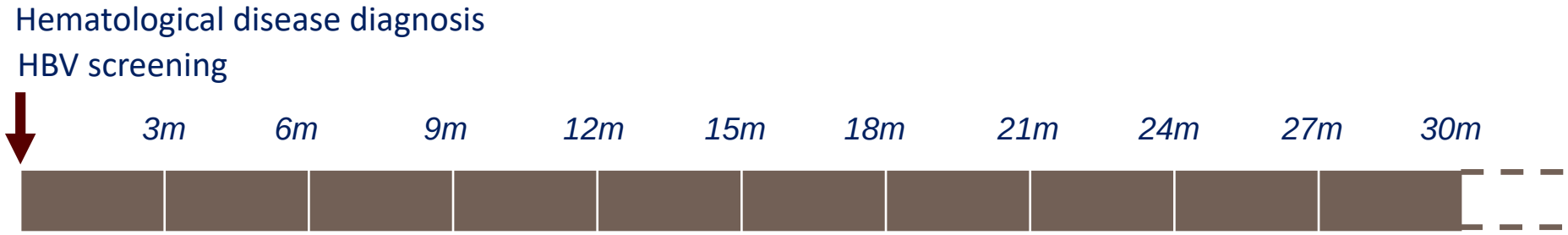
107 pts with oncohematological diseases

All HBsAg-neg/anti-HBc-pos

47 rituximab

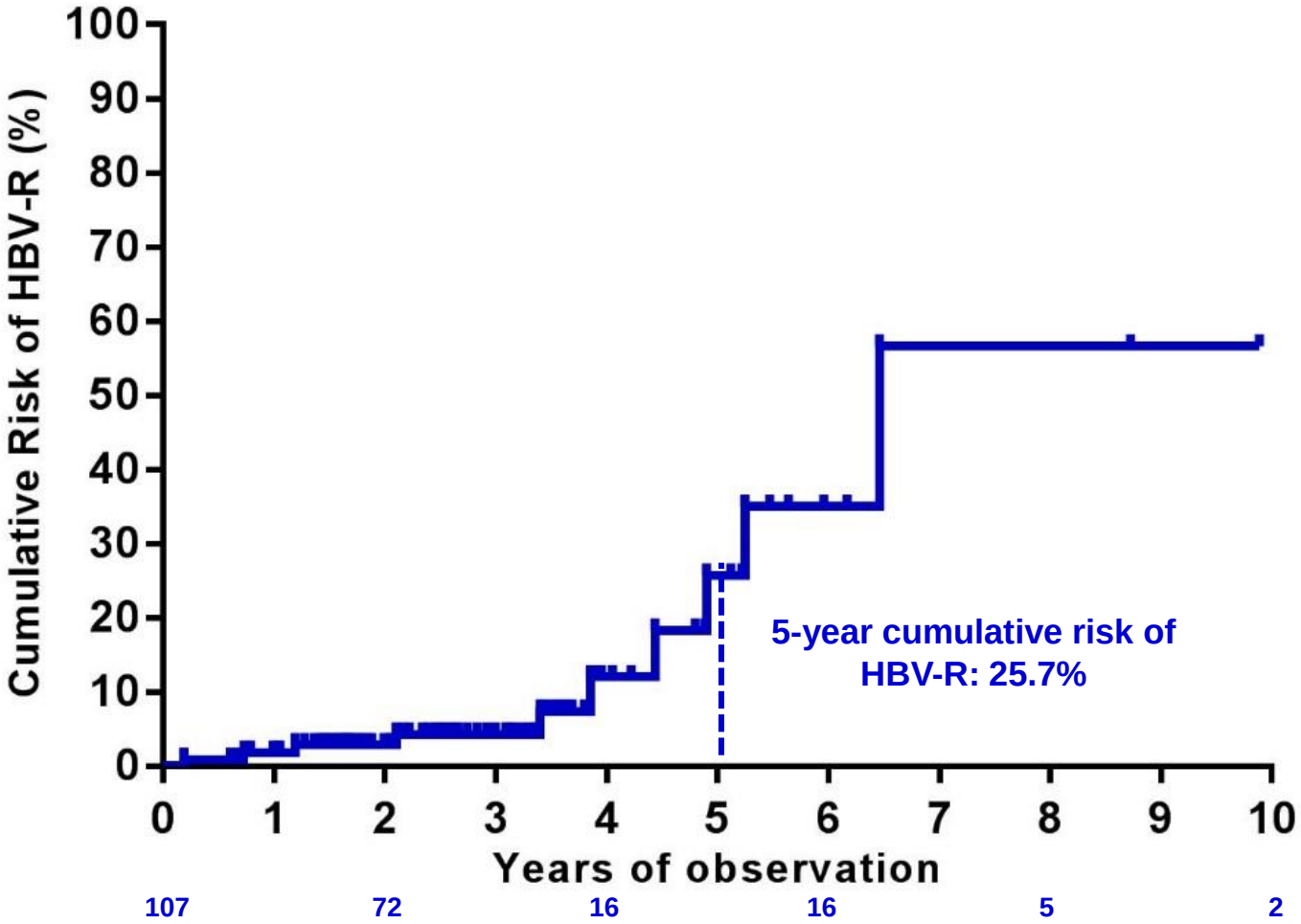
40 HSCT

27 other chemotherapeutics



All patients started antiviral prophylaxis as recommended by current guidelines and were monitored every 3 months

Despite the use of antiviral prophylaxis, HBV reactivation occurs in 12.1% of patients (with a 5-year cumulative rate of 25%)

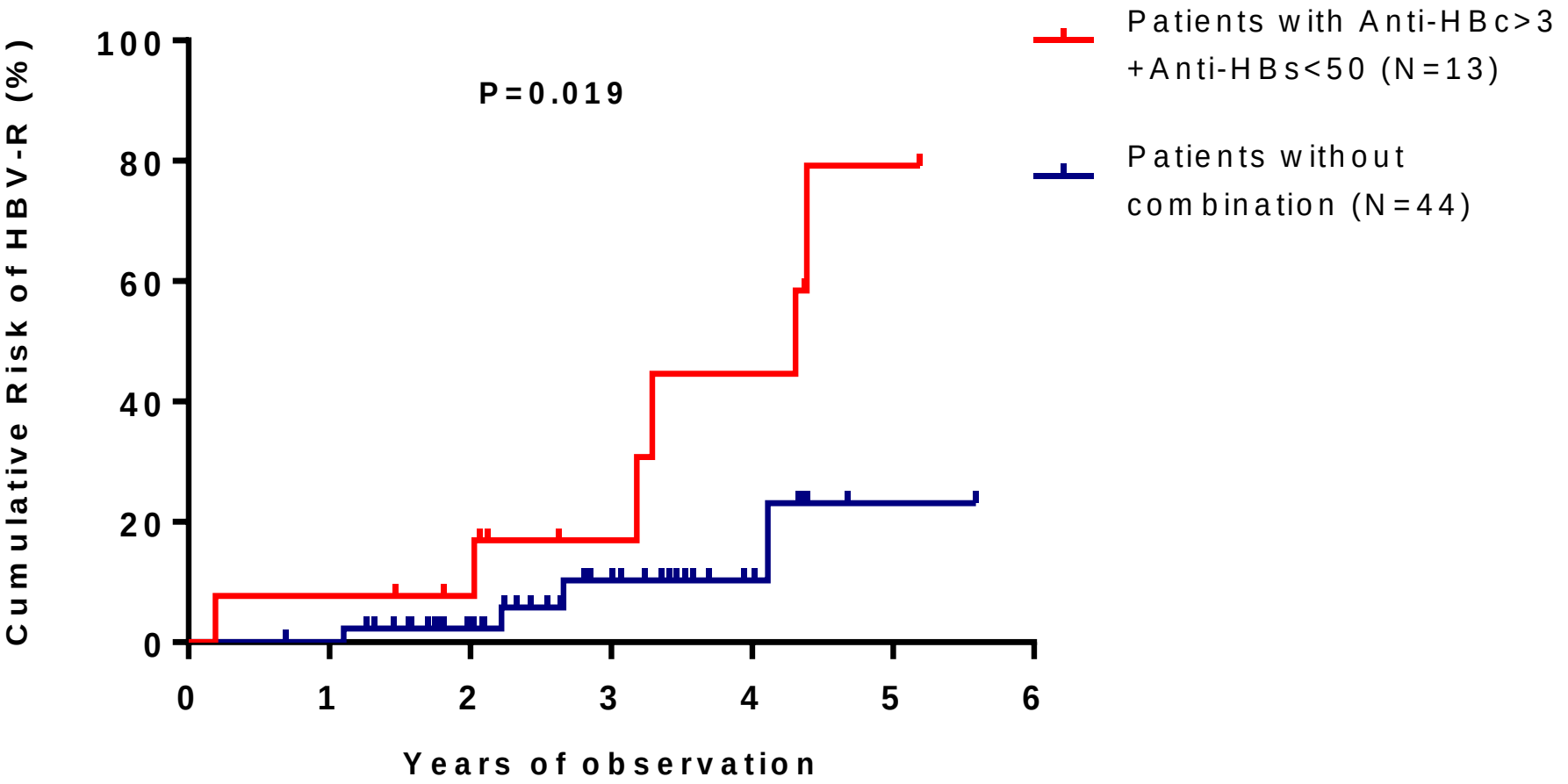


- A relevant proportion of patients experienced HBV reactivation after completing the recommended course of anti-HBV prophylaxis
  - **This suggests the need to reconsider proper duration of prophylaxis particularly in profound immunosuppression**.

Characteristics at HBV-R, N=13	
Serum HBV-DNA, Median (IQR) IU/ml	42 (23-5831)
Serum ALT > UNL, N (%):	6 (46.2)
- Serum ALT, Median (IQR) U/L	95 (81-986)
Immunosuppressive Regimen, N (%)	
Rituximab	2 (15.4)
Autologous HSCT	1 (7.7)
Allogenic HSCT	8 (61.5)
Other immune suppressive therapy	2 (15.4)
Timing of HBV-R	
HBV-R during LMV Prophylaxis, N (%)	5 (38.5)
HBV-R after LMV Interruption, N (%)	**8 (61.5)**
Months after LMV Interruption, Median (IQR)	3 (1-27)

Abbreviations: LMV; lamivudine, HSCT; hematopoietic stem cell transplantation

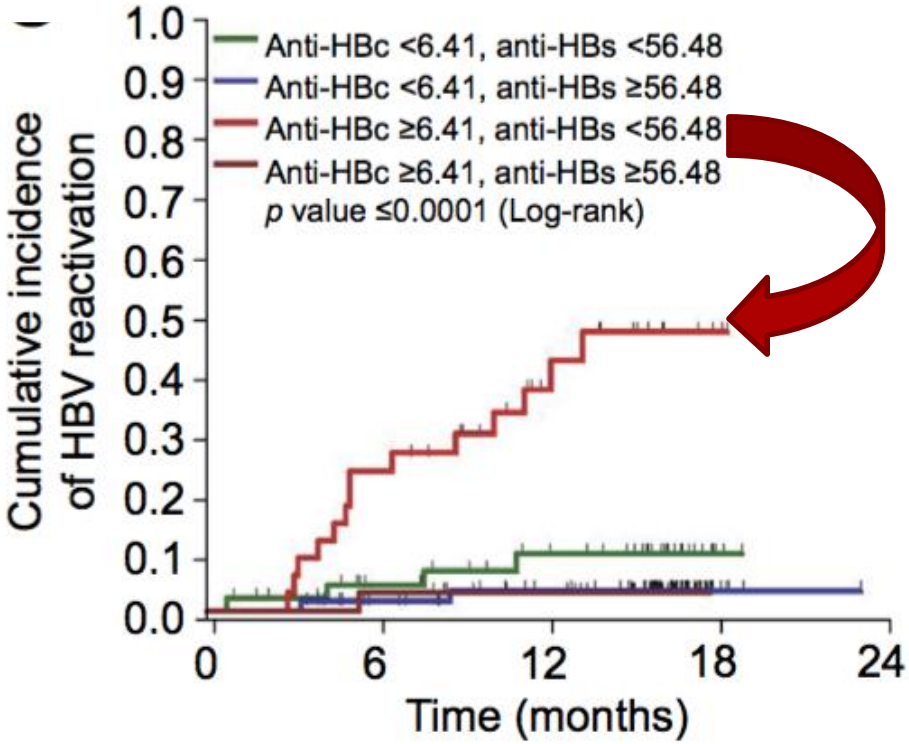
- **The analysis of serological markers during the entire monitoring reveals that an anti-HBc>3COI combined with anti-HBs persistently or declining to <50mIU/ml correlates with a higher risk to develop HBV-R**



(5years-cumulative risk of HBV-R: 79.2% in pts with anti-HBc>3COI+anti-HBs<50mIU/ml vs 23.1% without this combination, P=0.019)

Quantification of HBV core antibodies may help predict HBV reactivation in patients with lymphoma and resolved HBV infection

Yang et al., J Hepatol 2018



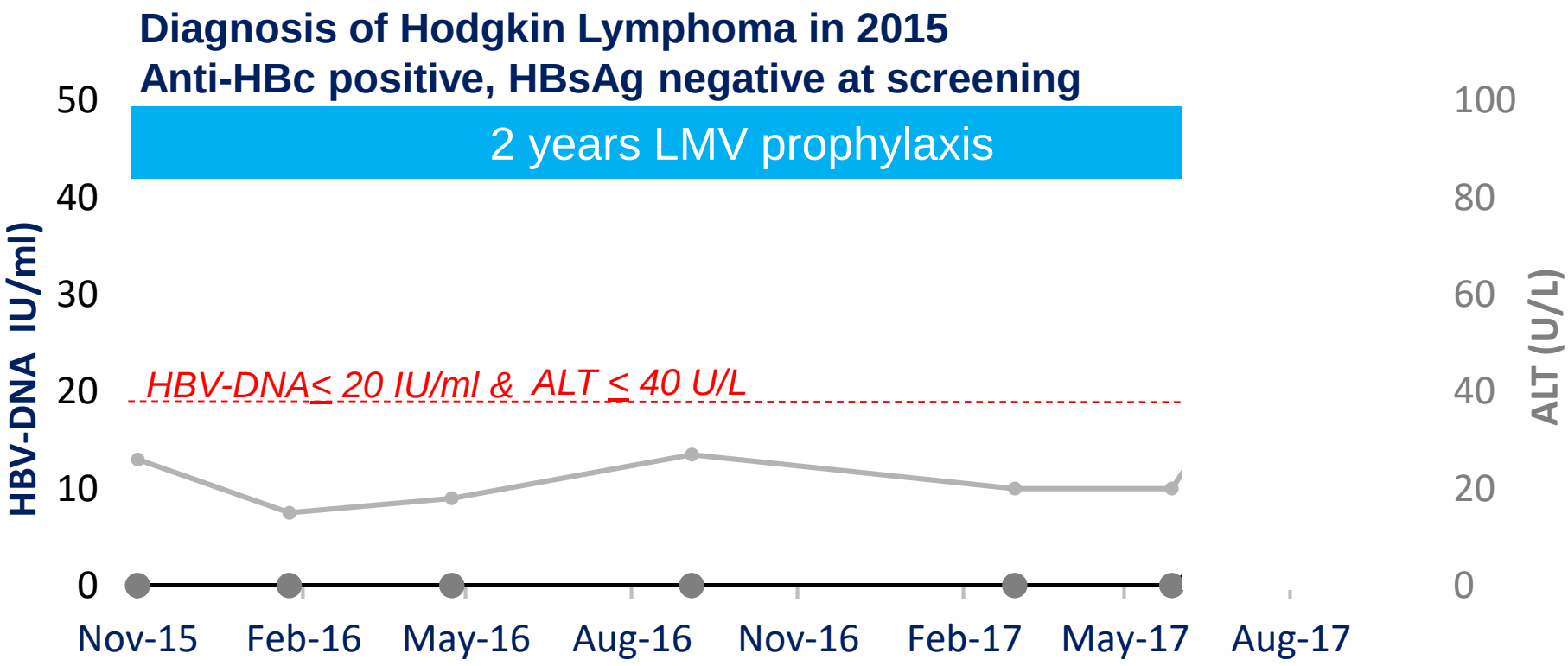
N° at risk				
Anti-HBc <6.41, anti-HBs <56.48	72	60	51	5
Anti-HBc <6.41, anti-HBs ≥56.48	51	39	31	2
Anti-HBc ≥6.41, anti-HBs <56.48	36	26	13	2
Anti-HBc ≥6.41, anti-HBs ≥56.48	33	32	26	0

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

Highly-sensitive HBsAg assays & HBV reactivation

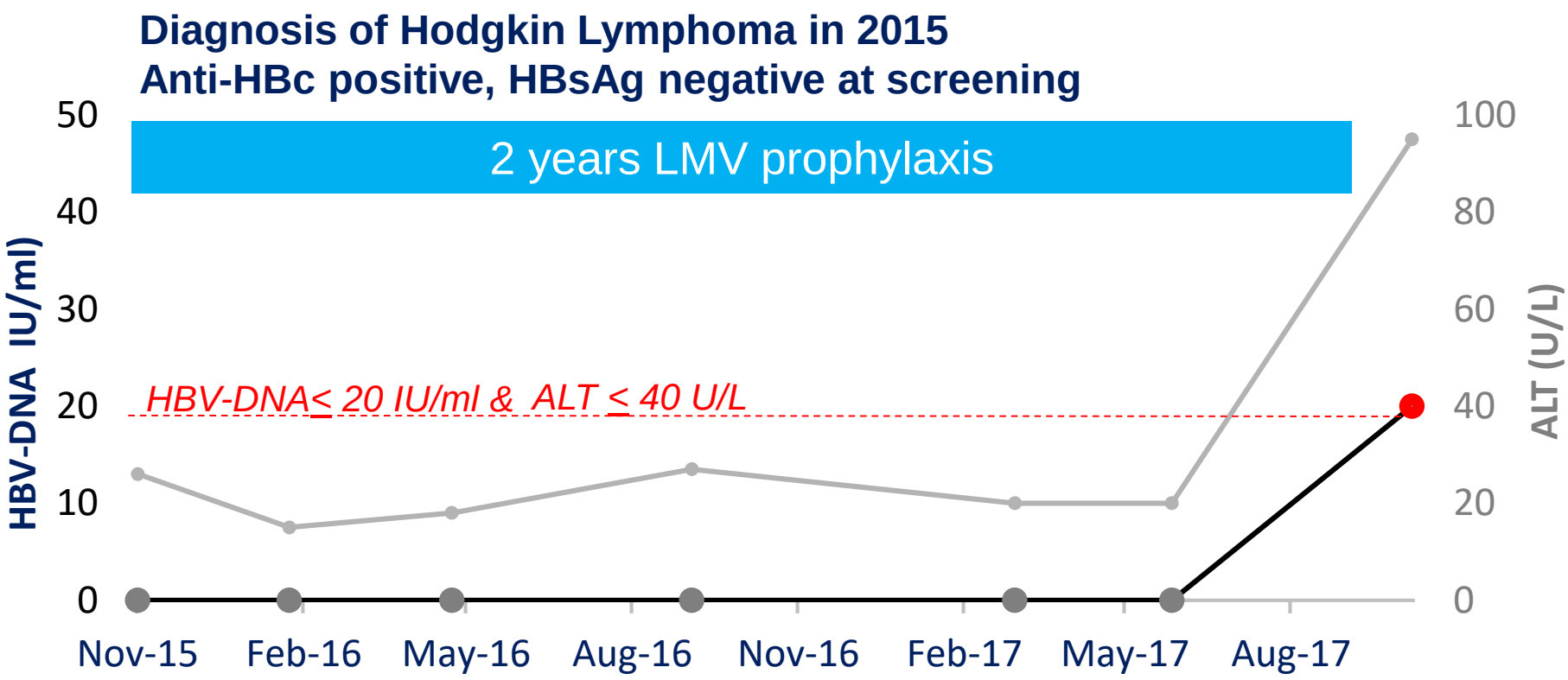
- Limit of detection of the classical assays: 50mIU/ml
- Limit of detection of the highly sensitive assays: 5mIU/ml (1log higher sensitivity)

Clinical Case from oncohematological setting



Under 2 years antiviral prophylaxis: persistent undetectable serum HBV-DNA and persistent negativity to HBsAg by classical assays

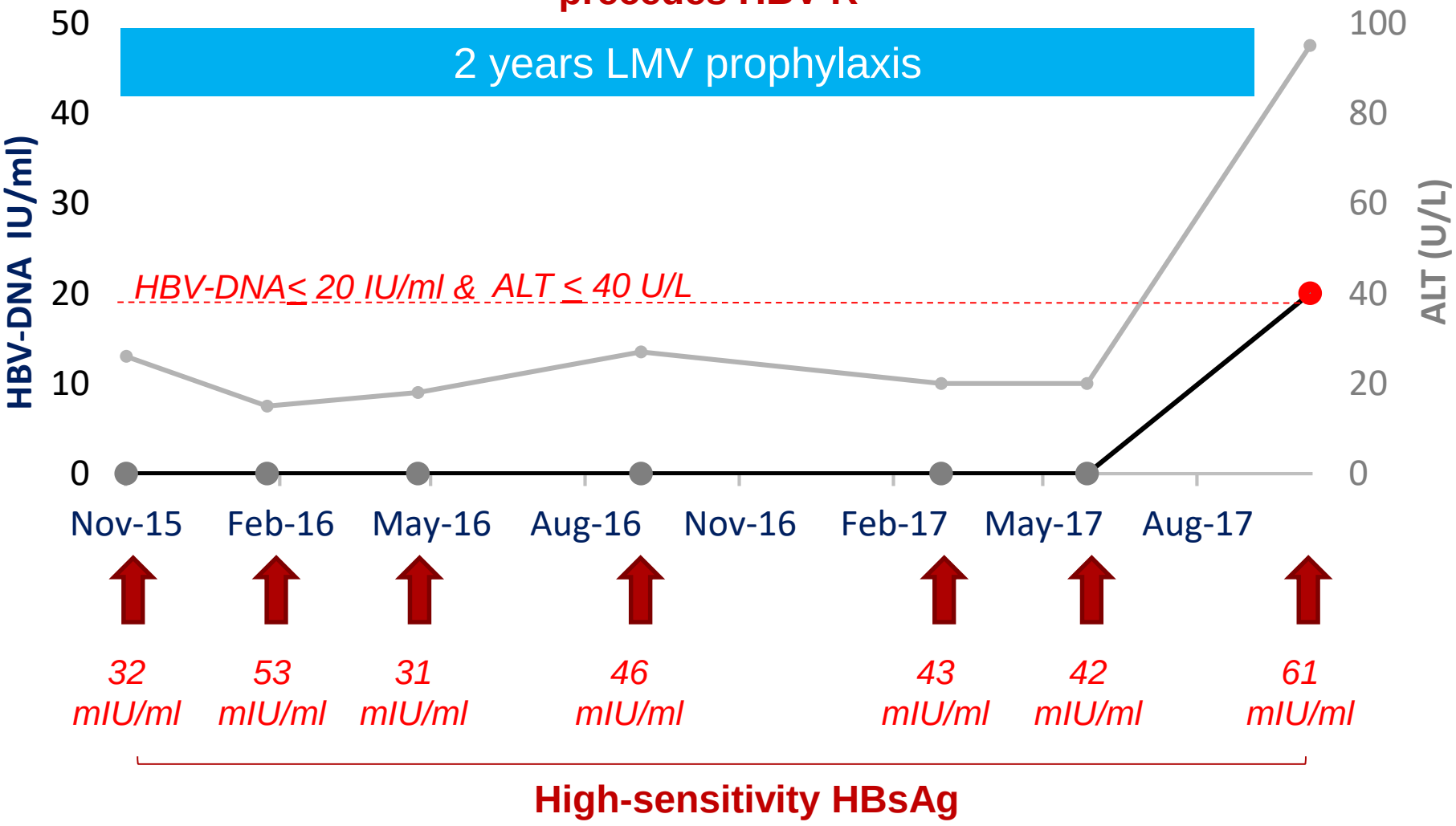
Clinical Case from oncohematological setting



Immediately after prophylaxis withdrawal:
increase in serum HBV-DNA and ALT flare

Clinical Case from oncohematological setting

Despite a persistent undetectable HBV-DNA, repeatedly HS-HBsAg >5mIU/ml (but not detected by routinely used qHBsAg assay) precedes HBV-R



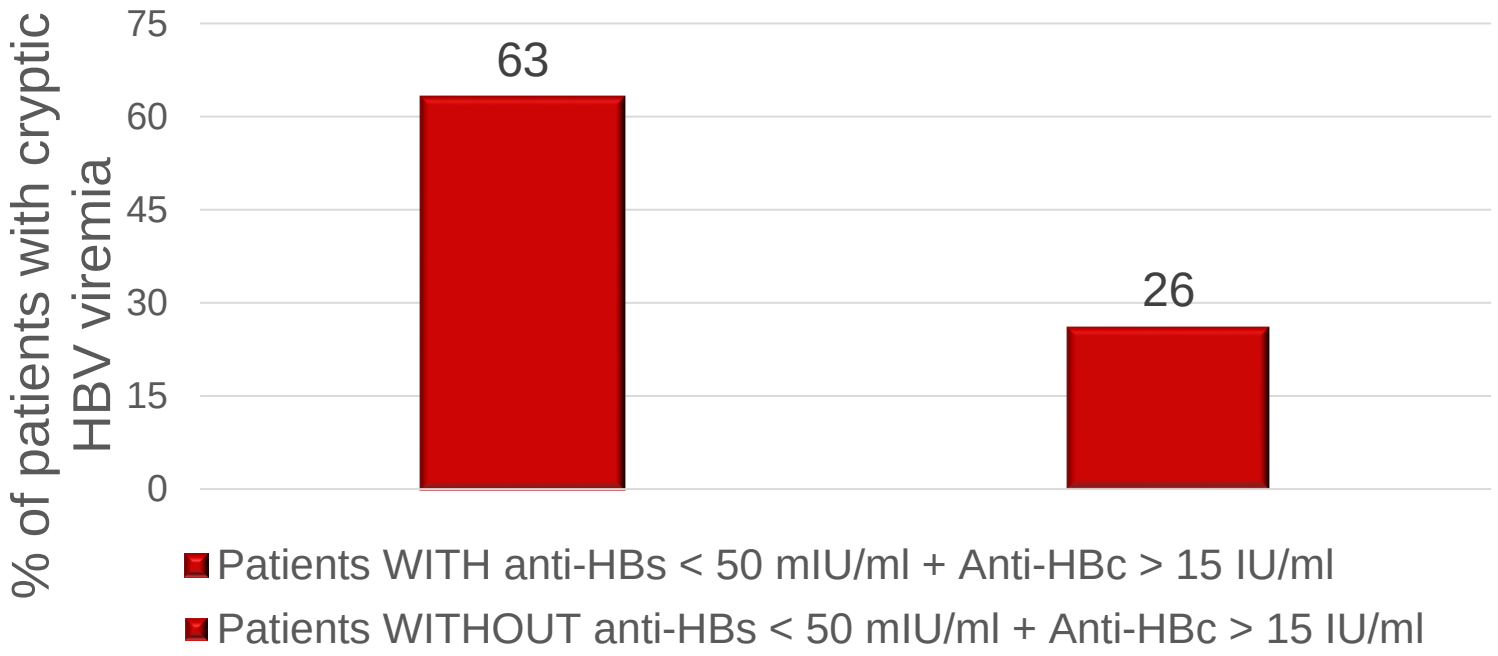
- **Novel biomarkers in the setting of HIV-1 infection**

- By analyzing a cohort of 81 HIV-infected anti-HBc-positive/HBsAg-negative patients, cryptic HBV viremia is detected in 29.6% of patients with a median (IQR) of 4 (1-15) COI.

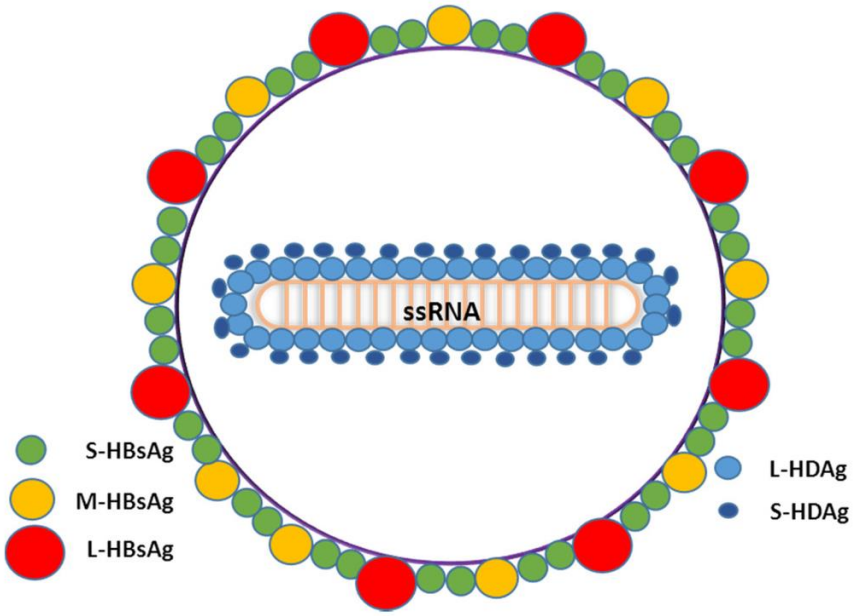
- No impact of different anti-HBV drugs on cryptic HBV viremia is observed (% of patients with serum HBV-DNA≥1 IU/ml: 27% for TAF, 28% for TDF and 37% for LMV, p=0.7).

- Moreover, a positive correlation is found between HBV-DNA and HIV-RNA (Rho:0.26, p=0.02).

- Anti-HBs<50mIU/ml combined with anti-HBc>15COI is predictive of cryptic HBV viremia (63% of patients with anti-HBs<50IU/ml+antiHBc>15COI has HBV-DNA≥1 IU/ml vs 26% without this combination, p=0.046, OR: 4.7[1.1-21.7]).



HDV: the smallest RNA virus, causing the most severe forms of hepatitis



Virological markers of HDV replication:

- **Serum HDV RNA**
- **HDAg**

Clinical Case

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

Serological profile at March 2019	
HBsAg	+
HBeAg	-
Serum HBV-DNA	<10IU/ml
HBsAg	9860 IU/ml
Anti-HDV IgG	+
HDAg	**-**

Clinical Case

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

Serological profile	
HBsAg	+
HBeAg	-
Serum HBV-DNA	<10IU/ml
HBsAg quantitativo	9860 IU/ml
Anti-HDV IgG	+
HDAg	**-**
Serum HDV-RNA	**2,696,930 copies/ml**

HEPATOLOGY

HEPATOLOGY, VOL. 64, NO. 5, 2016

First International External Quality Assessment for Hepatitis Delta Virus RNA Quantification in Plasma

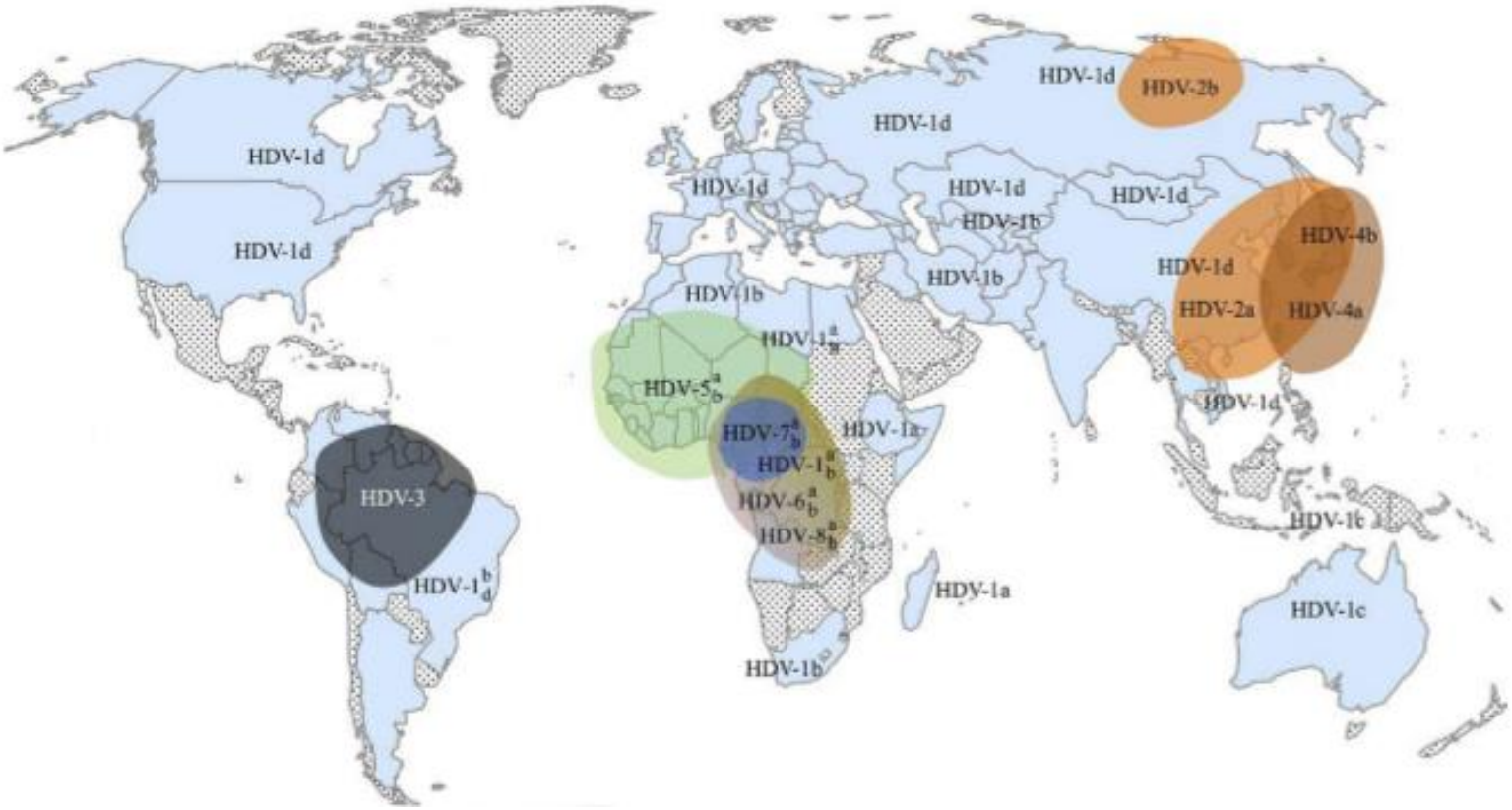
Frédéric Le Gal,[1,2] Ségolène Brichler,[1–3] Roland Sahli,[4] Sylvie Chevret,[5,6] and Emmanuel Gordien[1–3]

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.

- Owing to its error-prone way of replication, the genome of HDV is highly variable.
- Today, **8 genotypes** (with up to 35% divergence between different genotypes), have been described.

  - so far no genotyping assays are available for HDV



Le gal et al., Hepatology 2017

- **Genotype 1 infections have a lower survival rate and more adverse outcomes than infections with genotype 2**

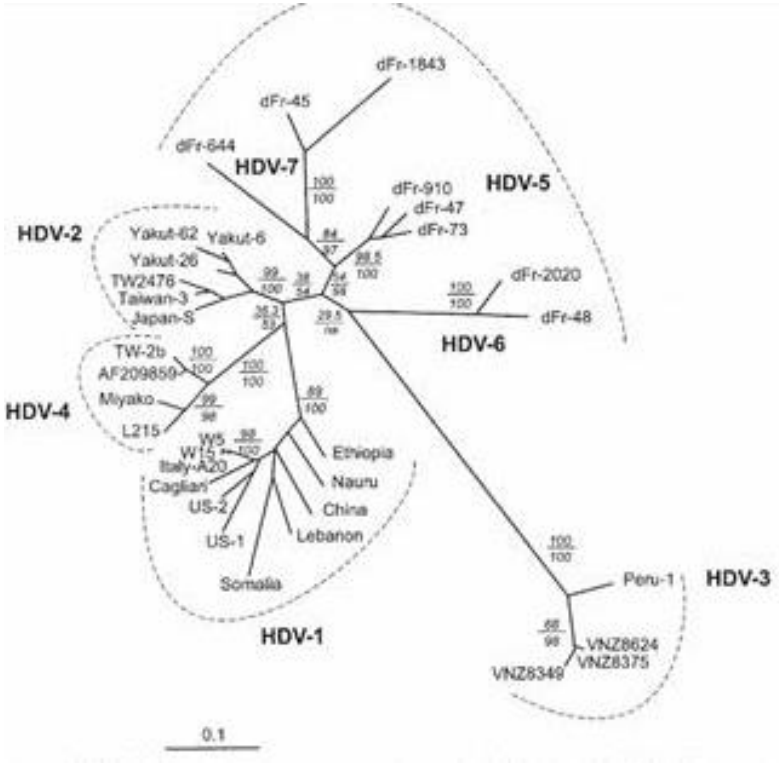
  Su et al., Gastroenterology 2017

- **Genotype 3 is the most divergent of all genotypes and is often associated with severe liver disease and outbreaks of fulminant hepatitis in South America**

  Casey et al., JID 1996

- **Genotype 3 seems to be more responsive to Interferon-alpha**

  Borzakov et al., IJID 2016



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