



ANR-17-RHUS-0003

International Workshop on Viral Biomarkers
Lyon, September 7th 2023

Are serum HBV RNAs and HBcrAg non-invasive markers of intrahepatic cccDNA?

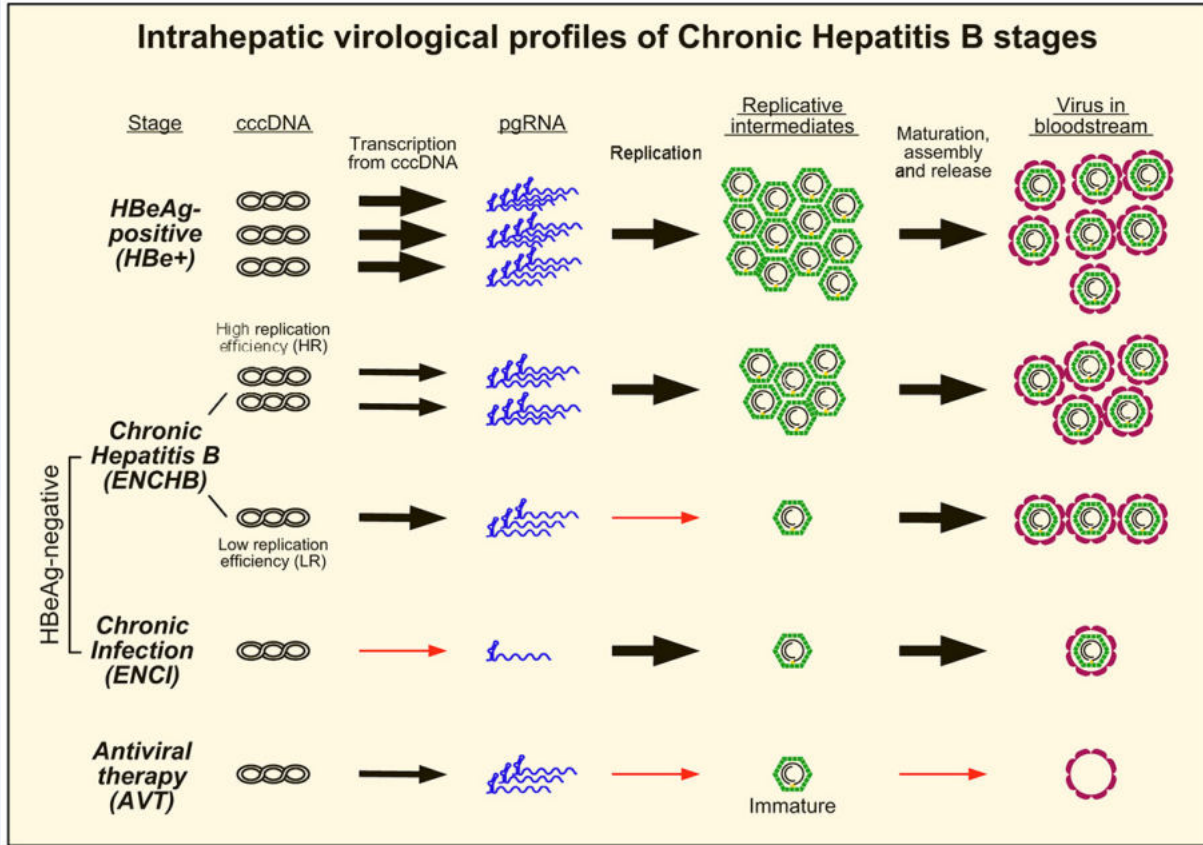
Barbara Testoni



Why do we need a biomarker for cccDNA transcriptional activity?

Possible dissociation between cccDNA levels and cccDNA transcriptional activity...
(3.5Kb RNA/cccDNA ratio)

...during CHB natural history

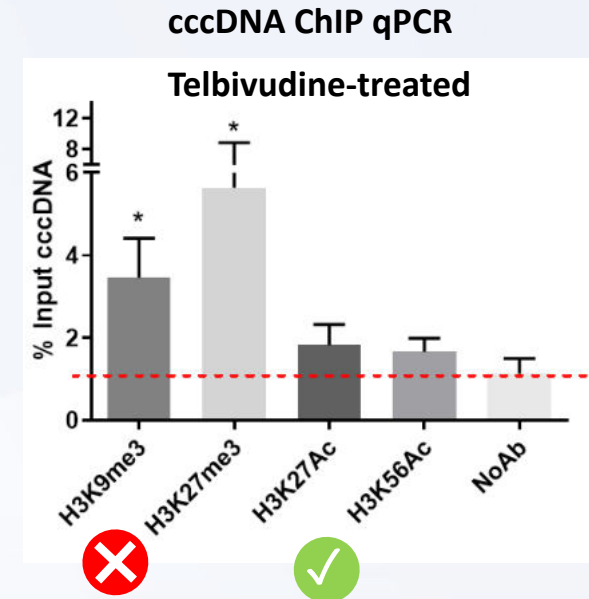
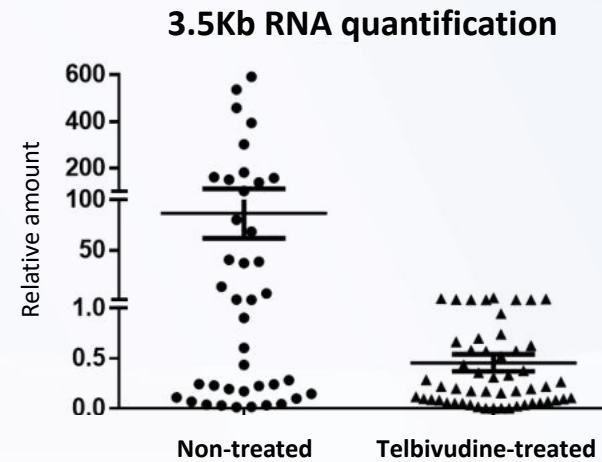


Volz, Gastroenterology 2007
Zhang, JCI 2016
Lebossé, Testoni, JHepatology 2017

Adapted from Suslov, JHepatology 2020

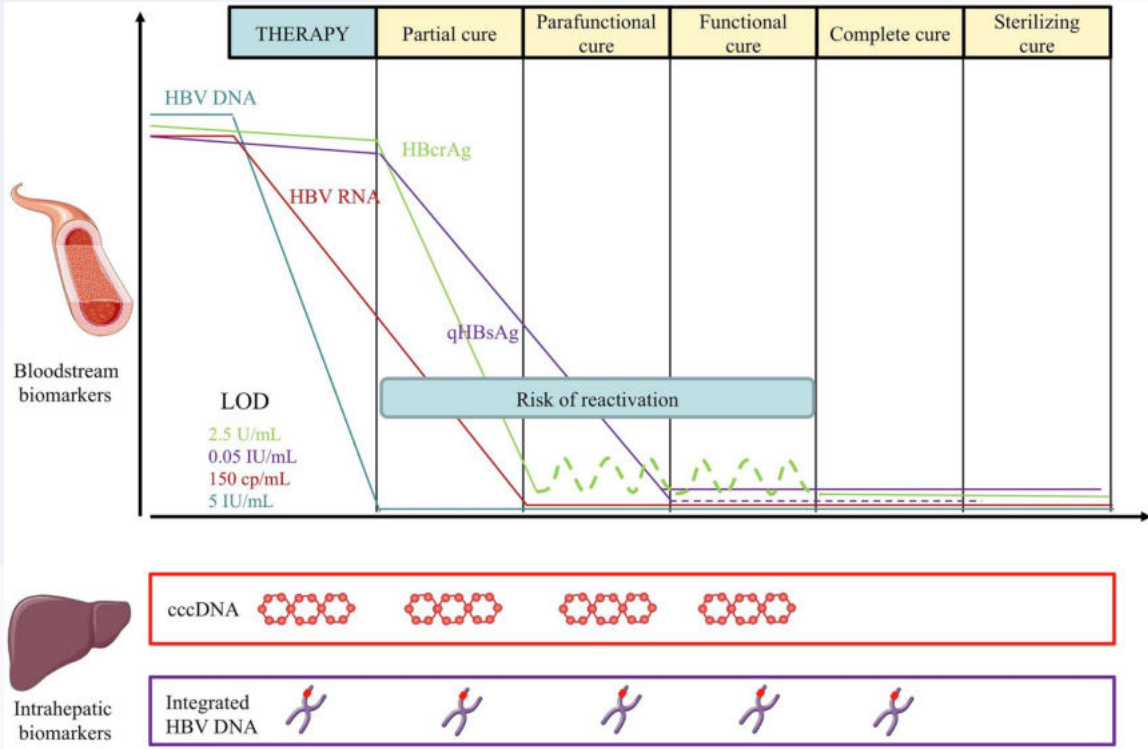
...during antiviral therapy

Long term telbivudine-treated patients



Lebossé, Sci Rep 2020
Balagopal, J Infect Dis 2020
Balagopal, JCI Insight 2020

Why do we need a biomarker for cccDNA transcriptional activity?



HBV complete cure

cccDNA clearance → Measure cccDNA levels

HBV functional cure

cccDNA « inactivation » → Measure 3.5Kb RNA/cccDNA

Gold standard: intrahepatic assessment of both

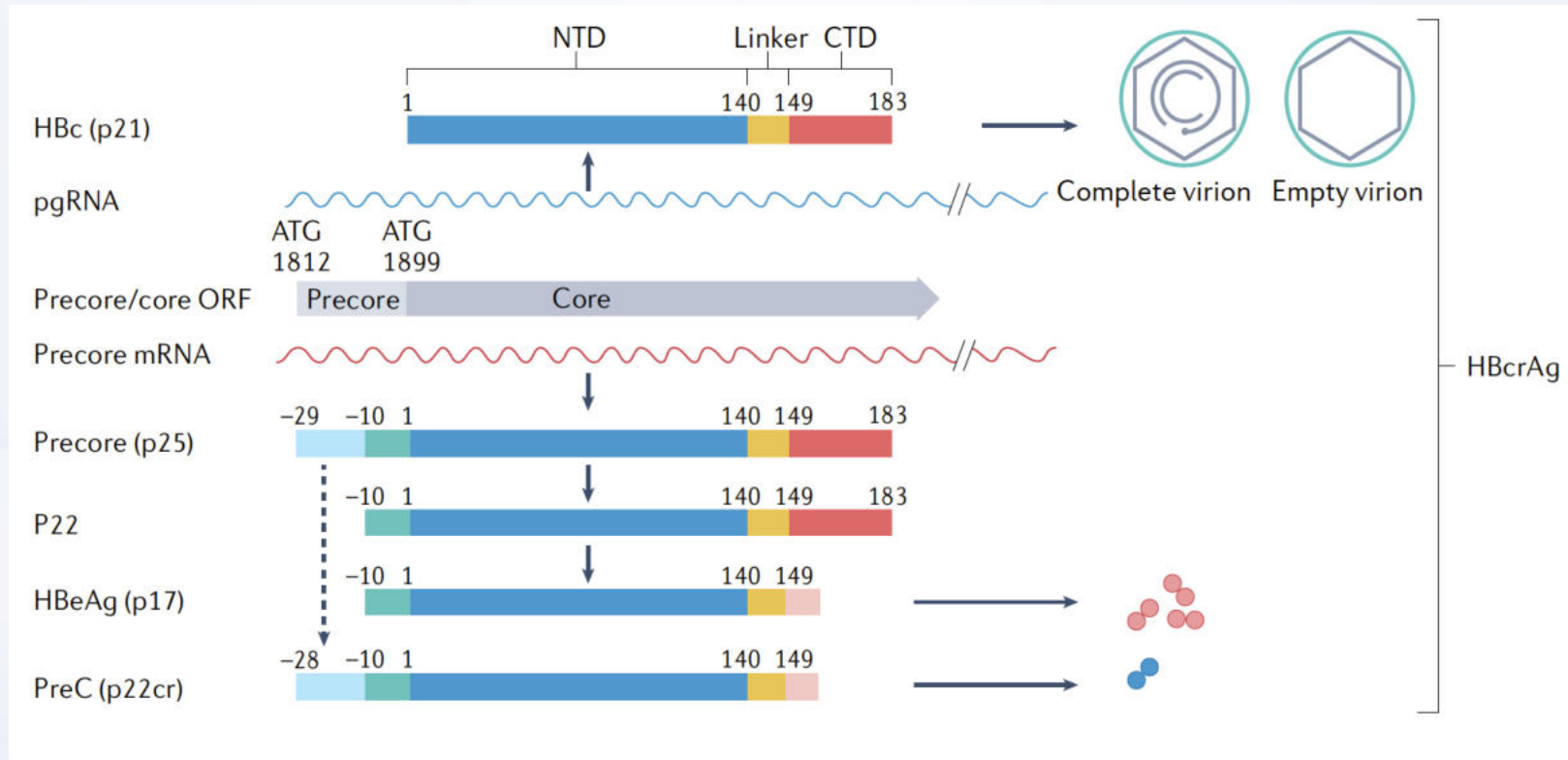
- cccDNA quantity
- cccDNA activity

Limitations: invasive technique (*FNA should be considered!!*)
 sampling bias (tiny portion of the whole liver)
 technical challenges

HBcrAg – where does it come from?

Composite marker

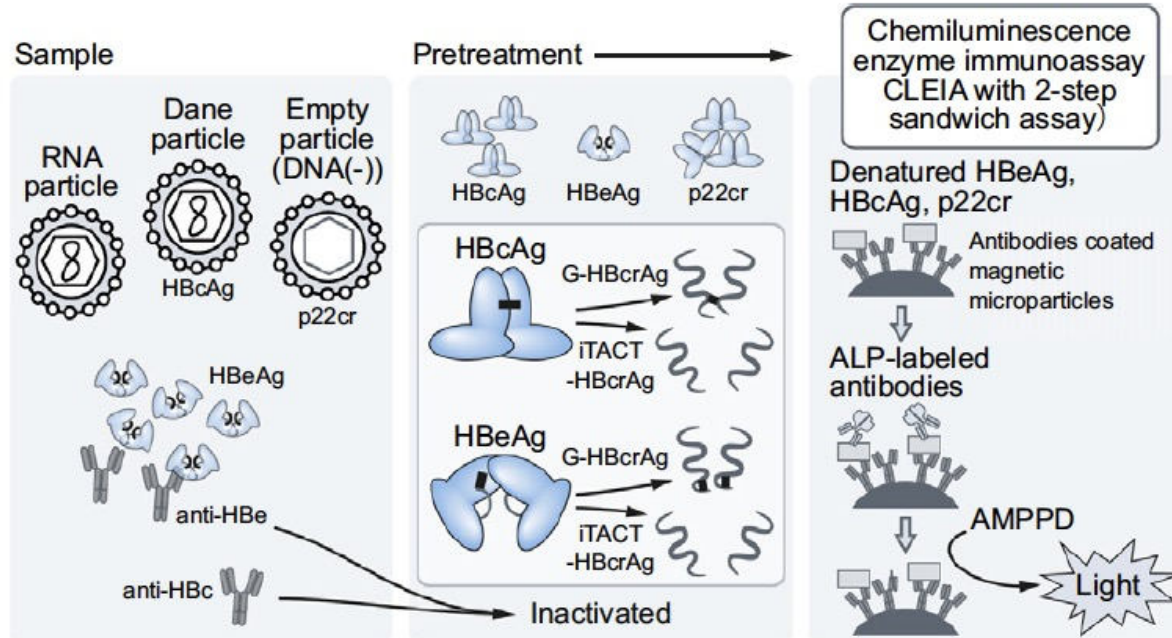
Translated from overlength HBV RNA, thus only produced from cccDNA



Kramvis, Nat Rev Gastroenterol Hepatol 2022

HBcrAg – available assay

CLEIA® HBcrAg assay kit (Lumipulse System, Fujirebio, Inc.)



Capture: mixture of 3 monoclonal antibodies reacting with **denatured HBcAg, HBeAg and other precore/core proteins**

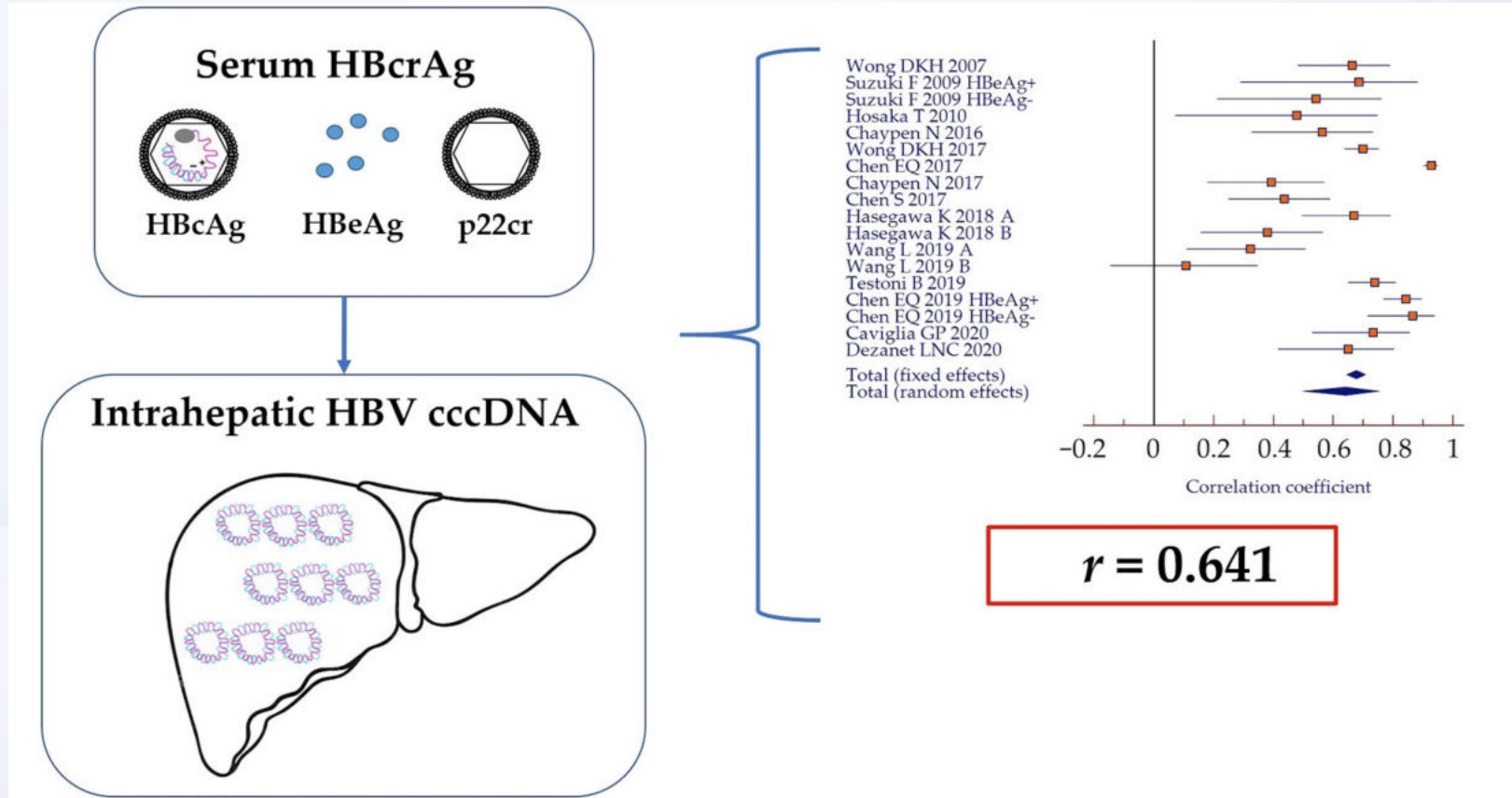
Pre-treatment: detergents

Detection: Alkaline phosphatase conjugated monoclonal antibodies against **denatured HBcAg, HBeAg, and p22cr**

Assay	Pretreatment process		TAT (include pretreatment)	LOQ	
	Procedure	Main denaturants			Incubation
G-HBcrAg	Manual	Detergents	60°C for 30 min	>60 min	2.8 Log U/ml*
iTACT-HBcrAg	Automatic (on-board)	Acid, detergents, reducing agent	37°C for 6.5 min	35 min	2.1 Log U/ml

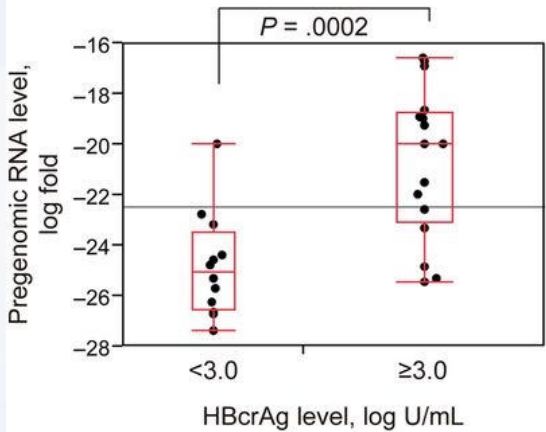
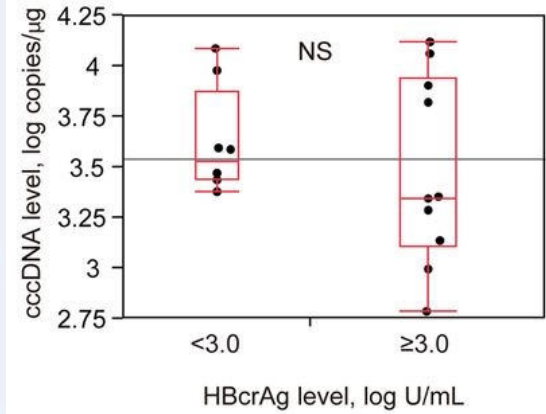
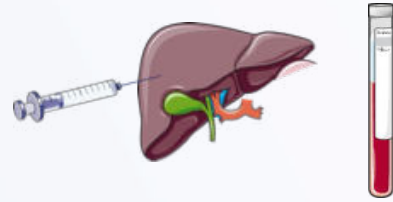
*LOQ used in this study

HBcrAg is a biomarker of liver cccDNA pool



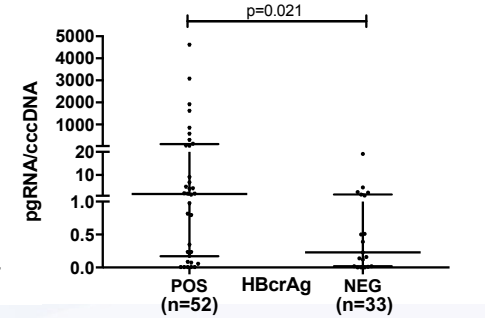
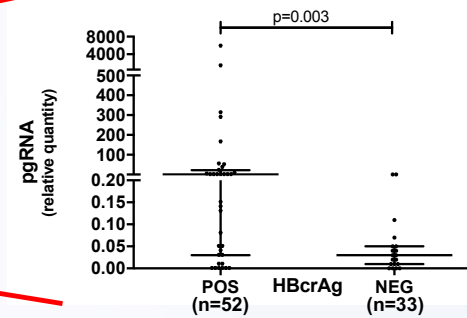
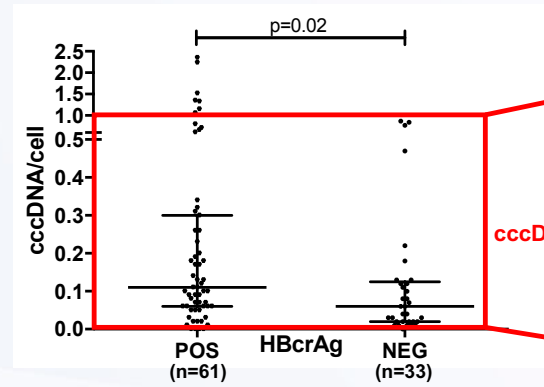
HBcrAg and intrahepatic cccDNA transcriptional activity

109 Asian NUC-treated CHB patients



Honda, JID 2016

130 Caucasian untreated CHB patients

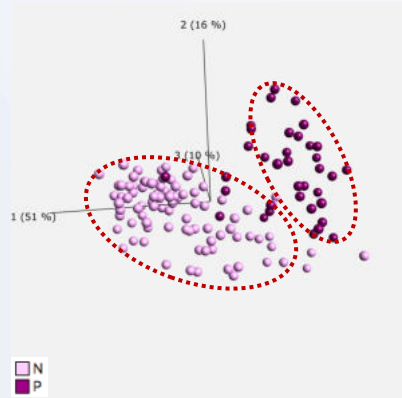


Testoni, JHepatol 2019

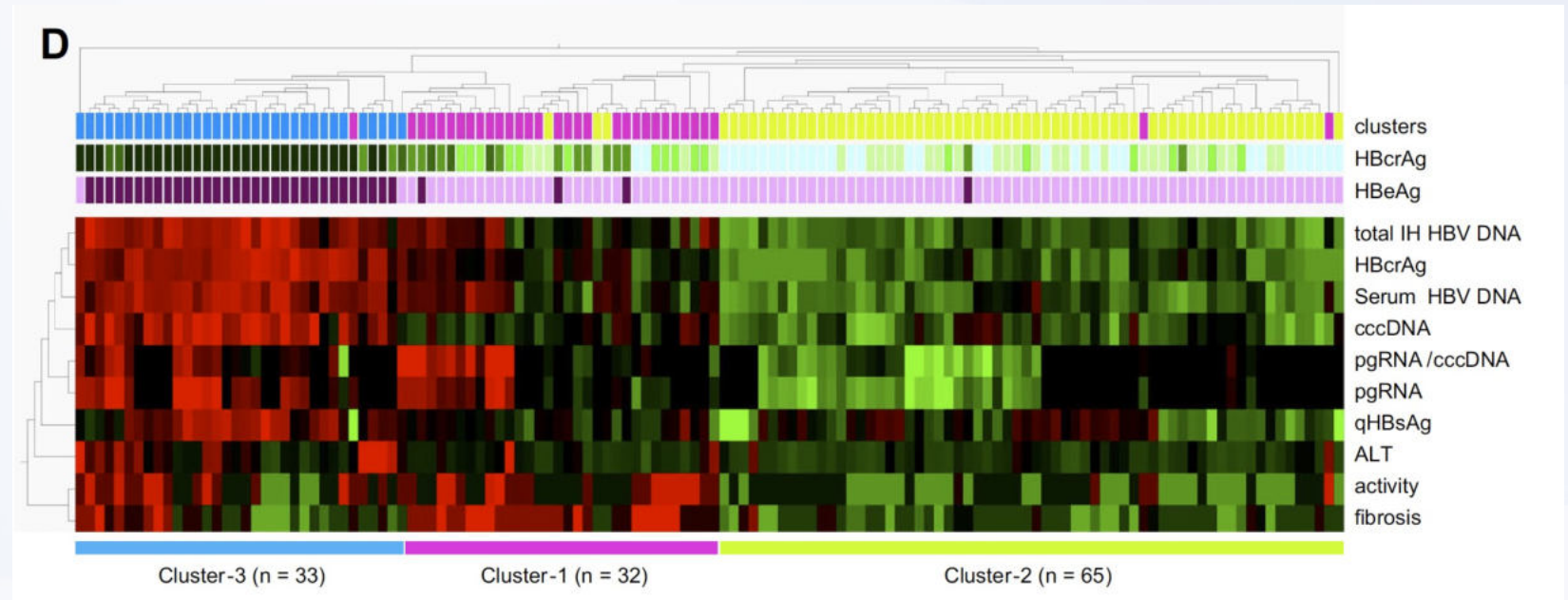
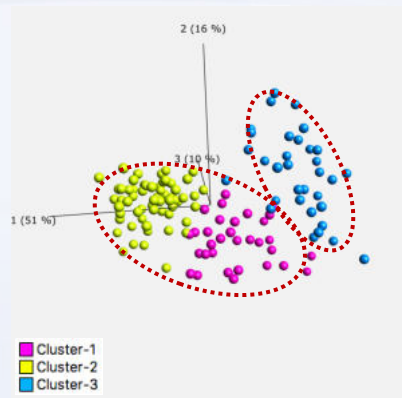
HBcrAg identifies a “highly active” group of HBeAg(-) patients

PCA analysis

HBeAg



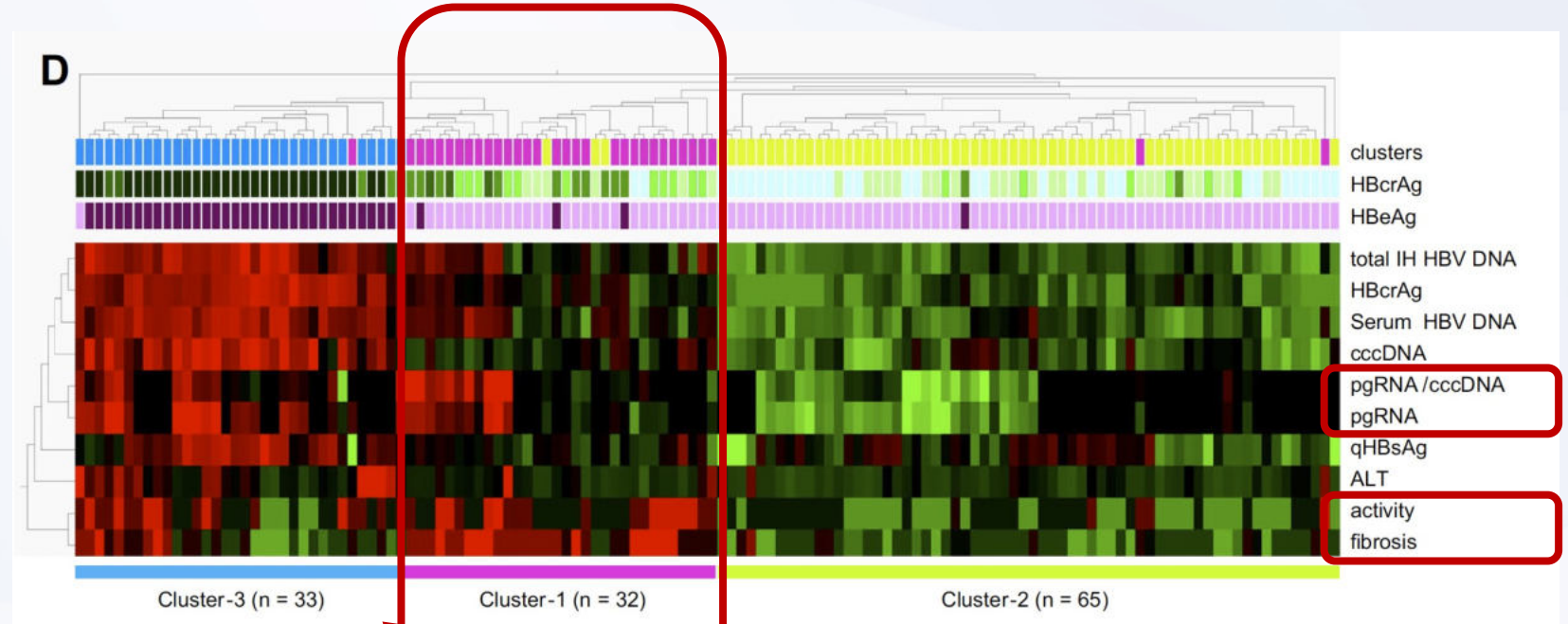
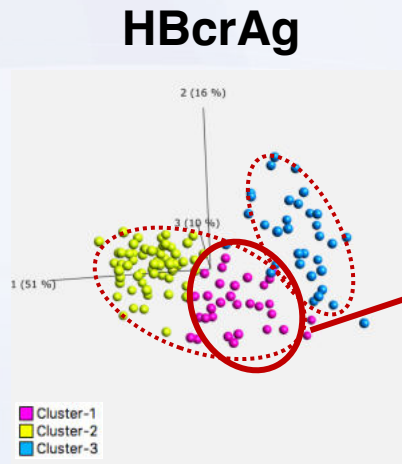
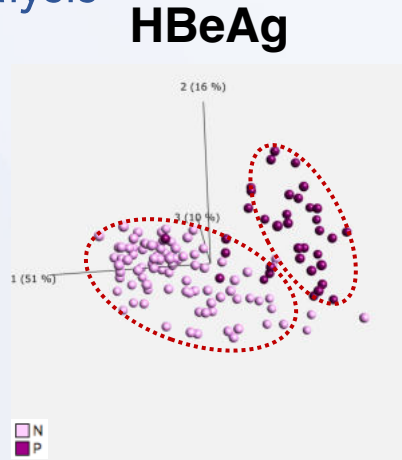
HBcrAg



Testoni, JHepatol 2019

HBcrAg identifies a “highly active” group of HBeAg(-) patients

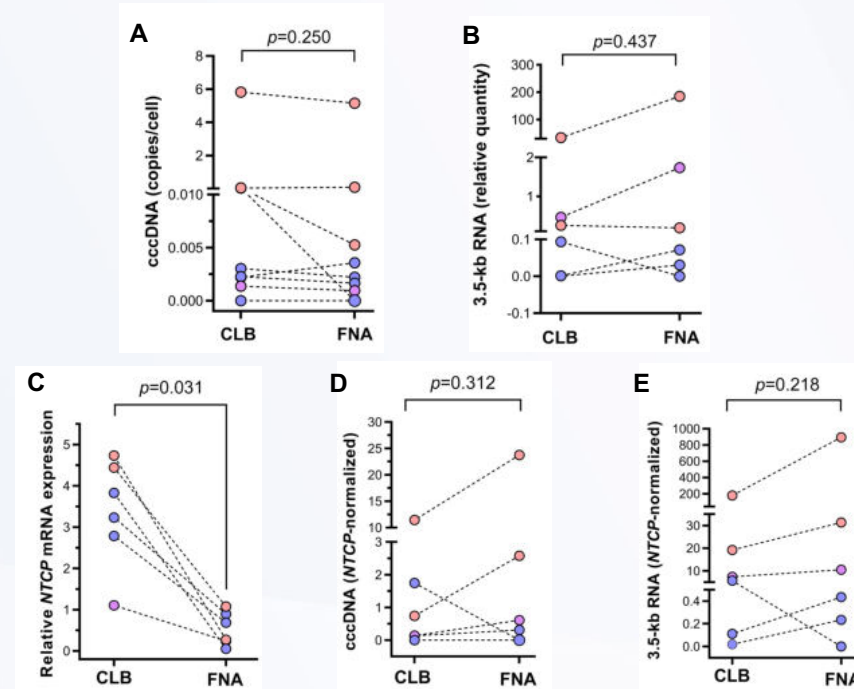
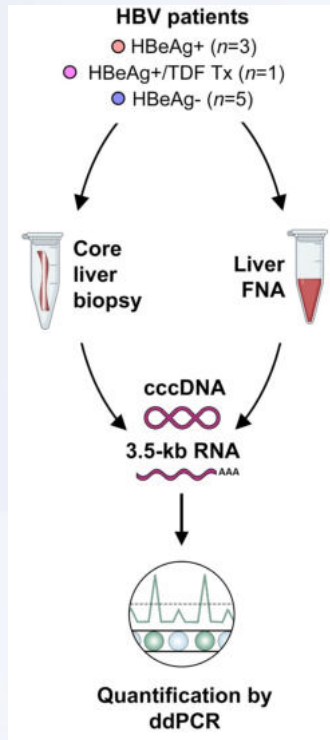
PCA analysis



High cccDNA transcriptional activity
High fibrosis, necroinfl. activity
Could not be identified by serum HBV DNA and HBsAg alone

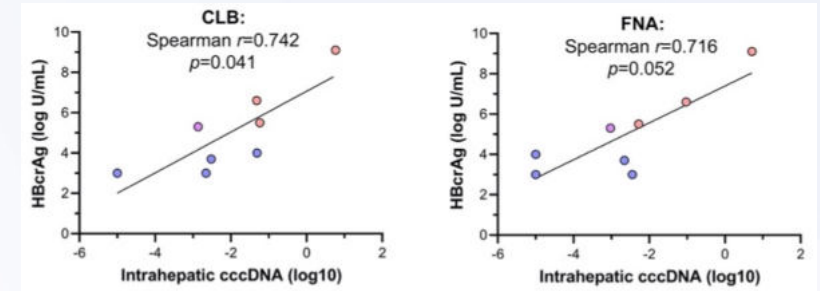
Testoni, JHepatol 2019

cccDNA levels quantified from FNAs correlate with HBcrAg



FNAs allow quantification of intrahepatic HBV markers by ddPCR

cccDNA levels quantified by FNA or CLB show comparable correlation with HBcrAg



Testoni, Roca Suarez*, JHEP Reports, in press*

HBcrAg - summary

Correlates with intrahepatic cccDNA pool and cccDNA transcriptional activity

Might help in discriminating « active » from « inactive » HBeAg(-) patients

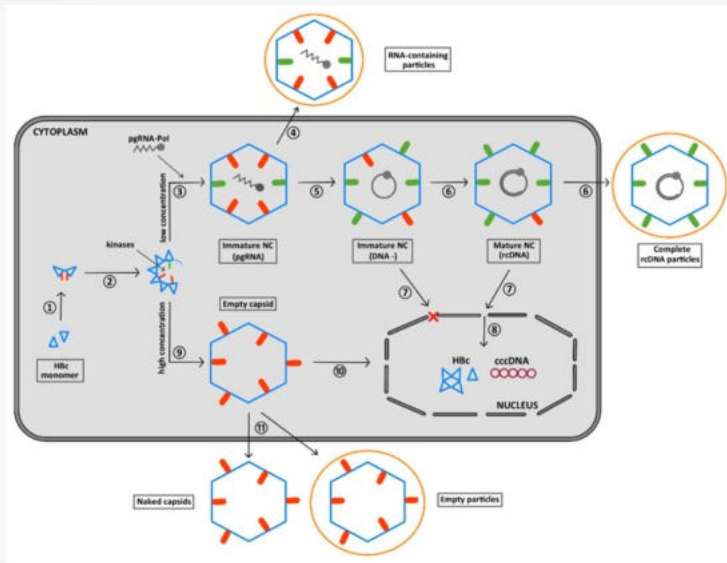
Most of HBcrAg(-) patients still have transcriptionally active cccDNA

Need for better sensitivity?

Limited use in HBeAg(+) patients

Abbott tests to discriminate HBcAg from P-HBcAg

Phosphorylation of HBc-CTD fine tunes interaction with nucleic acids

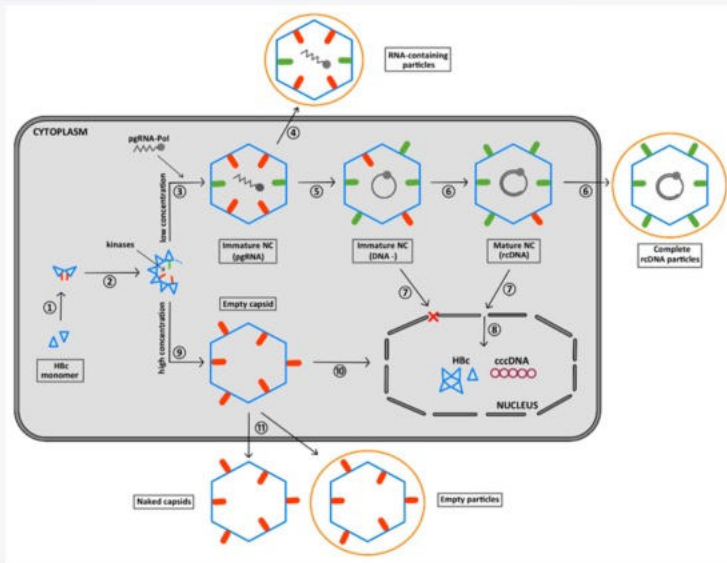


De Rocquigny, Viruses 2020

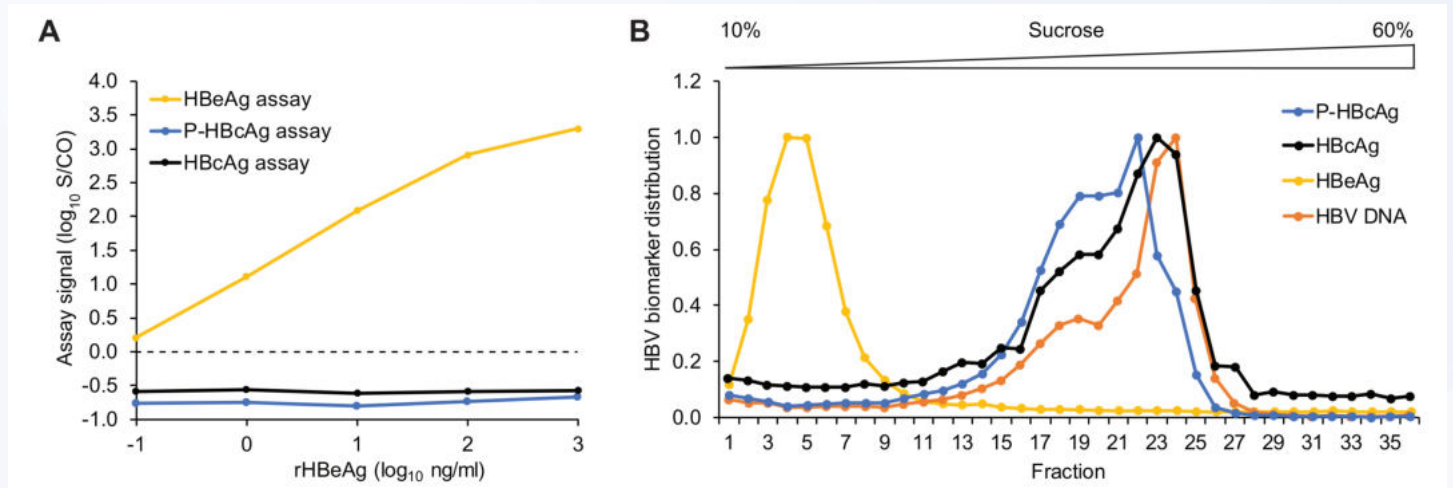
Abbott tests to discriminate HBcAg from P-HBcAg

Phosphorylation of HBc-CTD fine tunes interaction with nucleic acids

Chemiluminescent microparticle automated immunoassays for:
HBcAg (HBV DNA-containing particles)
P-HBcAg, non-HBV DNA-containing particles



De Rocquigny, Viruses 2020



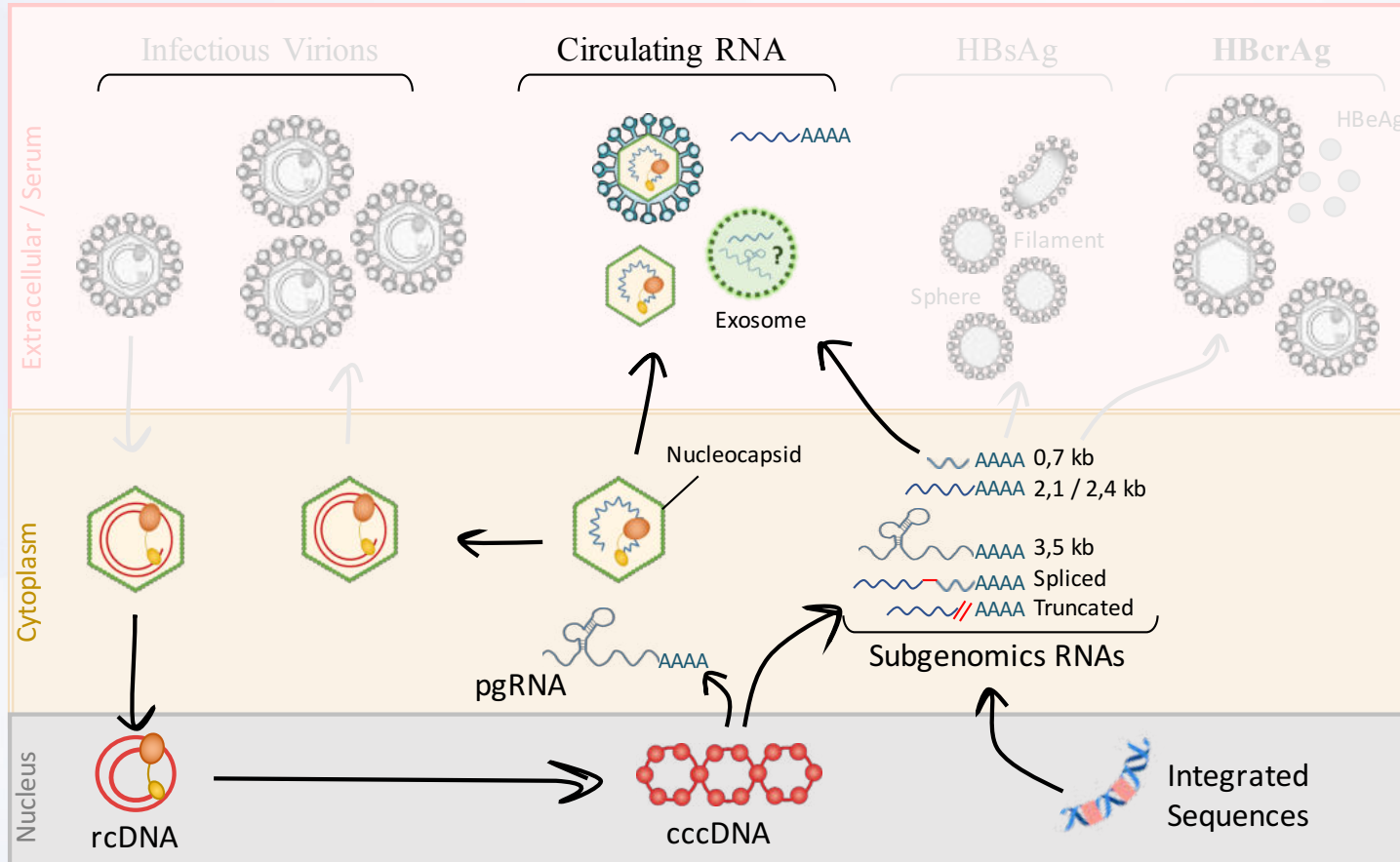
Geissler, J Clin Virol 2023

HBcAg reflects mostly viremia

P-HBcAg can be used to monitor cccDNA levels and transcriptional activity?

P-HBcAg can be used to differentiate classes of CAMs in combination with serum HBV RNA
 (e.g. CAM-E would reduce RNA but not P-HBcAg)

Serum HBV RNA – where do they come from?



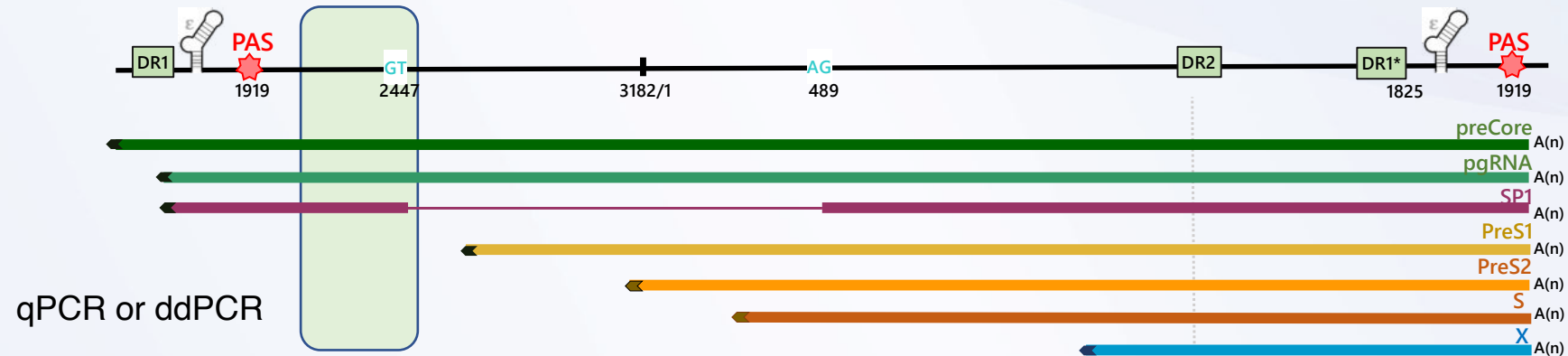
3.5Kb RNA is only transcribed from cccDNA

Encapsidated pgRNA is the predominant form of circulating HBV RNA

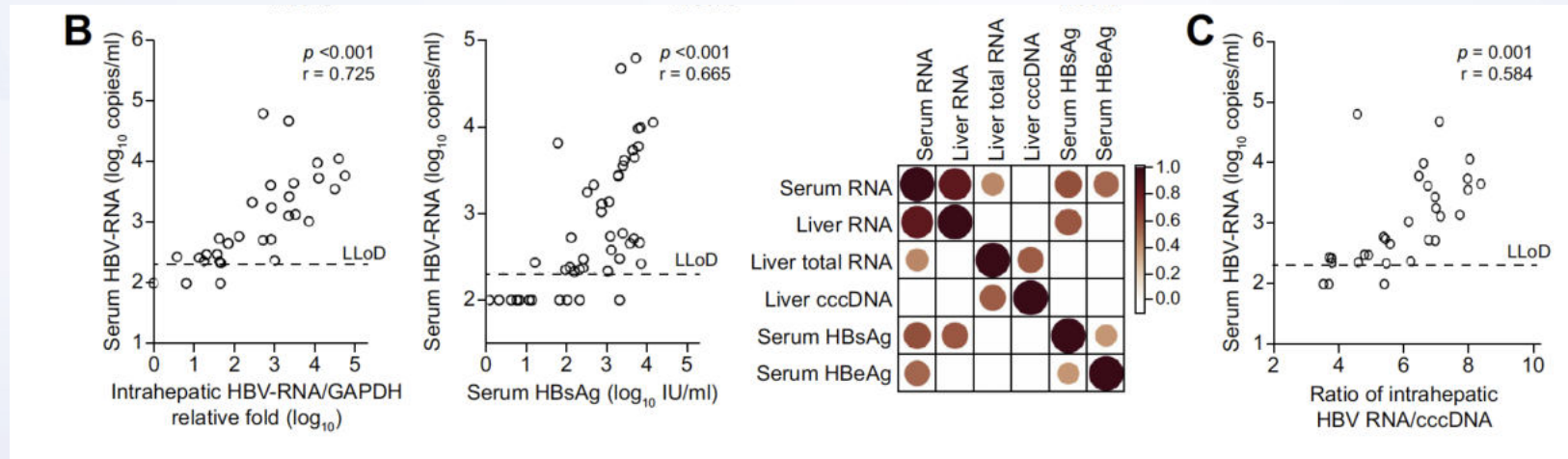
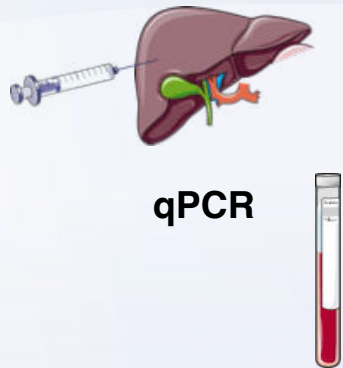
Wang, *JHepatol* 2016
 Jansen, *JID* 2016
 & others

Adapted from Testoni, *Sem Adv Liver Dis* 2017

Serum HBV pgRNA correlates with liver cccDNA transcriptional activity



47 Asian NUC-treated (>1 year) CHB patients

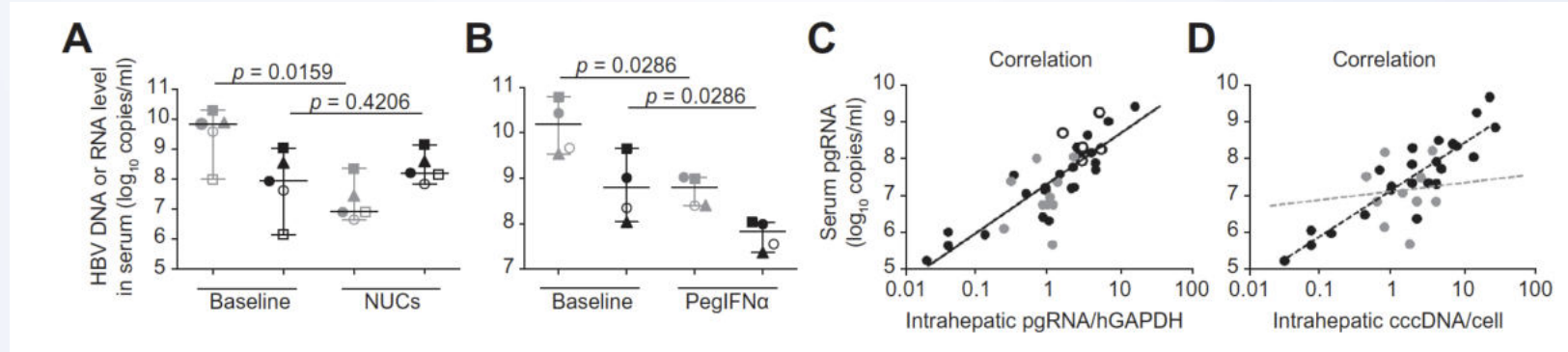


Serum HBV RNA correlate with liver cccDNA transcriptional activity

Hu-Hep mouse model

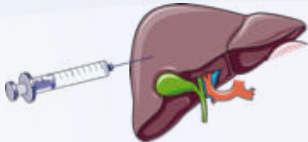


qPCR

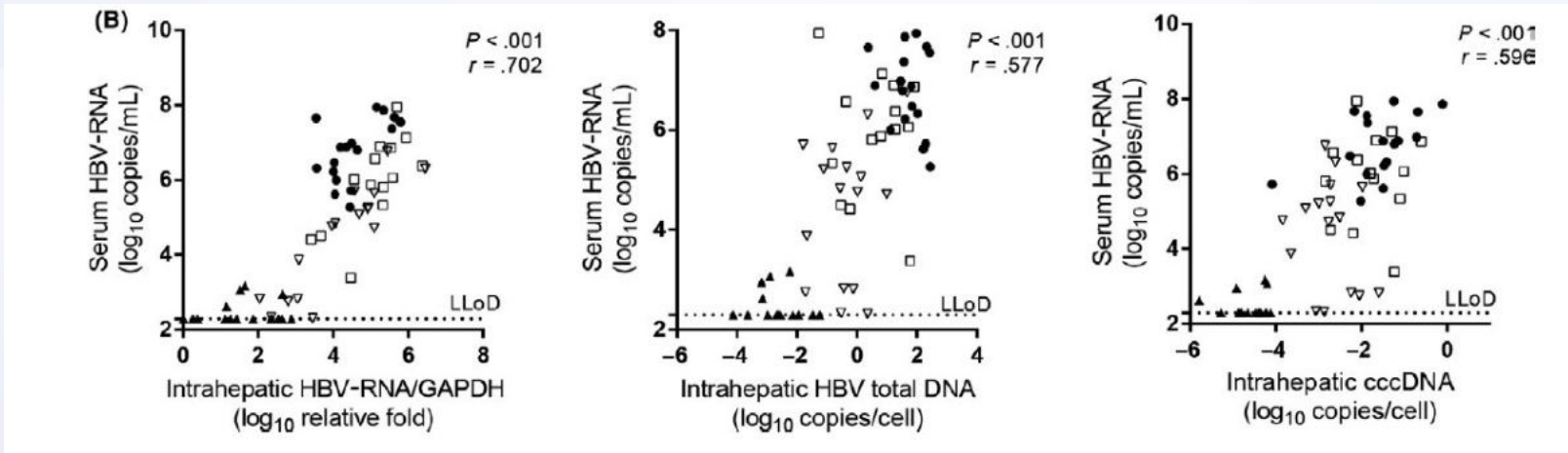


Giersch, *J Hepatol* 2017

102 treatment naïve Asian CHB patients

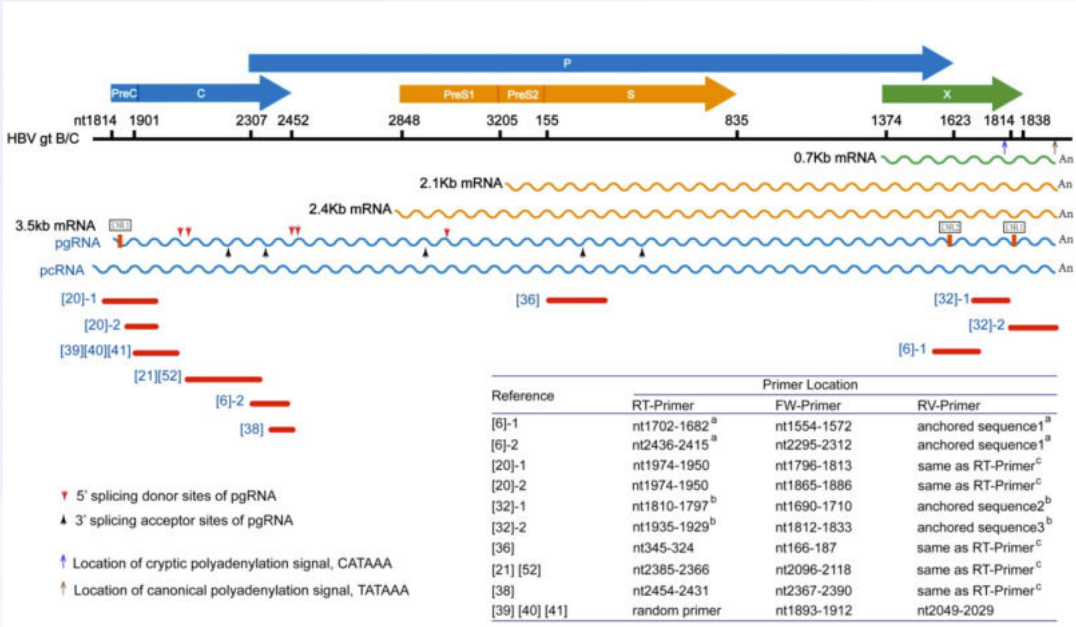


ddPCR



Wang, *JVH* 2018

Complexity of serum HBV RNA and assays for their quantification!



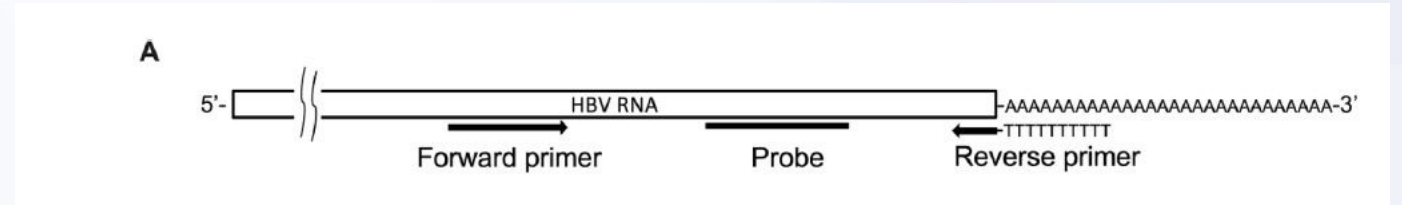
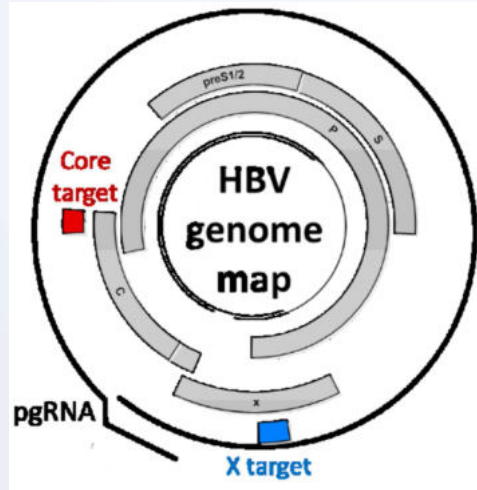
Liu, Hepatology 2018

Table 1 | Methods for quantification of HBV RNA in serum

Method	Details	Reverse transcription primer	Primer sites	LLOQ and LLOD
RT-qPCR	RNA isolation (including DNase treatment) and subsequent PCR method with specific primers either detecting pre-genomic or all HBV RNAs ^{52,76,165,166}	HBV specific	Precore, X, C or S region	2.55 log ₁₀ copies/mL (LLOQ) ¹⁰ ; 1.85 log ₁₀ copies/mL (LLOD) ⁶³ 2.6 log ₁₀ copies/mL (LLOD) ⁷⁵
Droplet digital PCR	Droplet digital PCR ^{53,167,168}	HBV specific	all regions	100 copies/mL = 2 log ₁₀ copies/mL (LLOD) ⁷⁹
3' Rapid amplification of cDNA ends (RACE)-based	Oligo (dT) primer plus a unique artificial anchored sequence to generate cDNA ^{63,64,169}	Oligo(dT) primer	Poly(A) tail	2.6–3.4 log ₁₀ copies/mL (LLOD) ^{80,81}
QuantGene assays	Hybridization-based and via branched DNA signal amplification technology—measurement via luminometer ⁵⁴	NA	X region	NA
Indirect	HBV (DNA + RNA) minus DNA determined by real-time PCR ^{170,171} Serum HBV pgRNA minus HBV pcRNA determined by real-time PCR ¹⁷²	HBV specific	Precore and C region	2.2–2.3 log ₁₀ copies/mL (LLOD) ^{170–172}
Commercial RNA assays (currently research use only)				
Abbott ^a	Serum HBV RNA, real-time PCR ⁷⁴	NA	NA	10 copies/mL (LLOD, V2)
Roche ^{b173}	Serum HBV RNA, real-time PCR	NA	NA	10 copies/mL (LLOQ); 10–10 ⁹ copies/mL (linear range)

Kramvis, Nat Rev Gastroenterol Hepatol 2022

Abbott RealTime RUO assay and cobas® HBV RNA assay



Primers and probes located across 3' end canonical polyadenylation signal (lost in integrated HBV DNA)

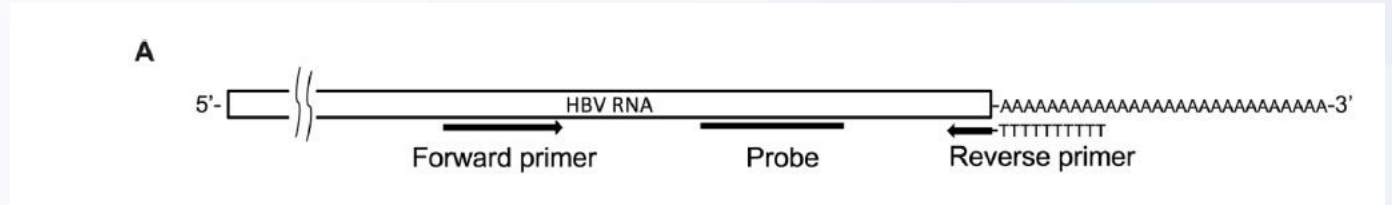
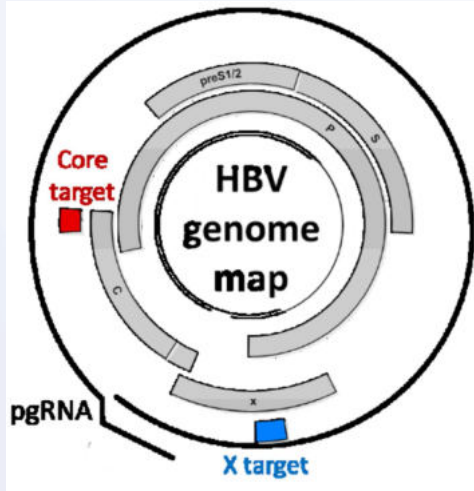
Scholtès, *J Clin Virol* 2022
Jackson, *J Med Virol* 2022

Primers and probes are designed to conserved regions within the 5' end of the X gene and the 3' end of the core gene

Targets are independently detected

Butler, *Hepatology* 2018;
Anderson, *CID* 2021
Anderson, *Hepatol Commun* 2023

Abbott RealTime RUO assay and cobas® HBV RNA assay



Primers and probes located across 3' end canonical polyadenylation signal (lost in integrated HBV DNA)

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Lyon, Dec 7th 2018

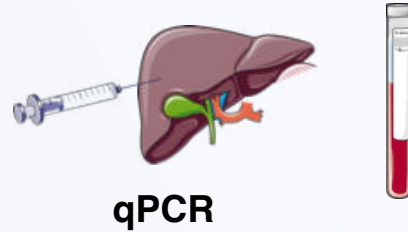


Serum HBV RNA correlates with liver cccDNA transcriptional activity

122 untreated CHB patients

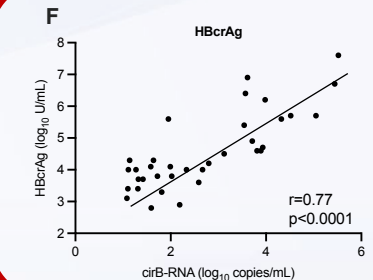
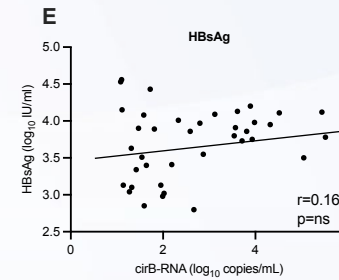
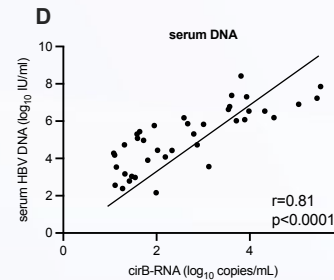
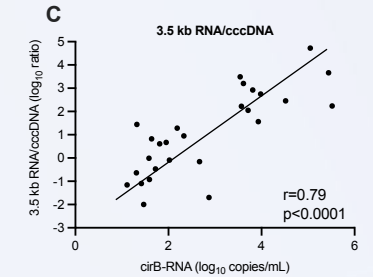
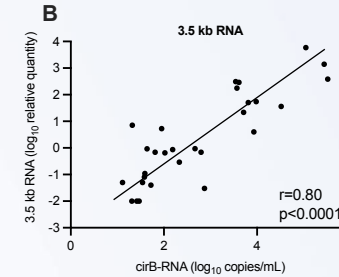
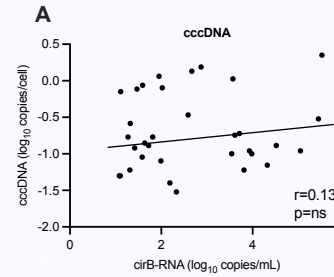
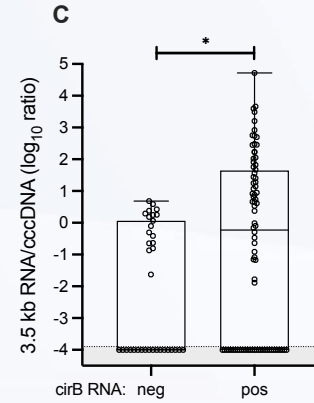
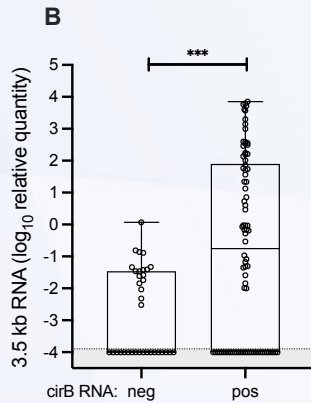
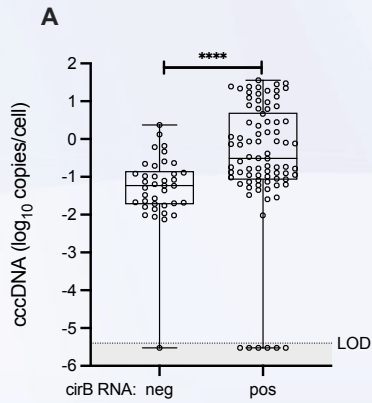
Genotype A/D

Mild fibrosis and necroinfl. activity



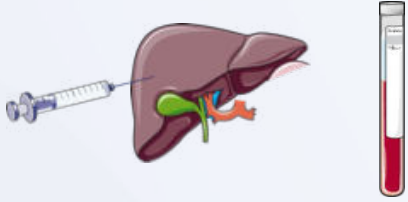
cobas® HBV RNA

89 untreated HBeAg(-) CHB patients



Testoni, in revision

Serum HBV RNA correlates with liver cccDNA transcriptional activity



ddPCR cobas® HBV RNA

30 long term NUC-treated CHB patients
(ECOGREFE French prospective cohort)

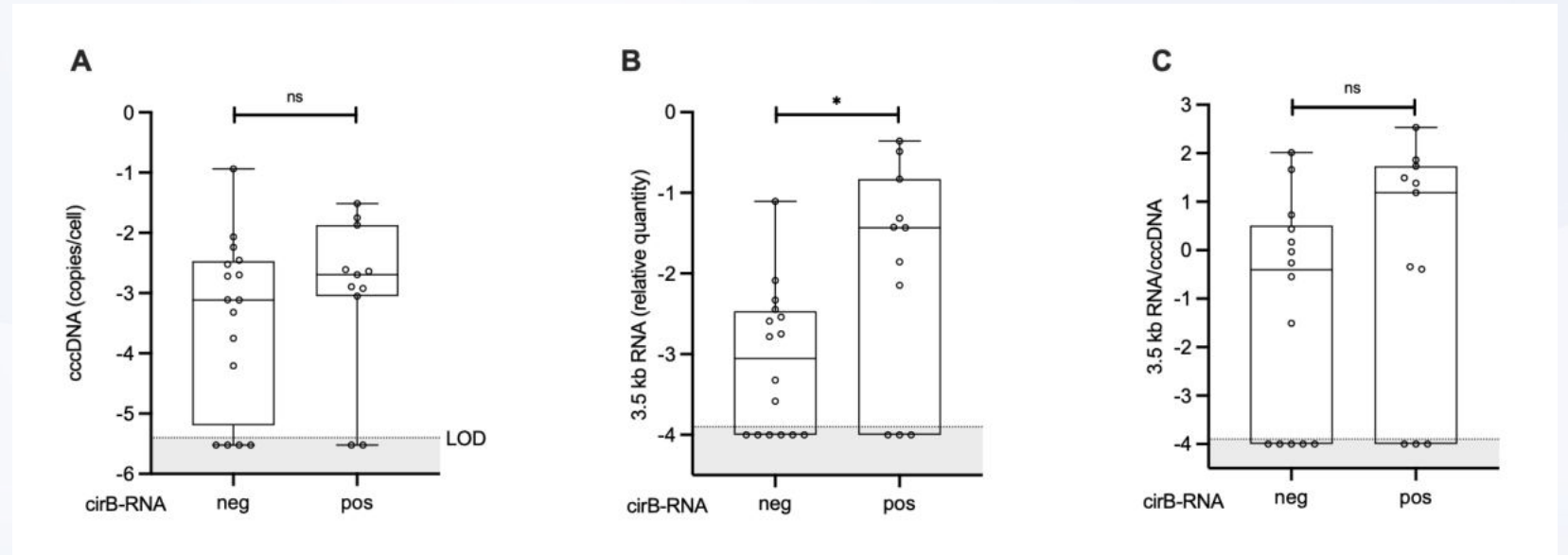
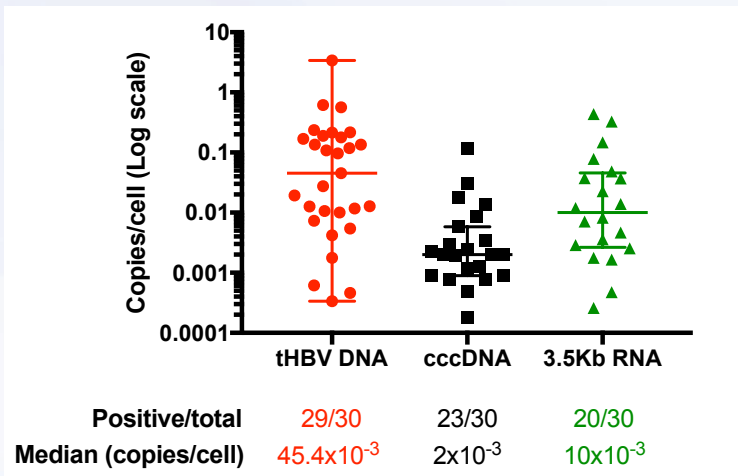
Villeret, *JHEP Reports* 2023

Eligible for liver transplantation (LT)

27/30 HBeAg(-)

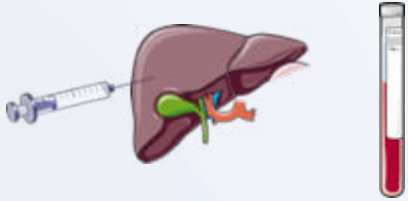
Undetectable viral load

Native liver



Testoni, in revision

Serum HBV RNA correlates with liver cccDNA transcriptional activity



ddPCR

cobas® HBV RNA

90 biopsies from CHB patients w/o liver cancer or cirrhosis

188 liver biopsies from treatment-naïve, chronically infected HBV patients from The Gambia (sub-sahran Africa)

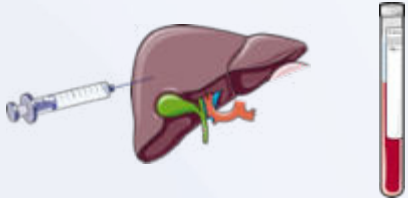
belonging to a subset of the PROLIFICA cohort participants who underwent liver biopsy and histopathological evaluation (Shimakawa et al. 2018)

Mostly **genotype E**
90% men
90% HBeAg(-)

	Median (Q1-Q3)
Age (years)	36 (30-40)
ALT (IU/L)	30 (23-50.3)
VL (log ₁₀ IU/ml)	3 (2.2-4.2)
HBsAg (log ₁₀ IU/ml)	4 (3.5-4.4)



Serum HBV RNA correlates with liver cccDNA transcriptional activity

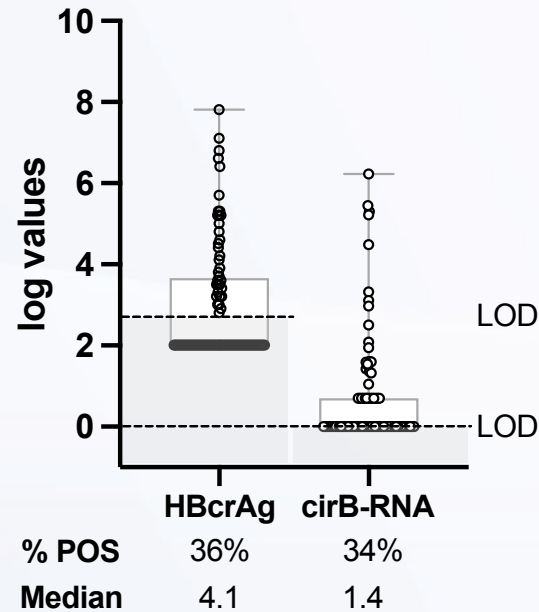
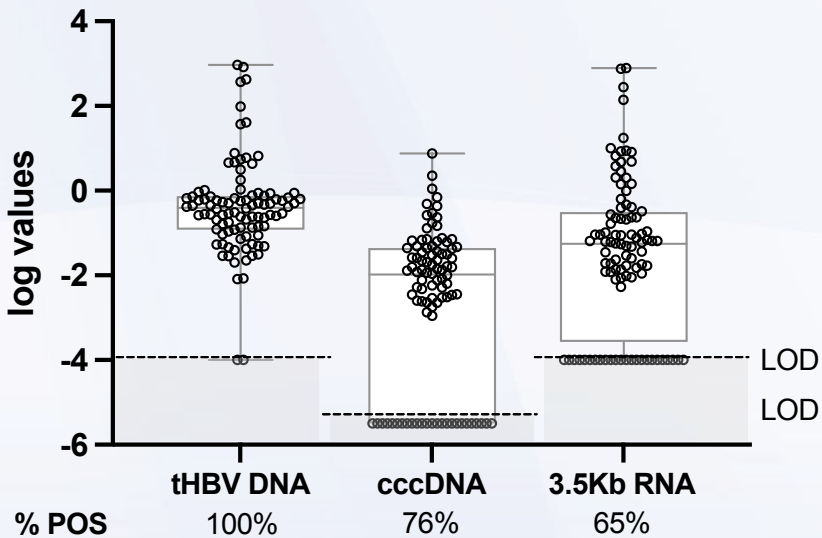


188 liver biopsies from treatment-naïve, chronically infected HBV patients from The Gambia (sub-sahran Africa)

belonging to a subset of the PROLIFICA cohort participants who underwent liver biopsy and histopathological evaluation (Shimakawa et al. 2018)



ddPCR cobas® HBV RNA



	HBcrAg	VL	qHBsAg	cccDNA
CirB-RNA	R=0.9 p<0.0001	R=0.5 p=0.02	R=0.3 p=ns	R=0.6 p=0.02

Spearman's correlation, a threshold = 0.5

Serum HBV RNA - summary

Correlates with intrahepatic cccDNA transcriptional activity in untreated and NUC-treated CHB patients

Interpretation of results may differ according to the assay used for RNA quantification

Standardization required!!!

Conclusions and open questions

HBcrAg and serum HBV RNA promising surrogate markers of cccDNA transcriptional activity, what about their combination?



HBcrAg & serum HBV RNA associated to HBsAg may help stratifying patients for the probability of achieving functional cure and for managing the duration of therapy

Need of knowledge from translational studies coupling intrahepatic and serum markers correlation!!

→ *FNA could allow longitudinal studies of both cccDNA pool/activity and immune correlates*

Biomarkers have to be selected according to the therapeutic strategy:

→ *Target engagement vs cccDNA biomarker!!!*

Acknowledgments



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Dr. Alessandro Loglio

Università degli Studi in Palermo

Prof. Antonio Craxi

Dr. Vito Di Marco

French LT centers:

- Paul Brousse Hospital:
Pr SAMUEL Didier,
Dr ROCHE Bruno
- Montpellier Hospital:
Pr PAGEAUX Georges-Philippe
- Grenoble-Alpes Hospital:
Pr LEROY Vincent
- Nice Hospital:
Pr ANTY Rodolphe

Hepatitis viruses and pathobiology of chronic liver diseases

Pr. Fabien Zoulim

Delphine Bousquet
Guillaume Giraud
Xavier Grand
Sarah Heintz
Doohyun Kim
Hyoseon Tak

TCH

Françoise Berby
Isabelle Bordes

Project Manager

Bernadette Vaz

Epigenetics, microenvironment and liver cancer

Pr. Massimo Levrero

Vincenzo Alfano
Francesca Casuscelli di Tocco
Marie-Laure Plissonnier
Alexia Paturel

Roche Team

Marintha Heil
Aaron Hamilton



Clinic

Caroline Scholtes
Carrie-lynn Newsom



& all the patients!



@TestoniResearch



HBcrAg and intrahepatic cccDNA transcriptional activity

Table 2. Correlations between HBcrAg, qHBsAg, serum HBV-DNA and intrahepatic viral markers.

	Liver markers			
	tHBV-DNA	cccDNA	pgRNA	cccDNA transcriptional activity (pgRNA/cccDNA)
ALL¹				
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 hepatitis² (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 = 0.007	R = 0.18; p = n.s.
HBeAg- chronic hepatitis¹ (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 infection¹ (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 $\alpha = 0.05$.

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

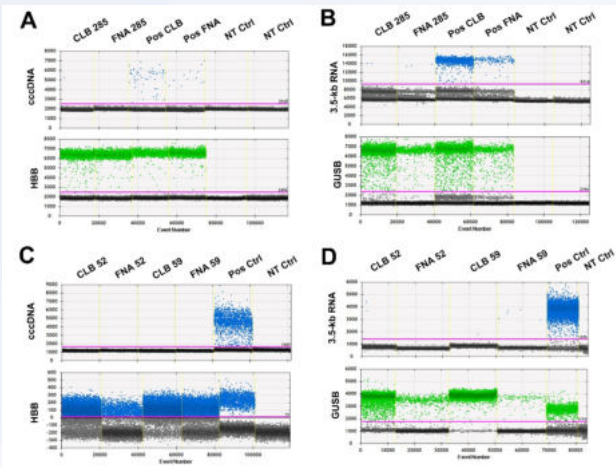
² HBeAg+ chronic infection category was composed by only 4 patients (see Table S1), therefore it was not included in the analysis.

Testoni, *JHepatol* 2019

Table 1. Patients' characteristics

ID	Age	Sex	Ethnic origin	Genotype	serum HBsAg (log IU/mL)	HBeAg	HBeAb	Serum HBV DNA (log IU/mL)	serum HBcrAg (log U/mL)	Serum HBV RNA (log IU/mL)	ALT (IU/L)	Ishak Fibrosis Stage (/6)	EASL category	Additional Notes
259	30	Male	Caucasian – European	D	4.88	Neg	Pos	2.19	3.7	n.d.	55	2	HBeAg- chronic hepatitis	Nil
262	30	Female	Asian – Bangladeshi	n.d.	4.34	Pos	Neg	9.88	9.1	8.8	100	3	HBeAg+ chronic hepatitis	Nil
265	33	Male	Asian – Bangladeshi	D	3.77	Neg	Pos	3.98	3	n.d.	34	1	HBeAg- chronic hepatitis	Nil
267	59	Male	Asian – Bangladeshi	n.d.	2.81	Neg	Pos	3.15	<2	d.n.q.	13	0	HBeAg- chronic infection	Nil
279	35	Male	Asian – Bangladeshi	C	1.41	Pos	Neg	5.16	6.6	3.5	65	3	HBeAg+ chronic hepatitis	Nil
280	35	Male	Asian – Pakistani	n.d.	3.89	Pos	Neg	1.30	5.3	2.8	54	2	HBeAg+ chronic hepatitis	On treatment (TDF)
281	42	Male	Afro Caribbean	n.d.	2.26	Neg	Pos	1.79	3	n.d.	35	1	HBeAg- chronic hepatitis	Nil
283	22	Male	Afro Caribbean	E	4.16	Pos	Neg	5.67	5.5	2.6	25	2	HBeAg+ chronic hepatitis	Nil
284	33	Male	Asian – Bangladeshi	A	4.42	Neg	Pos	4.64	4	1.8	19	1	HBeAg- chronic hepatitis	Nil
285	54	Female	Caucasian	–	–	–	–	–	–	–	69	n.d.	–	AIH; portal-based inflammation
52	67	Female	Caucasian	–	–	–	–	–	–	–	47	1	–	Cytolysis
59	52	Female	Caucasian	–	–	–	–	–	–	–	67	1	–	Cytolysis, steatosis 5%

IH, autoimmune hepatitis; ALT, alanin aminotransferase; d.n.q., detected not quantified; HBcrAg, hepatitis B core-related antigen; HBeAb, hepatitis B e antibody; n.d. not detected; TDF, tenofovir disoproxil fumarate



HBcrAg in resource-limited settings

> J Viral Hepat. 2021 May;28(5):837-843. doi: 10.1111/jvh.13489. Epub 2021 Mar 1.

Analytical validation of hepatitis B core-related antigen (HBcrAg) using dried blood spots (DBS)

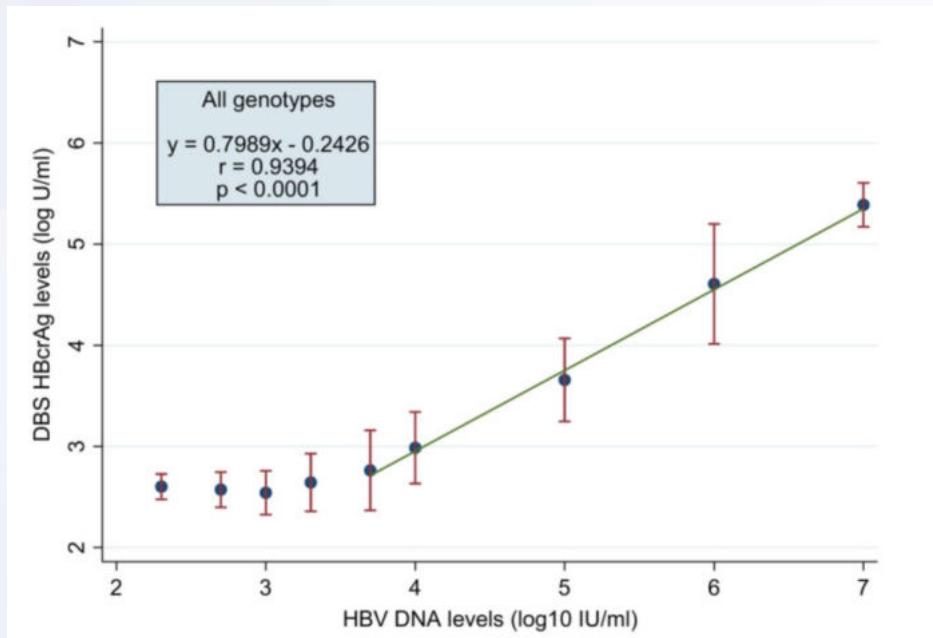
Yusuke Shimakawa ¹, Laura Vernoux ², Audrey Gabassi ³, Séverine Mercier-Delarue ³, Jeanne Perpétue Vincent ¹, François Simon ³, Sarah Maylin ³

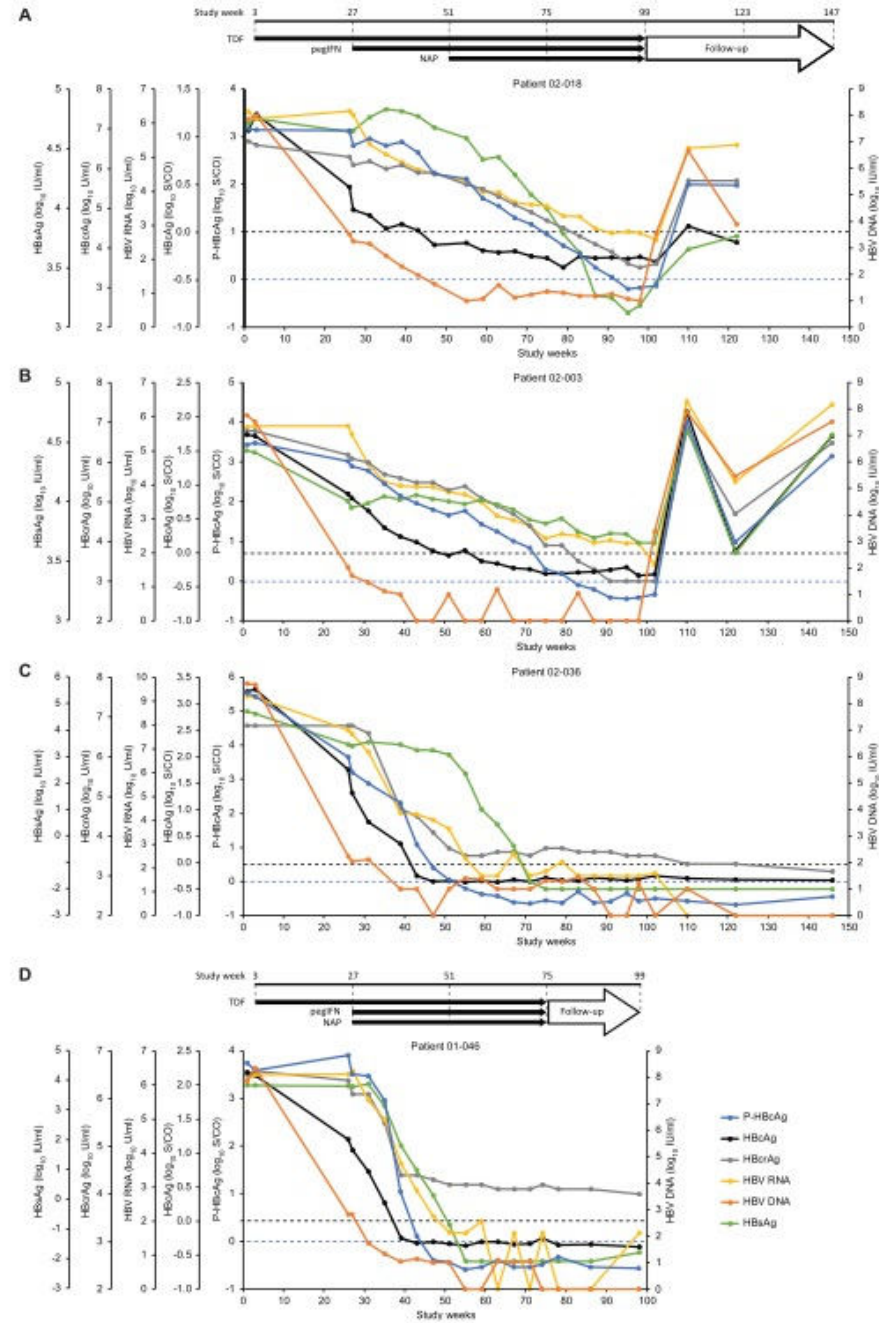
Minimally invasive

No cold chain

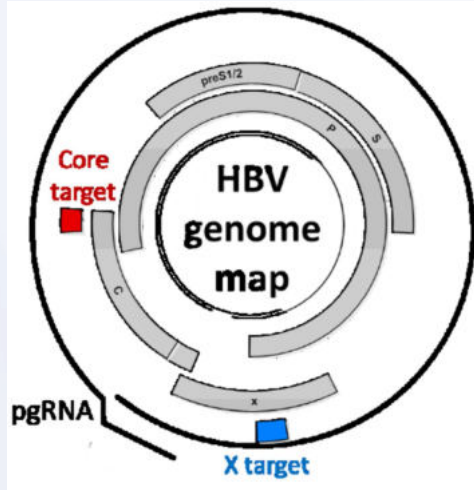
Genotype independent

Identify highly viremic individuals who need antiviral therapy – prevent mother to child transmission





Abbott RealTime 0.2 mL HBV RNA Research Use Only (RUO) assay



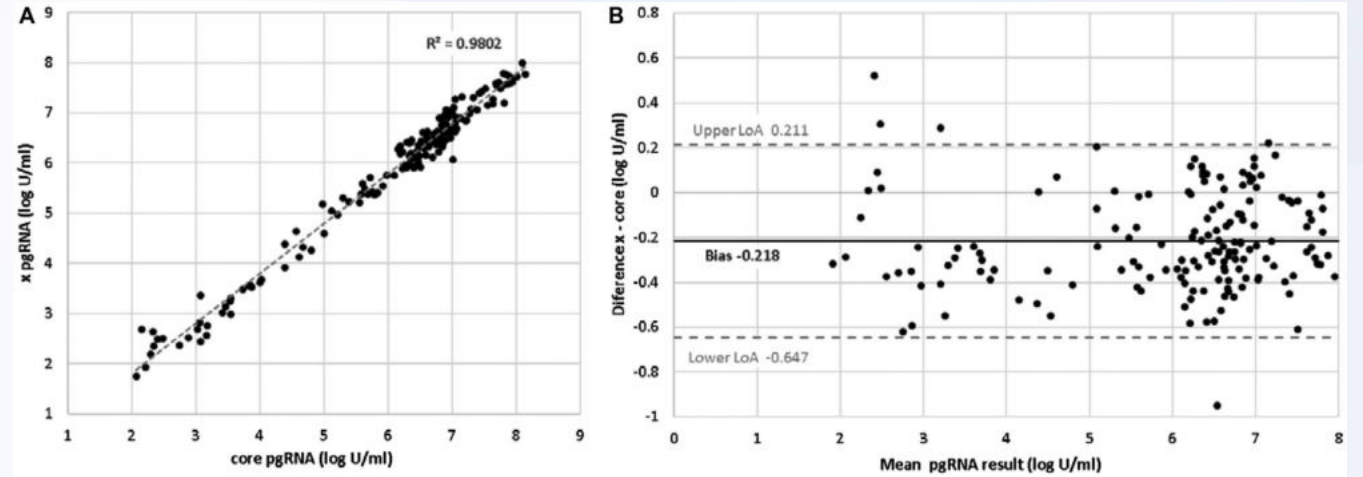
Primers and probes are designed to conserved regions within the 5' end of the X gene and the 3' end of the core gene

Targets are independently detected

Genotype independent

LOD X target 1.65 log U/mL and
Core target 1.67 log U/mL

Calibration of the HBV RNA assay was established using DNA-extracted HBV DNA secondary standards
1 U RNA equivalent to 1 IU of HBV DNA



results for X and core target detection among the samples tested were comparable

26 NUC-treated and 102 untreated CHB patients

longitudinal cohort of 684 individual patients (n = 1827 samples) with baseline and NA treatment time points

*Butler, Hepatology 2018;
Anderson, CID 2021*

Comparison between v1 and modified v2 assays showed increased sensitivity from 152 copies/mL with v1 to 10 (0.6 mL) and 22 (0.2 mL) copies/mL with v2, respectively (*Anderson, Hepatology Comm 2023*)