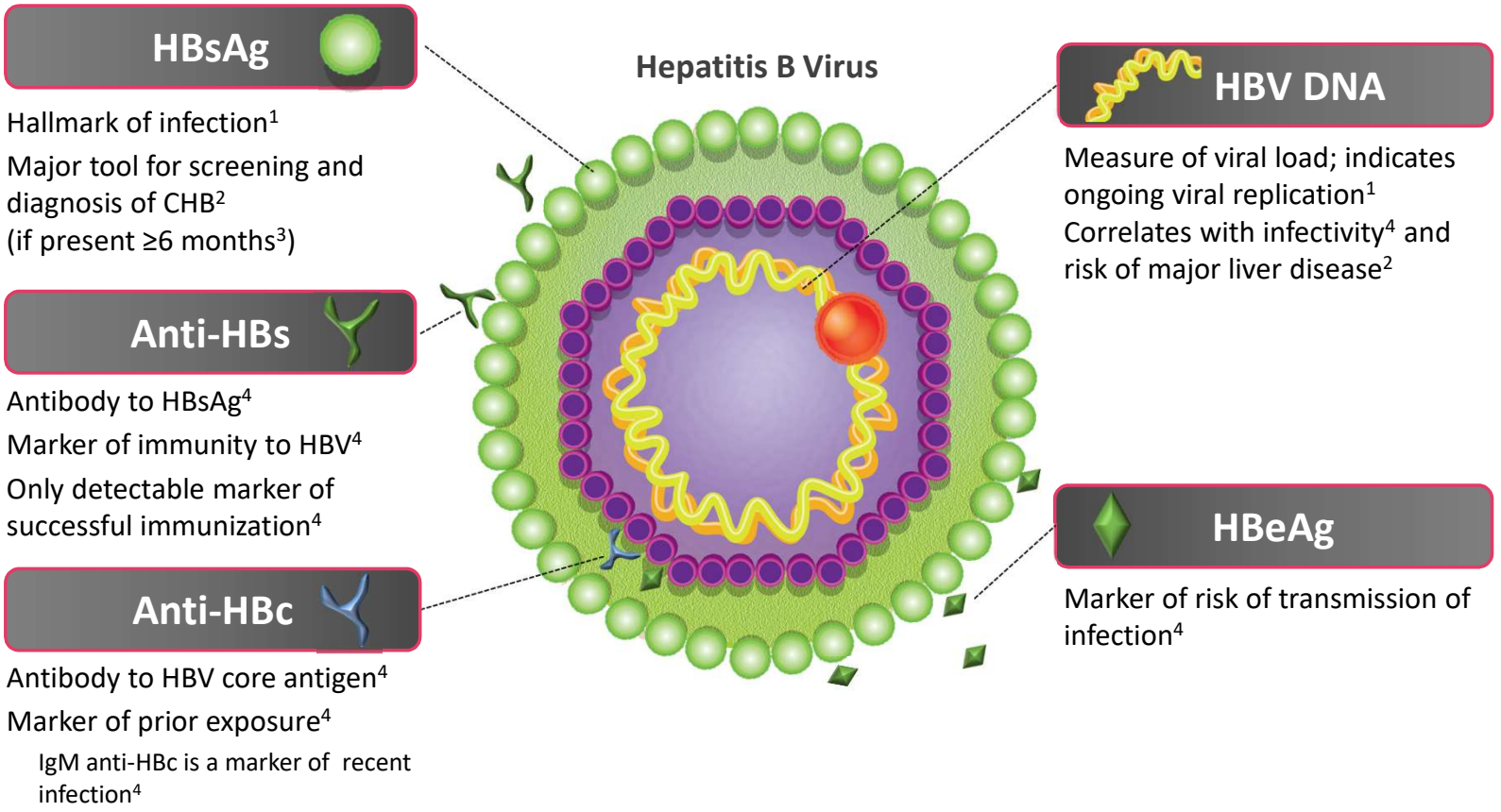
New non-invasive markers of HBV replication and transcription

Ivana Carey

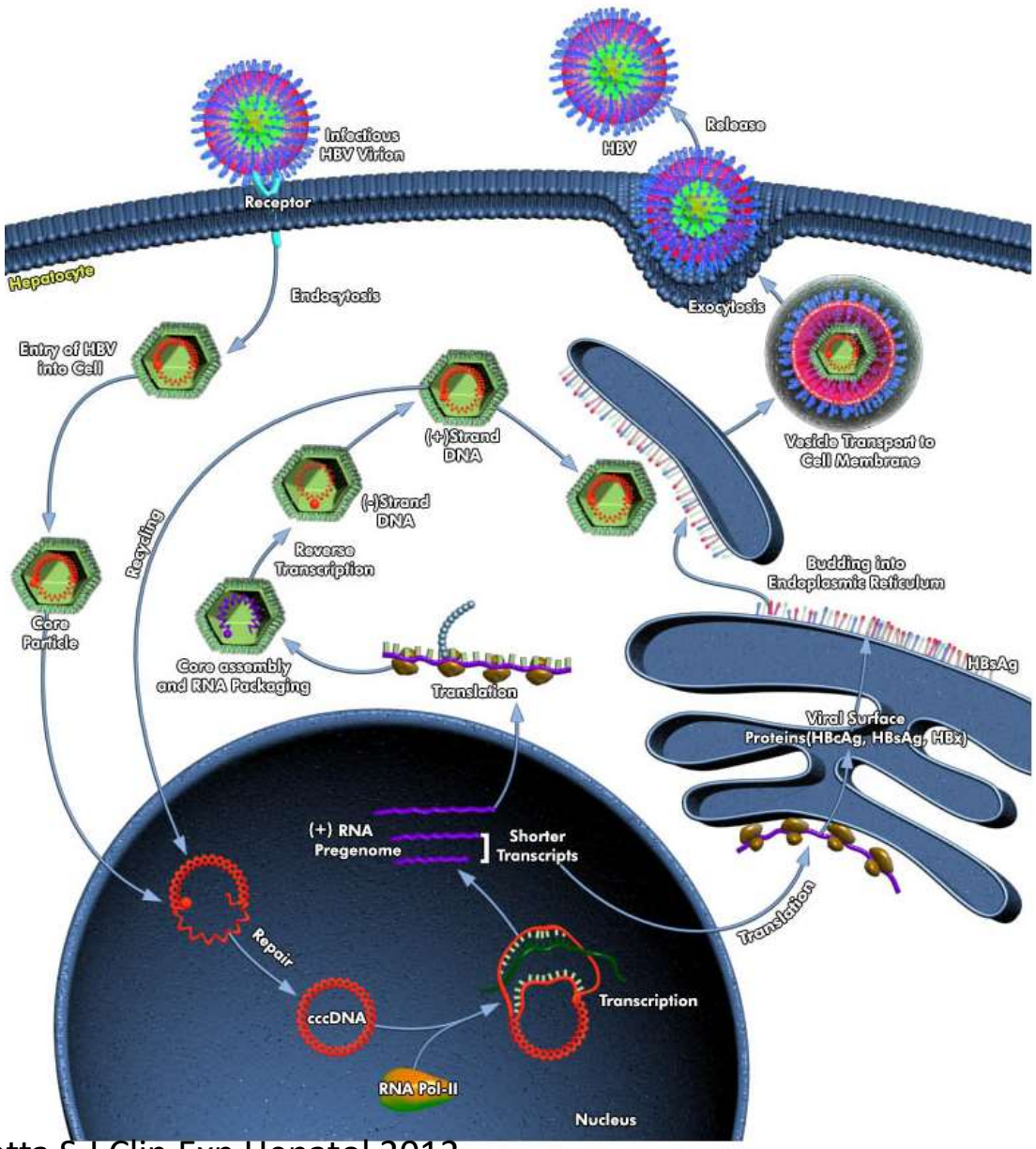
King's College Hospital London

Serologic Markers of HBV Infection



anti-HBs=antibody to HBsAg; anti-HBc=antibody to hepatitis B core antigen; IgM=immunoglobulin M.

1. Trepo C, et al. *Lancet.* 2014;384:2053-2063. 2. Niederau C. *World J Gastroenterol.* 2014;20:11595-11617. 3. CDC. *Morb Mortal Wkly Rep.* 2008;57:1-20;
4. Kao JH. *Expert Rev Gastroenterol Hepatol.* 2008;2:553-562.



Datta S J Clin Exp Hepatol 2012

What is currently measured from HBV replication cycle in circulation?

HBV DNA

- ✓ Viral DNA circulating in blood

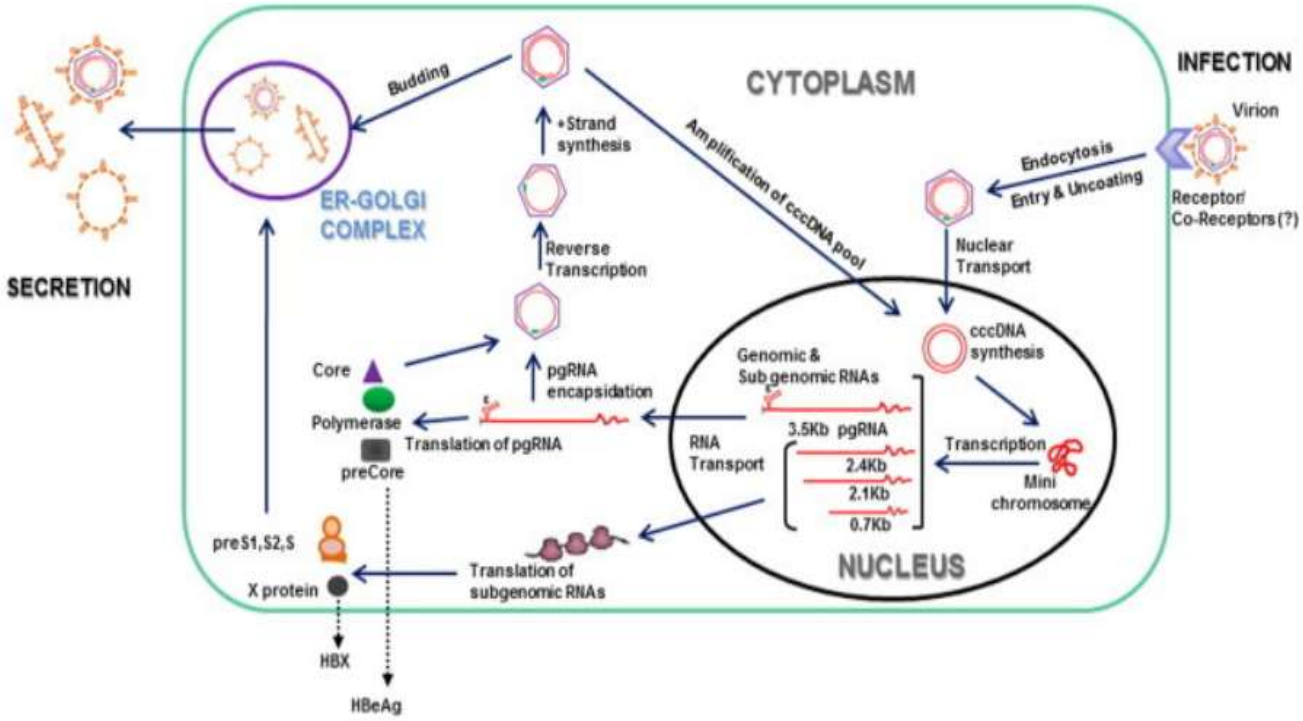
HBeAg status

- ✓ Viral antigen via pre-genomic RNA from cccDNA -requires wild type pre-core or basal core promoter (BCP) region sequence

HBsAg level

- ✓ Viral antigen – via sub-genomic RNA from cccDNA, but as well integrated DNA

What could be newly measured from HBV replication cycle in circulation?



Datta S J Clin Exp Hepatol 2012

HBcrAg

- ✓ Pre-core/core proteins via pre-genomic RNA from cccDNA

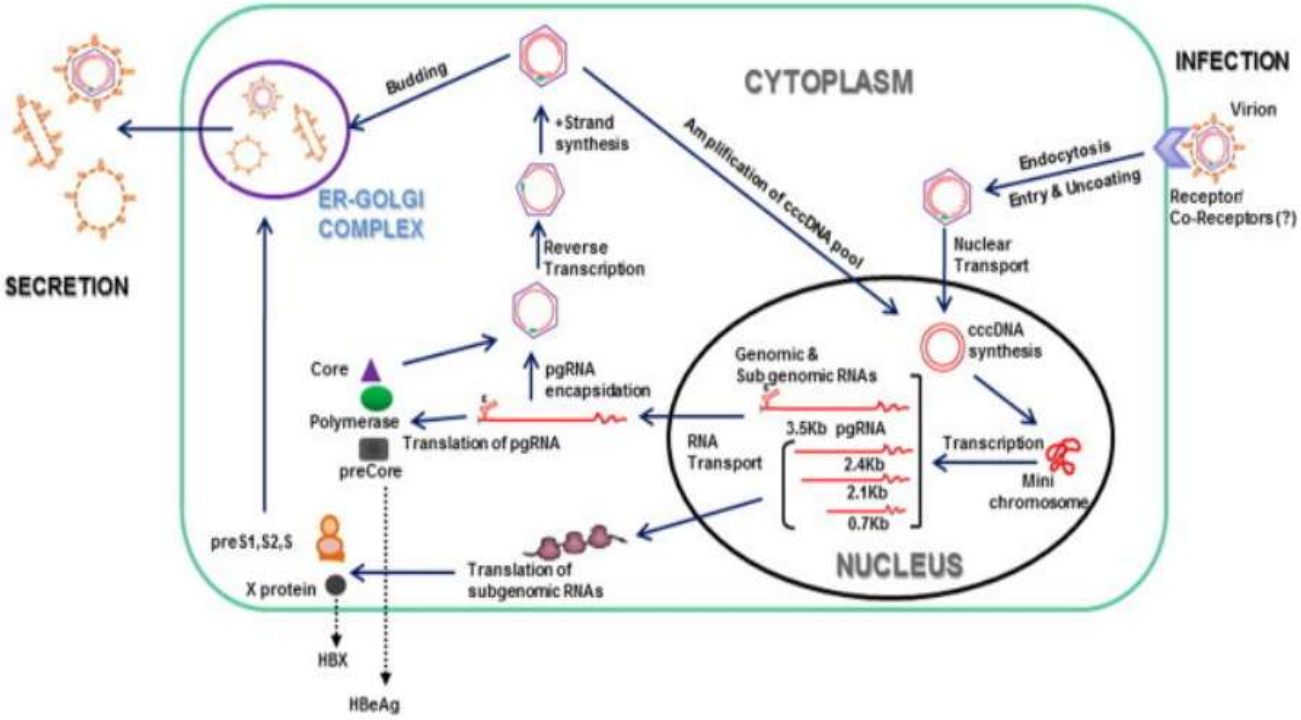
HBsAg protein fragments

- ✓ Sub-genomic RNA contains pre-S1/S2 and S mRNA and serves as a template for production of large, medium and small S proteins

HBV RNA

- ✓ Pre-genomic RNA (viral, core, pre-core, polymerase)
- ✓ Sub-genomic RNA (X and pre-S/S)

What could be measured from HBV replication cycle from the liver?



Datta S J Clin Exp Hepatol 2012

HBsAg expression in the liver

- ✓ In ER within cytoplasm – ground glass

HBV core antigen expression

- ✓ Nuclear and cytoplasmatic expression

Relaxed circular HBV DNA

- ✓ HBV DNA within cytoplasm and nuceleus

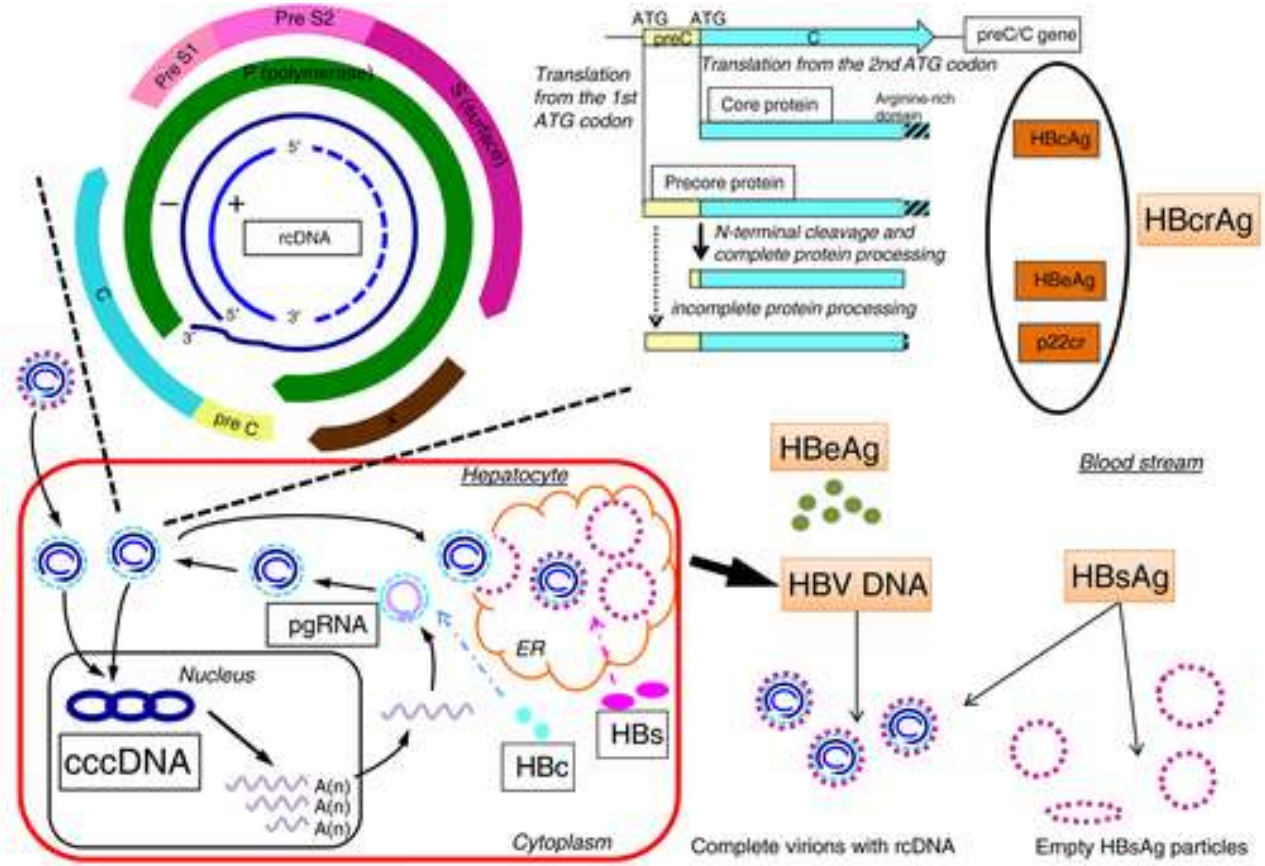
Covalently closed circular DNA

- ✓ Viral DNA within nucleus

HBV RNA

- ✓ Viral RNA within cytoplasm

Hepatitis B core-related antigen (HBcrAg)



cccDNA = covalently closed circular DNA, ER = endoplasmic reticulum, HBc = hepatitis B core protein, HBcrAg = hepatitis B core-related antigen, HBeAg = hepatitis B e antigen, HBs = hepatitis B surface protein, HBsAg = hepatitis B virus surface antigen, HBV = hepatitis B virus, p22cr = truncated 22kDa precore protein, pgRNA = pre-genomic RNA, rcDNA = relaxed circular DNA

HBcrAg

Originates from pre-genomic RNA pre-core/core gene

Composite of 3 related proteins

- HBcAg
- HBeAg
- p22cr – truncated

Mak L-Y AP&T 2017

HBcrAg assay performance

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

CLIA- Chemiluminescence-immunoassay

Mak L-Y AP&T 2017

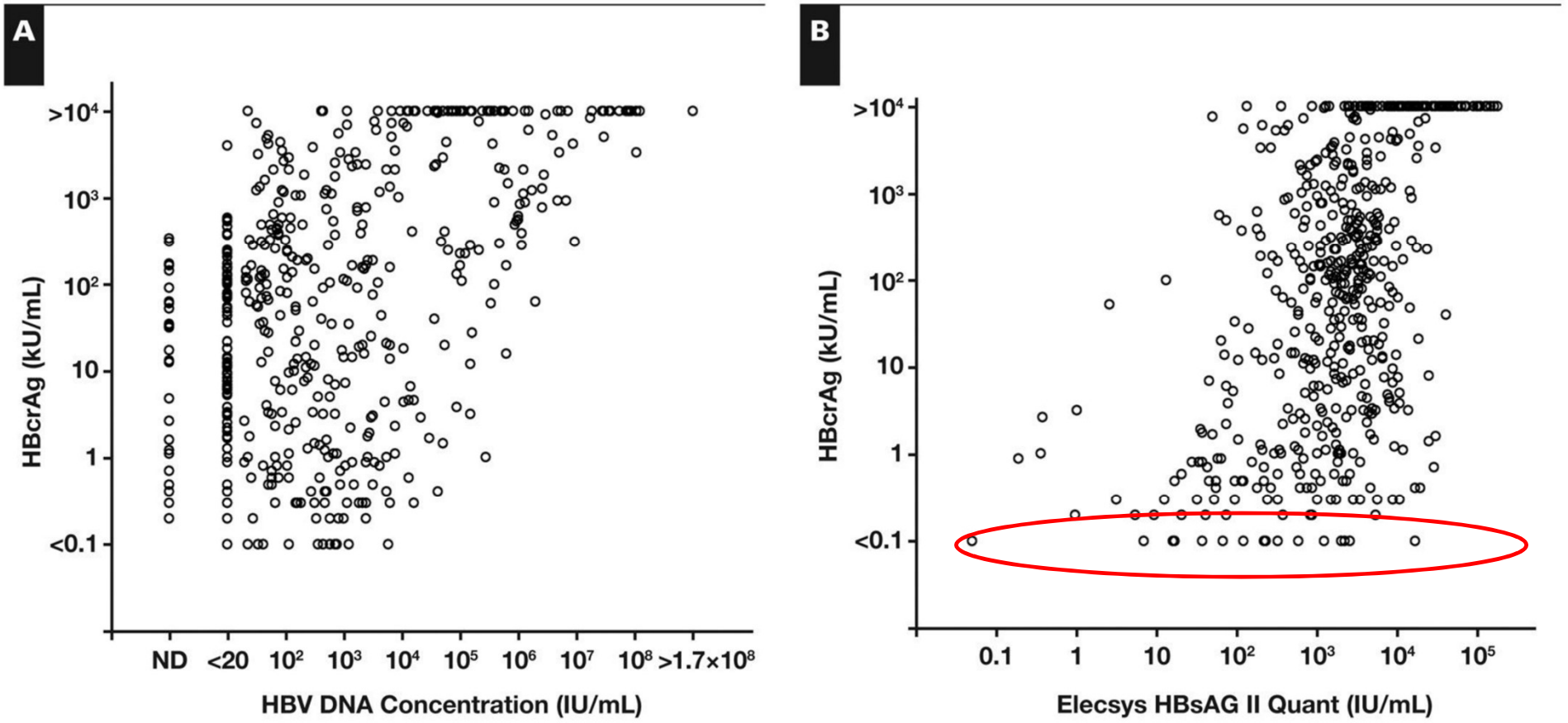
Clinical utility of HBcrAg

HBcrAg serum levels

- Reflecting HBV cccDNA transcriptional activity in hepatocytes[1]
- Additional tool for monitoring NUC treatment and predicting therapeutic efficacy[2]
- Predicting natural course of disease (HBeAg negative infection vs. hepatitis stages differentiation, HCC risk, HBeAg seroconversion)[3,4,5]

1. Wong J Clin Microbiol 2007;
2. Tanaka E. Hepatol Res 2012;
3. Maasoumy B Clin Microbiol Infect 2015;
4. Tada T J Hepatol 2016
5. Wang B. J Viral Hepat 2018

HBcrAg assay performance
(n=529 samples, 98% genotype C2)

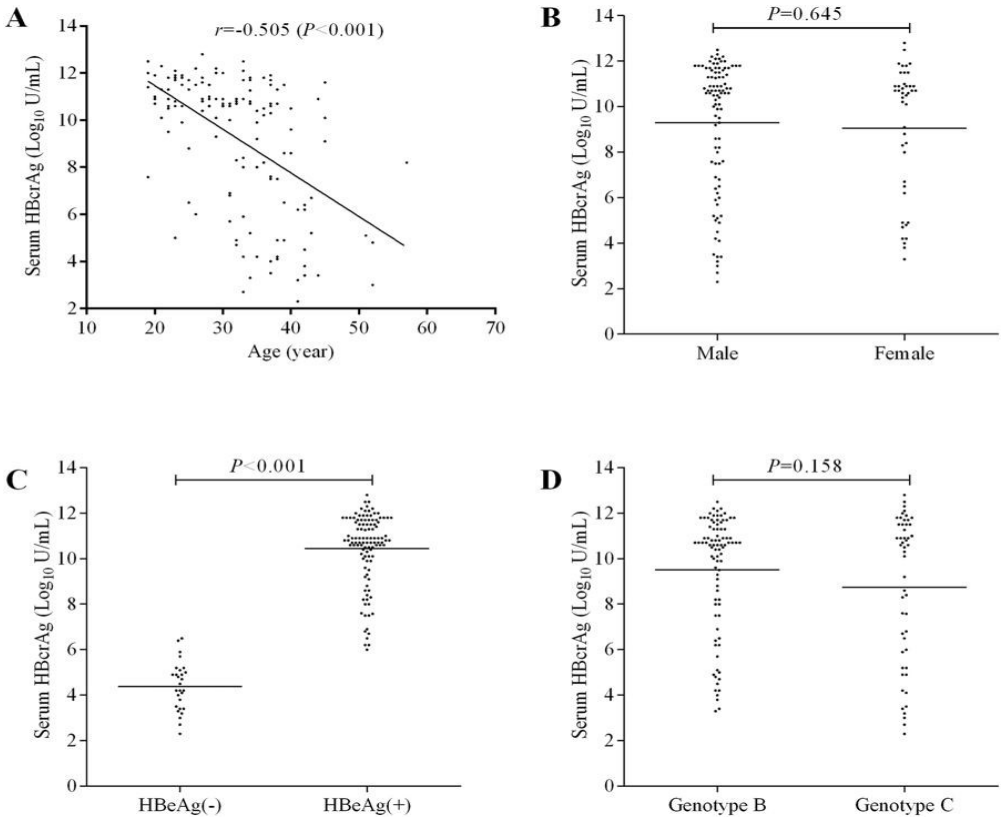


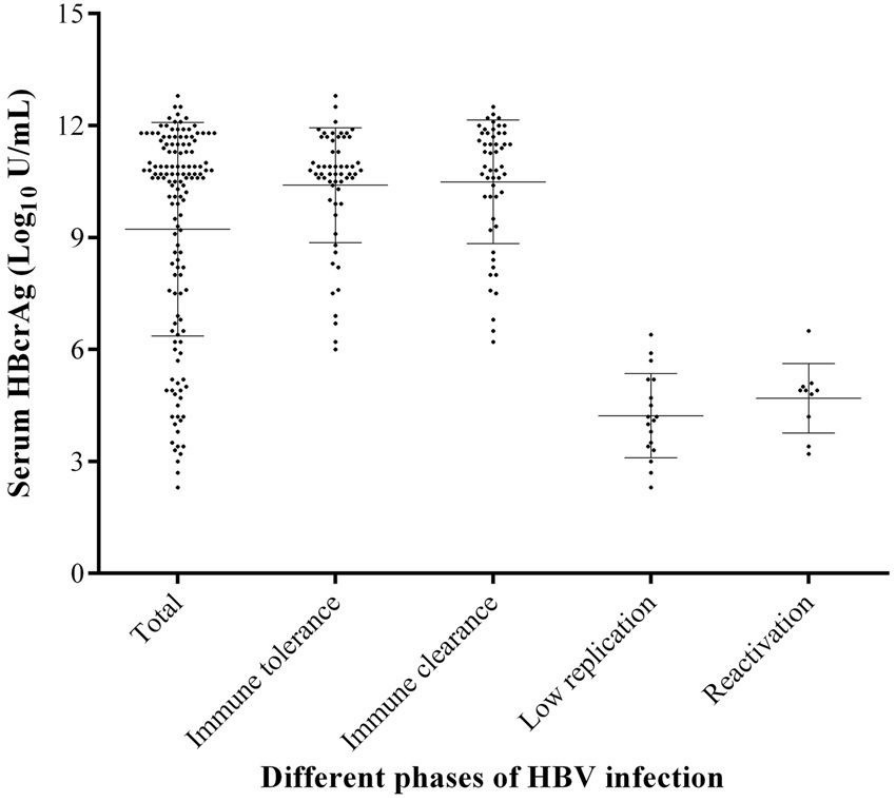
r=0.5198

r=0.5164

Park Y Am J Clin Pathol 2012

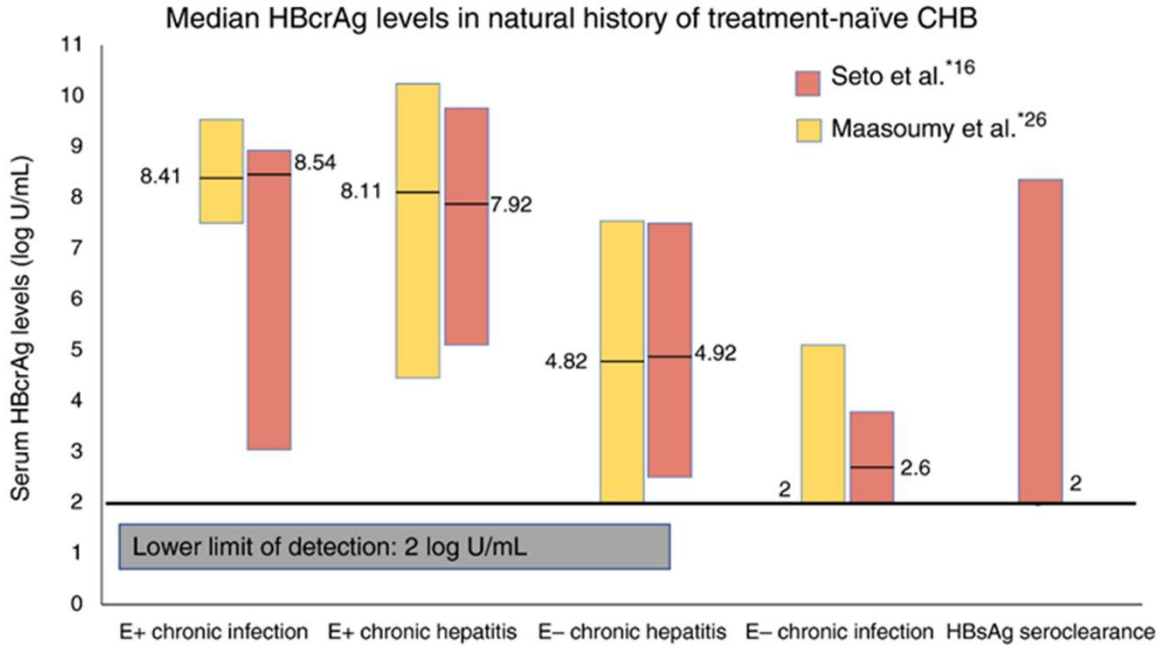
HBcrAg varies between disease stages





Chen E-Q Scientific Reports 2015

HBcrAg varies between disease stages



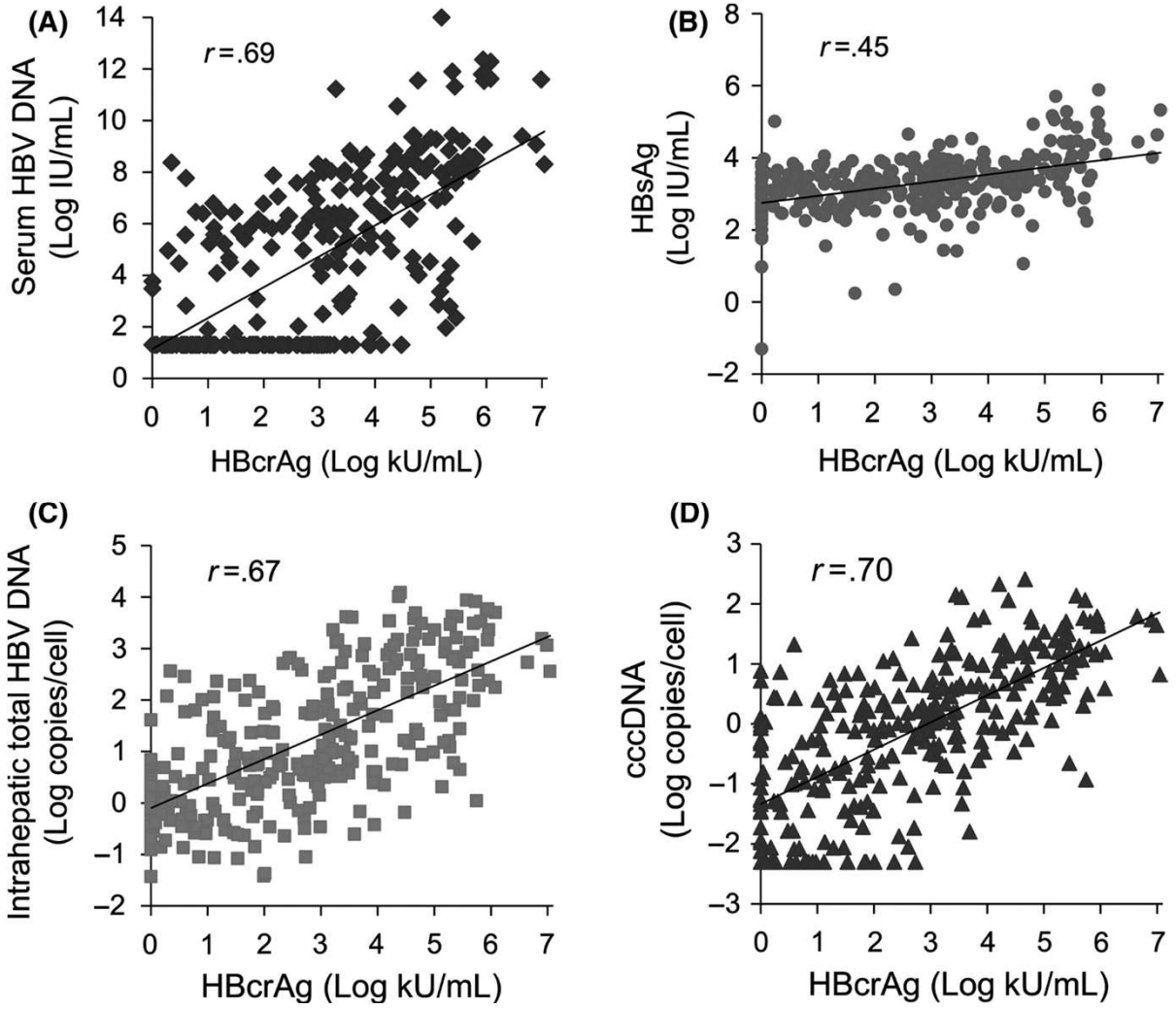
Correlation coefficients of HBV DNA, HBsAg and HBV RNA with HBcrAg					
	E+chronic infection	E+ chronic hepatitis	E– chronic hepatitis	E– chronic hepatitis	Ref.
HBV DNA	$r = .369$ ($P = .007$)	$r = .484$ ($P < .001$)	$r = .537$ ($P < .001$)	$r = .472$ ($P < .001$)	16
	$r = .45$ ($P = .013$)	$r = .66$ ($P < .0001$)	$r = .74$ ($P < .0001$)	$r = .18$ ($P = .054$)	26
HBsAg	$r = .286$ ($P = .040$)	$r = .406$ ($P = .017$)	$r = .245$ ($P < .001$)	$r = .388$ ($P < .001$)	16
	$r = .47$ ($P = .0095$)	$r = .53$ ($P < .0001$)	$r = .40$ ($P = .0045$)	$r = .47$ ($P < .0001$)	26

CHB = chronic hepatitis B infection, E+ = Hepatitis B e antigen positive, E– = Hepatitis B e antigen negative, HBcrAg = Hepatitis B virus core-related antigen, HBsAg = Hepatitis B surface antigen * References of corresponding findings linked to reference list of main text. The bars represent the range of values.

Seto WK Clin Microbiol Infect 2014

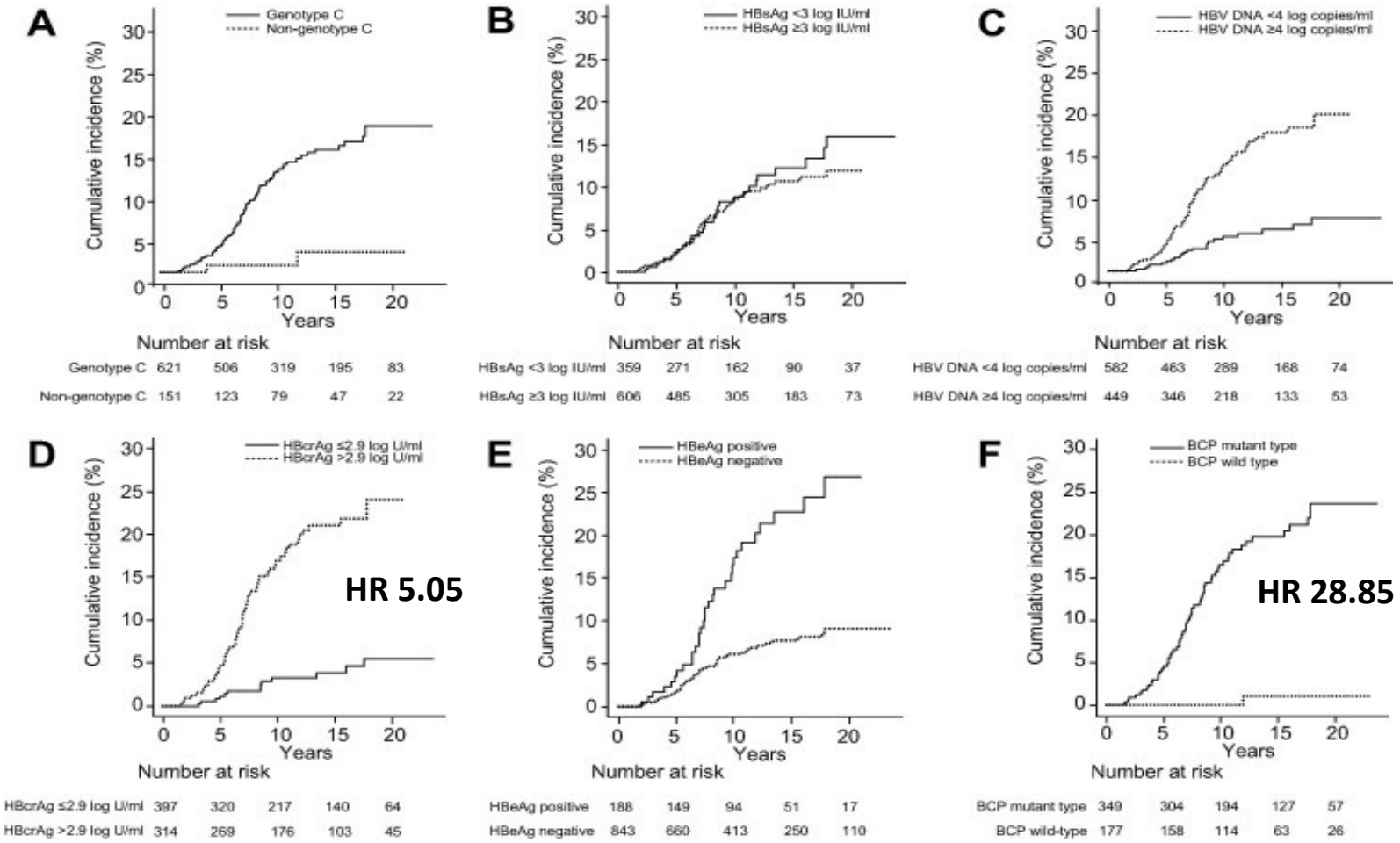
Maasoumy B Clin Microbiol Infect 2015

HBcrAg assay performance: correlations with intrahepatic markers



Wong J Liver Inter 2016

High HBcrAg is linked with increased risk of HCC



Tada T J Hepatol 2016

HBcrAg level can predict HBeAg seroconversion in HBeAg positive patients on NUC therapy

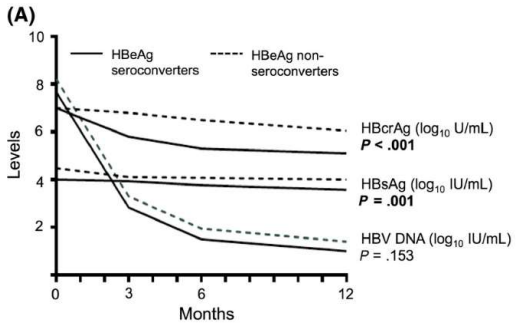
118 HBeAg+ patients treated with NUC monotherapy

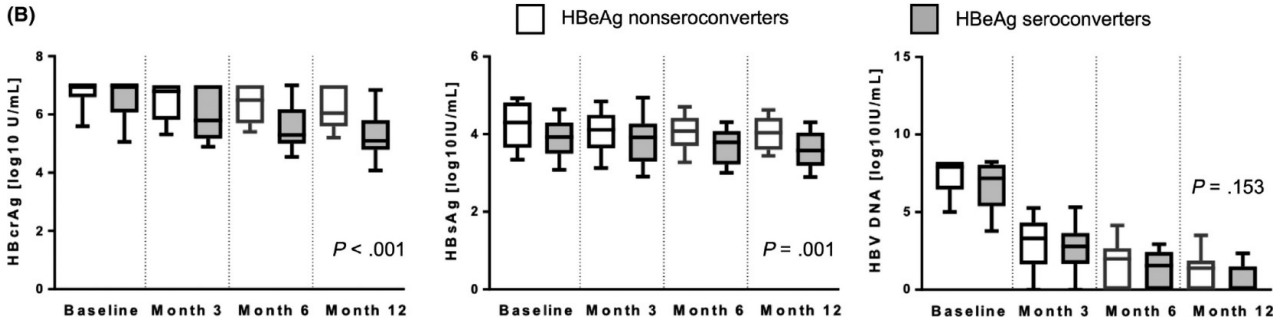
43 (36%) patients achieved HBeAg seroconversion

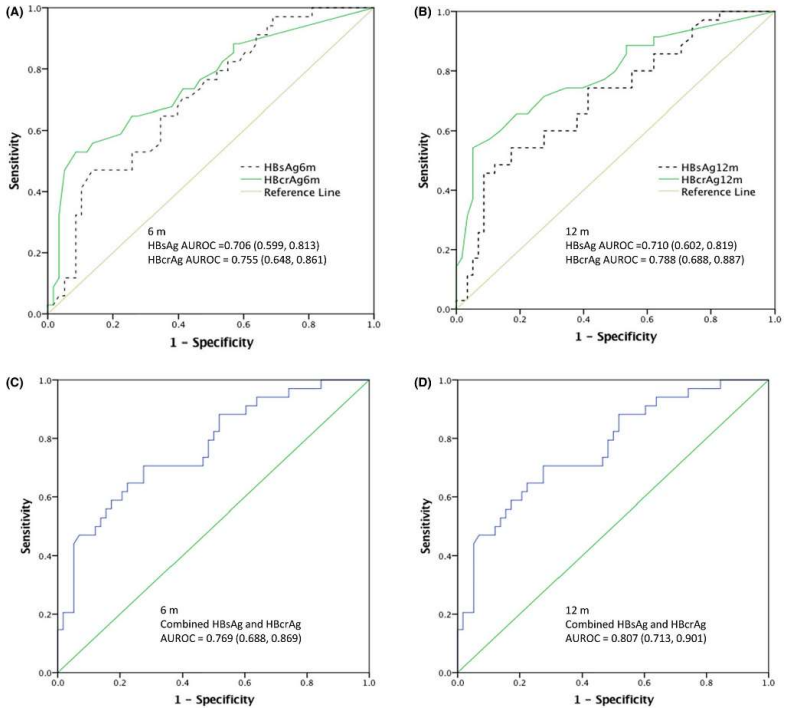
Lower HBsAg and HBcrAg were predictive of future HBeAg seroconversion on therapy

At 6 months: HBsAg level of >3.9 $\log_{10}$ IU/mL and HBcrAg level of >5.7 $\log_{10}$ U/mL predicting lack of HBeAg seroconversion

At 12 months: HBsAg level of >3.8 $\log_{10}$ IU/mL and HBcrAg level of >5.5 $\log_{10}$ U/mL predicting lack of HBeAg seroconversion





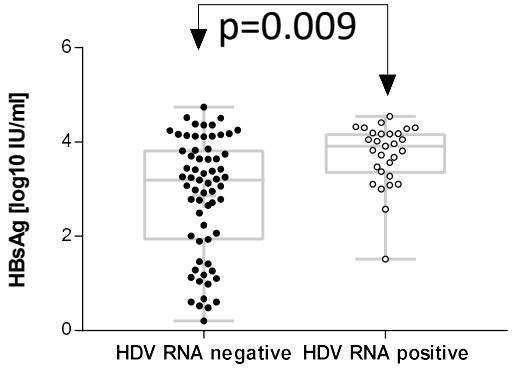


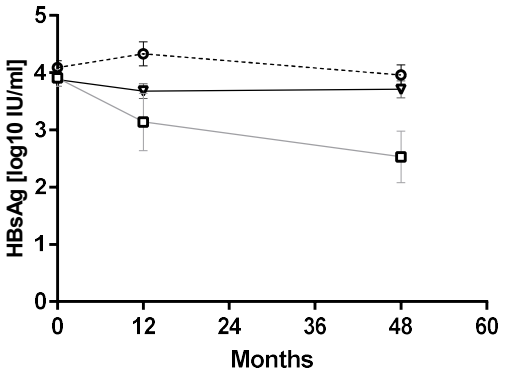
Wang B J Viral Hep 2018

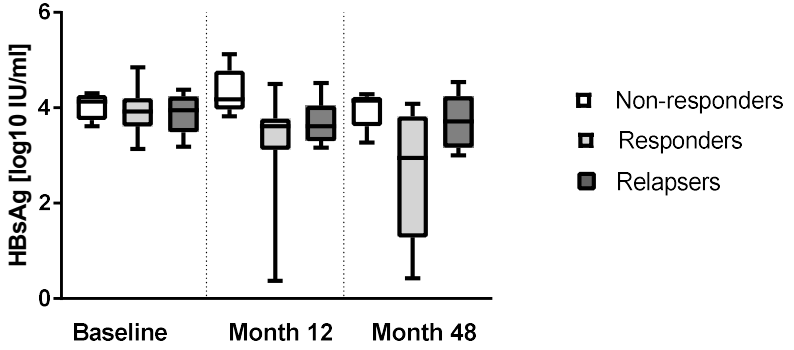
HBV/HDV co-infection and HBcrAg

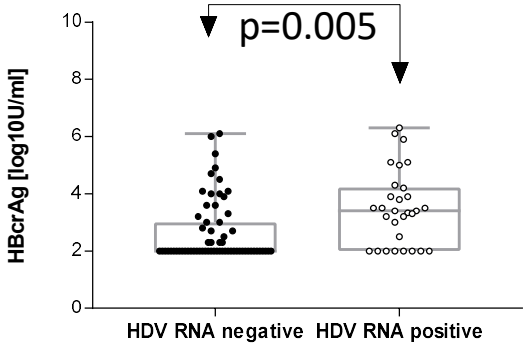
HBsAg and HBcrAg serum levels are lower in HDV RNA negative patients with HDV exposure in past history (anti-HDV total positive) (n=65) vs. HDV RNA positive patients (n=30)

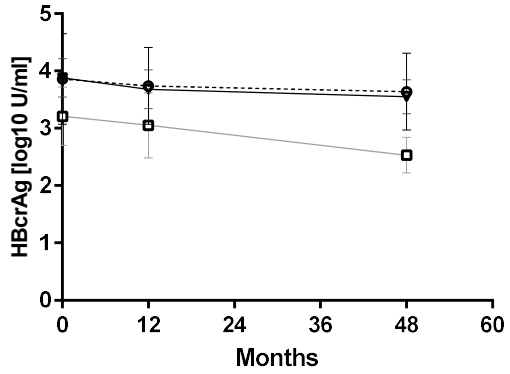
28 HDV RNA positive patients were treated with pegylated IFN and changes in HBsAg and HBcrAg serum levels differ according to therapy response: Response (n=11) vs. Relapse (n=12) vs. non-response (n=5)
Lower HBcrAg plasma levels were predictive of response

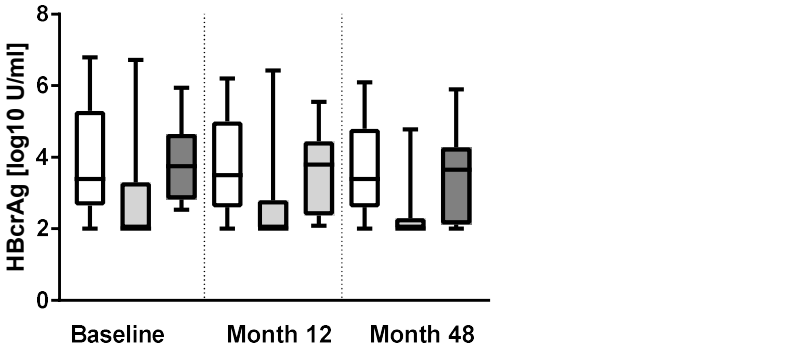








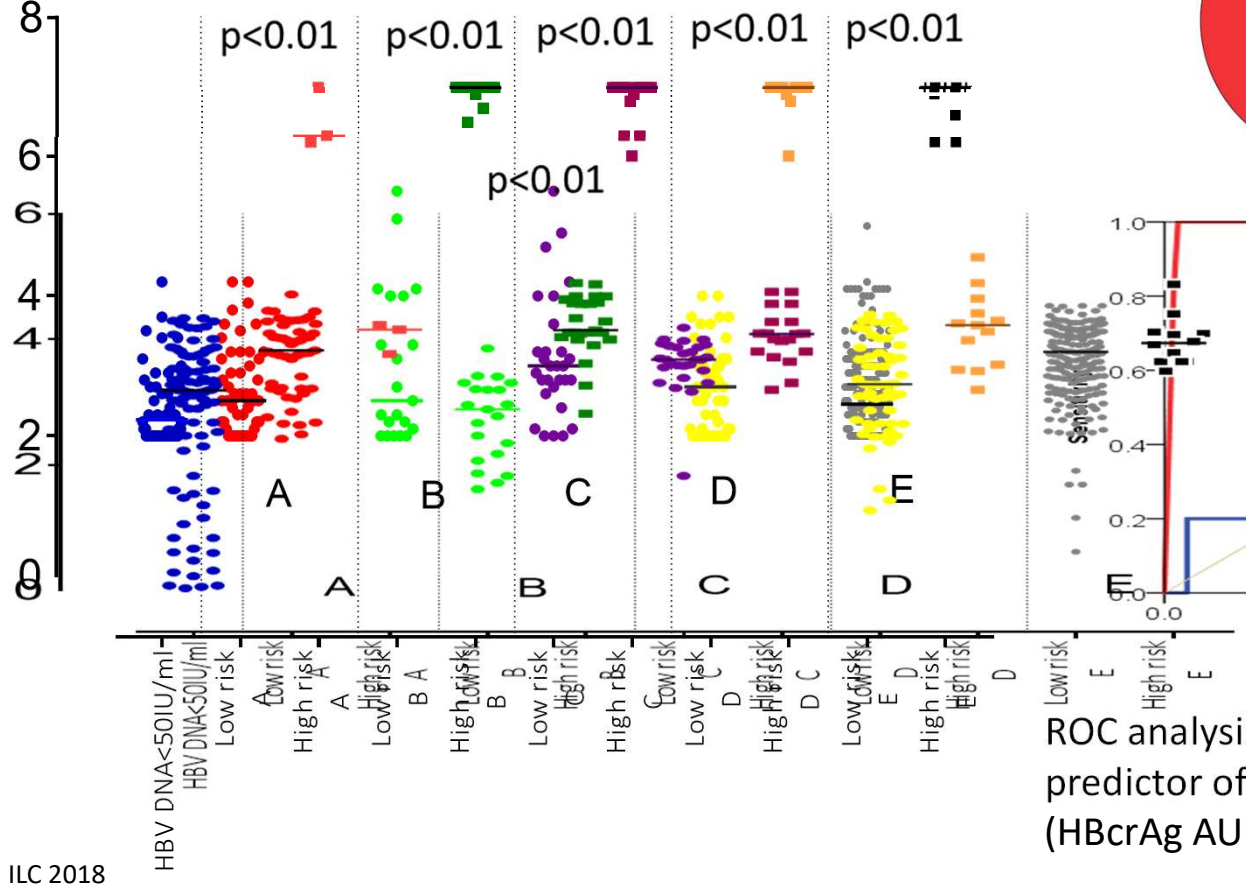


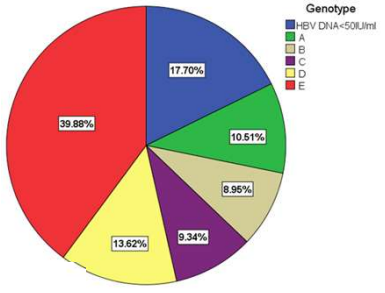


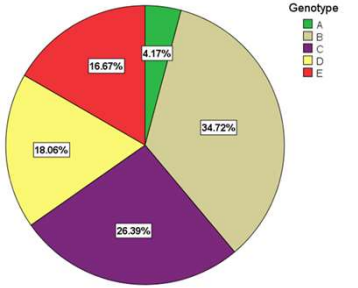
Carey I AASLD 2017

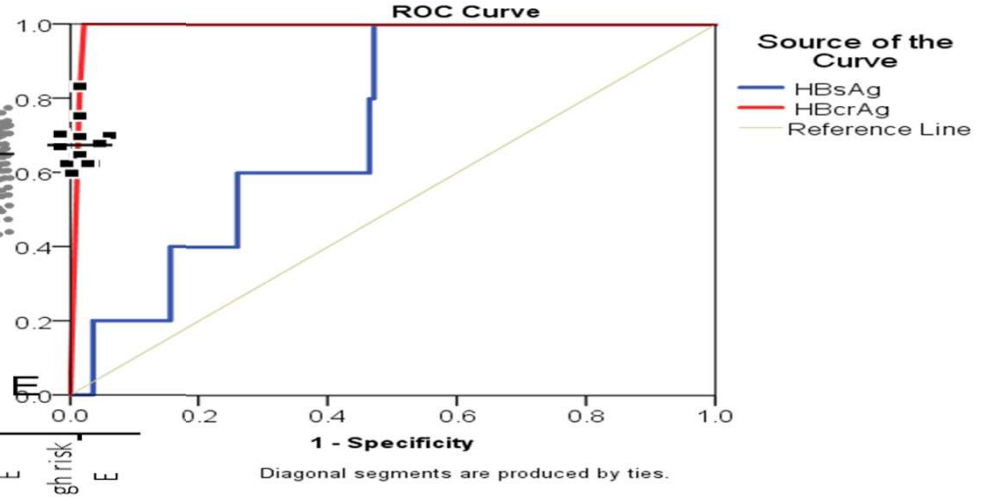
HBcrAg > 6 log10 U/ml predicts high risk of MTCT transmission

514 HBV pregnant patients – based on HBV DNA at 2nd trimester (24 weeks gestation) **high MTCT risk** (HBV DNA > 200 000 IU/ml) (n=72) vs. **low MTCT risk** (HBV DNA < 200 000 IU/ml) (n=442)





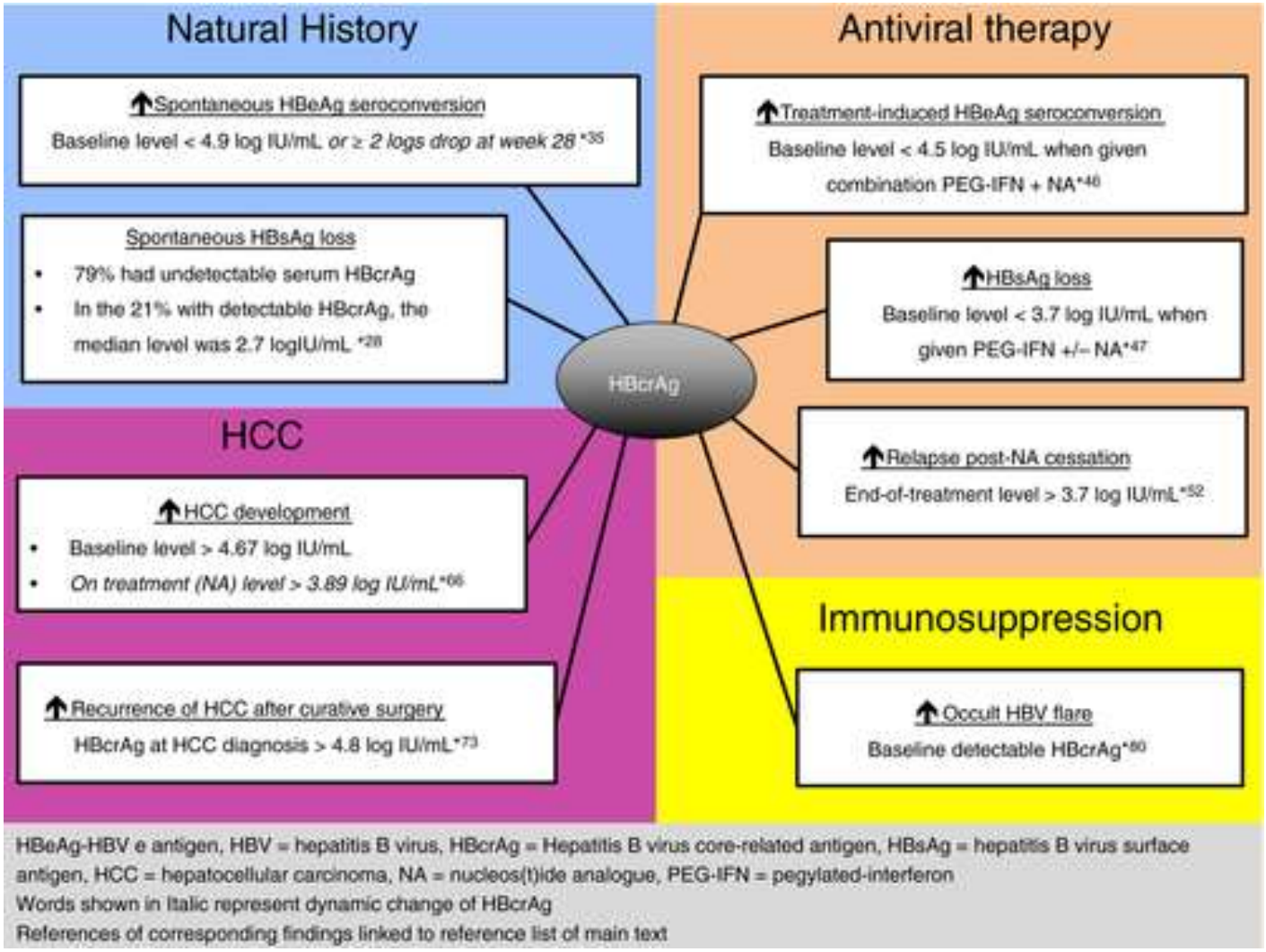




ROC analysis confirmed that HBcrAg is a more accurate predictor of high risk MCTC (based on HBV DNA) than HBsAg (HBcrAg AUROC=1.0 vs. HBsAg AUROC=0.777, both p<0.01)

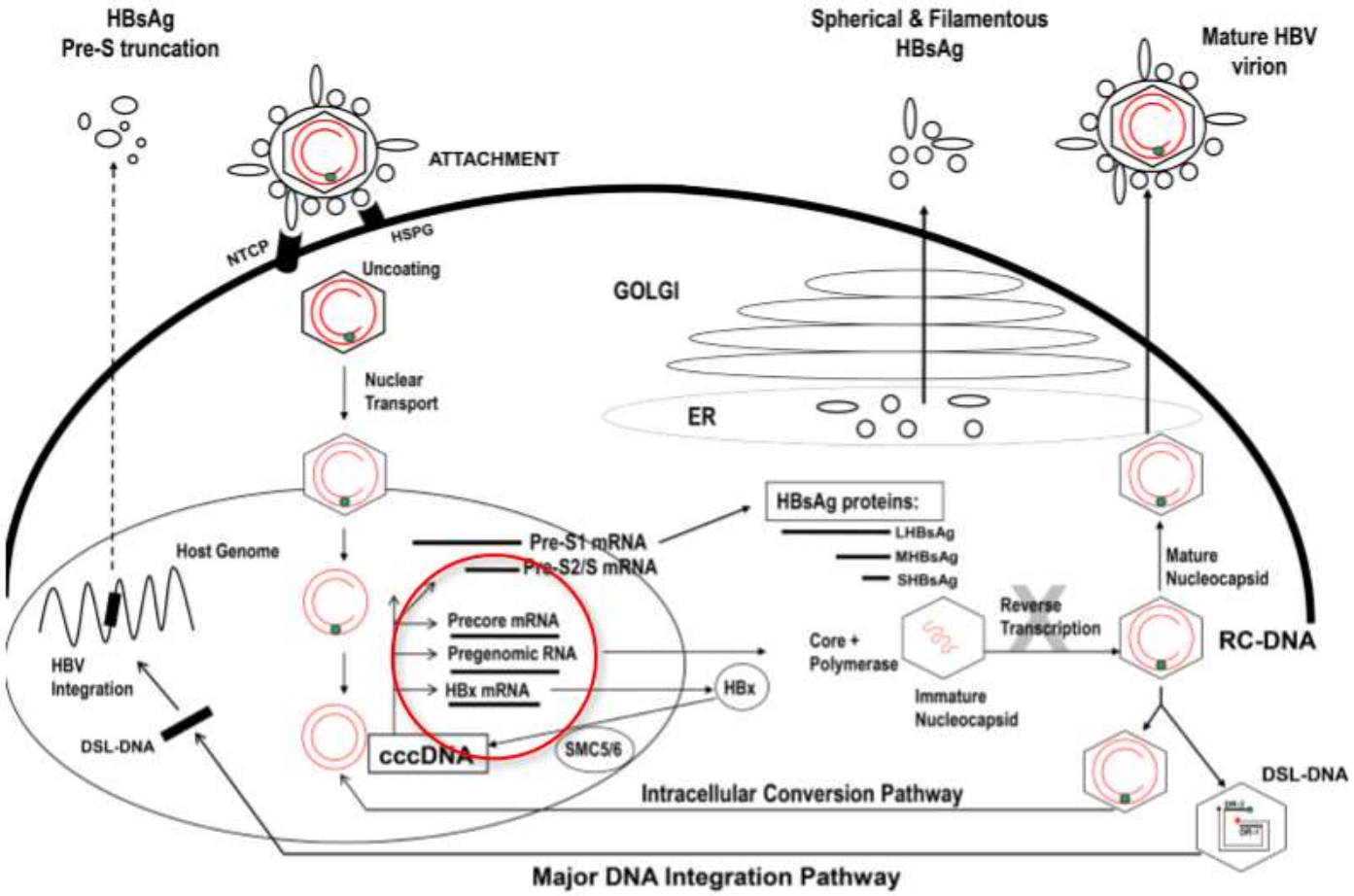
Carey I ILC 2018

HBcrAg plasma levels importance



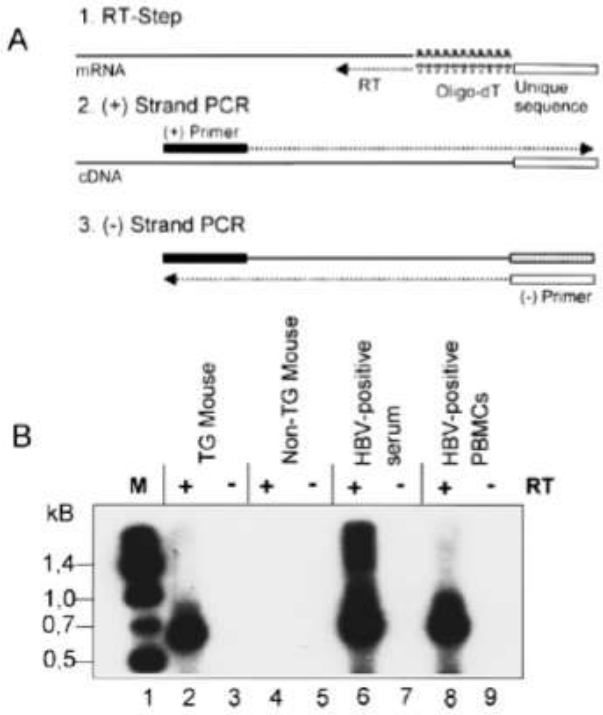
Mak L-Y AP&T 2017

HBV RNA – pgRNA and mRNA



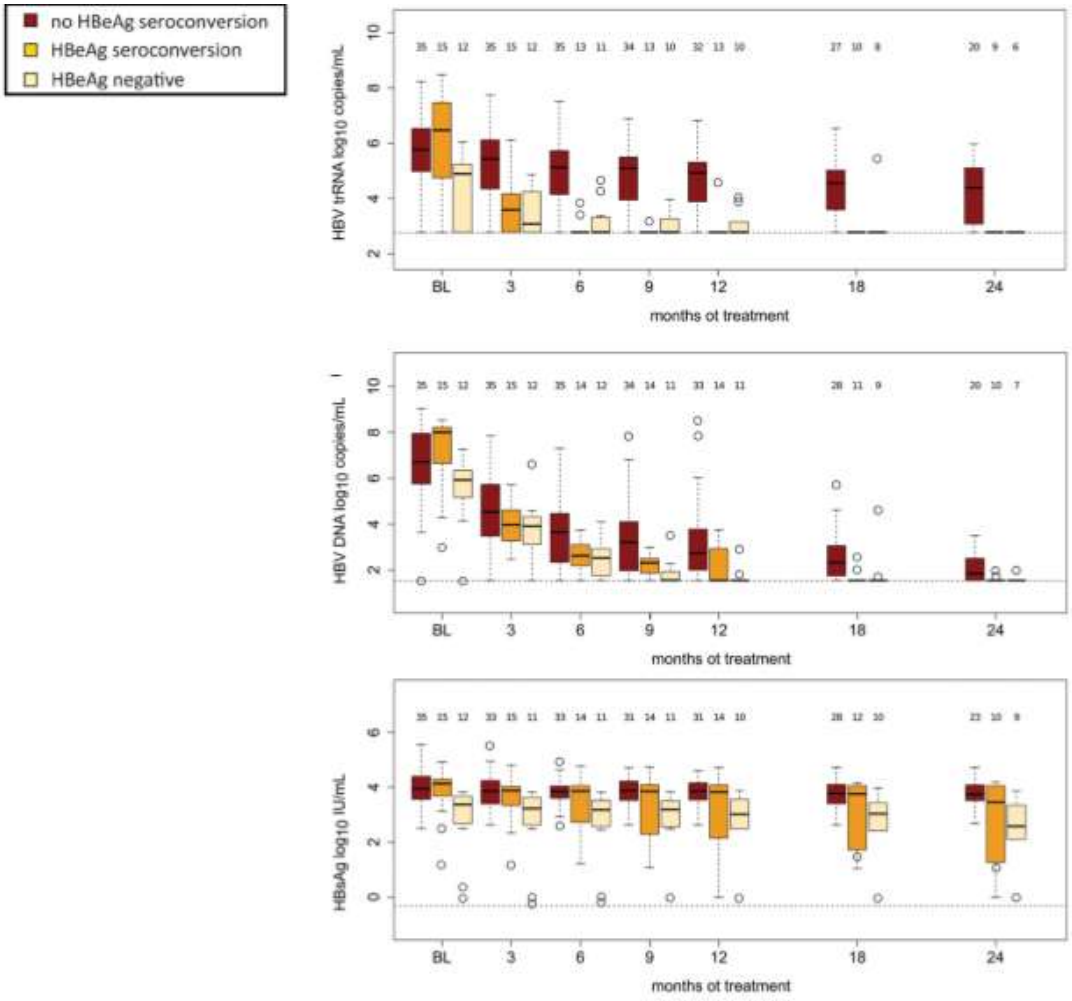
HBV RNA assays

- **Circulating HBV RNA was first described by Köck et al. (1996, *Hepatology*) in the serum of HBV-infected patients**
- **Rokuhara et al. (*2006, J Gastroenterol*): Serum HBV RNA as a potential new marker for monitoring lamivudine therapy; strong correlations between HBV RNA and HBV DNA**
- **van Bömmel et al. (Hepatology 2015): Strong correlation between quantitative serum HBV RNA dynamics and HBeAg loss in NUC-treated patients (RACE-PCR, rapid amplification of cDNA-ends with polymerase chain reaction)**



Köck et al. (Hepatology 1996) Hepatitis B virus nucleic acids associated with human peripheral blood mononuclear cells do not originate from replicating virus.

HBV RNA assays - response to NUCs



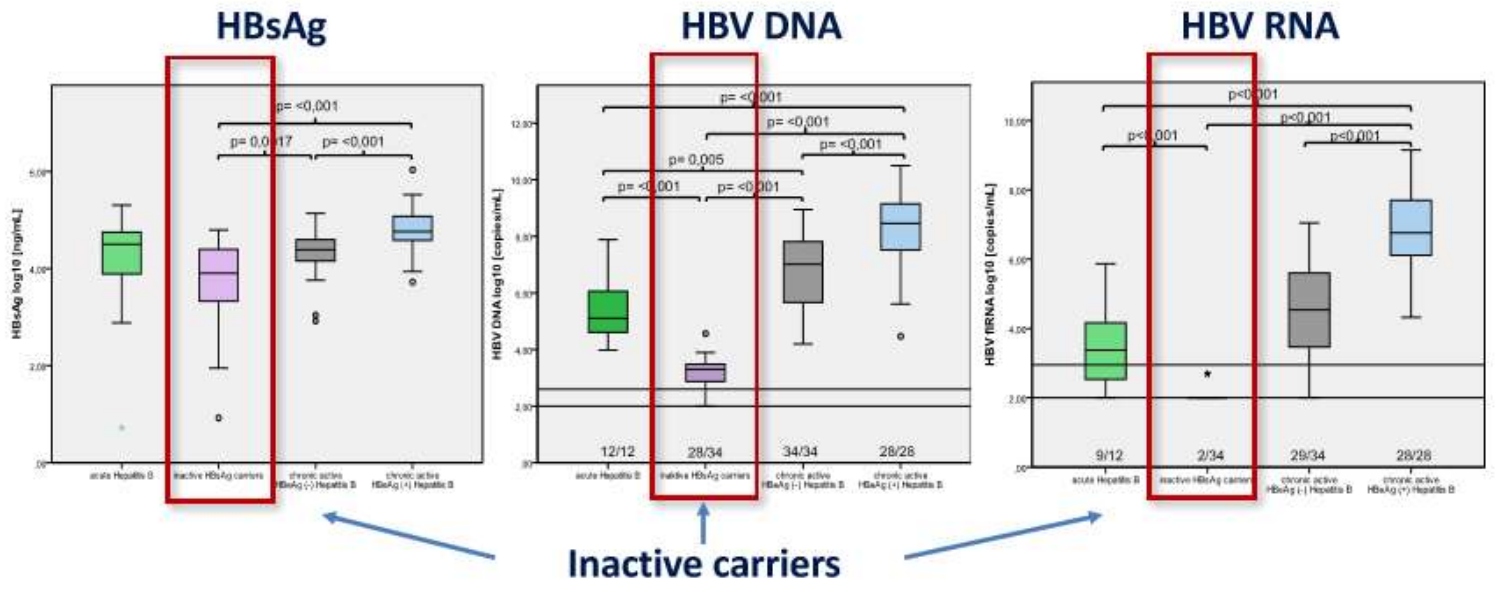
HBV trRNA (log copies/mL)

HBV DNA (log copies/mL)

HBsAg IU/mL

Van Bömmel F et al. Hepatology 2015; 61: 66

HBV RNA assays - different HBV phases



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.

Krauel Gut 2018 in press

Can pgRNA predict viral rebound after stopping NUCs?

Association of HBV RNA (pgRNA virion levels) and viral rebound after discontinuation of NUCs

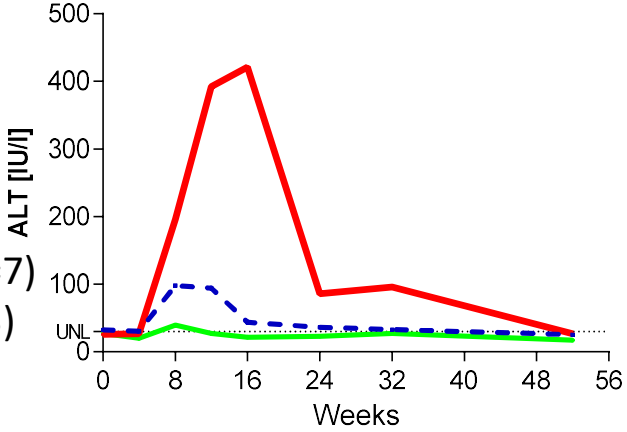
HBV RNA	Viral rebound (n)	No viral rebound (n)	Total (n)	*p value
Positive	21	0	21	
Below the LoQ	3	9	12	0.001
Total (n)	24	9	33	

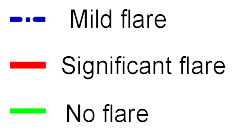
*Chi-Square test; n, number of CHB patients.

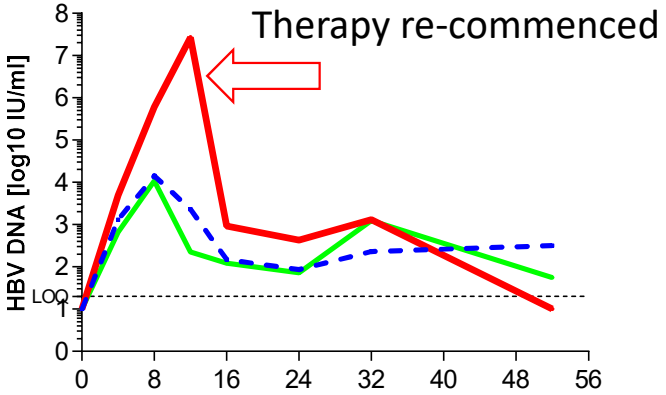
Wang J Hepatol 2016

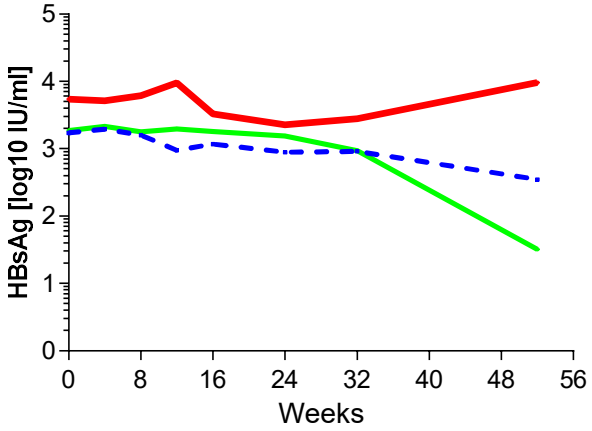
Can pgRNA predict viral rebound after stopping NUCs? - King's experience

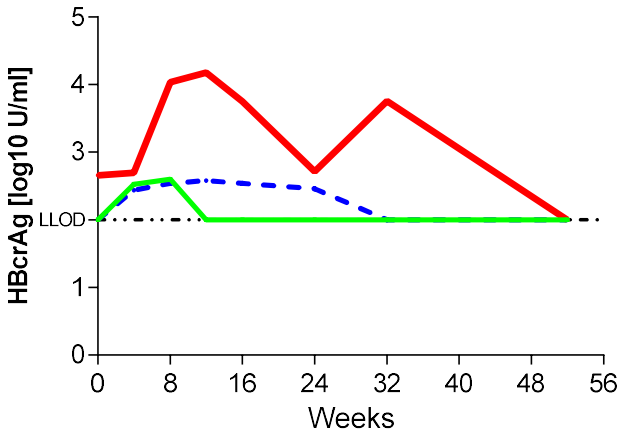
15 HBeAg negative patients
On NUC therapy for > 5 years
All HBV DNA < 20 IU/ml > 3 years
All stopped:
No flare – n=5
Mild flare – ALT>2UNL < 5 UNL (n=7)
Significant flare – ALT>10UNL (n=3)

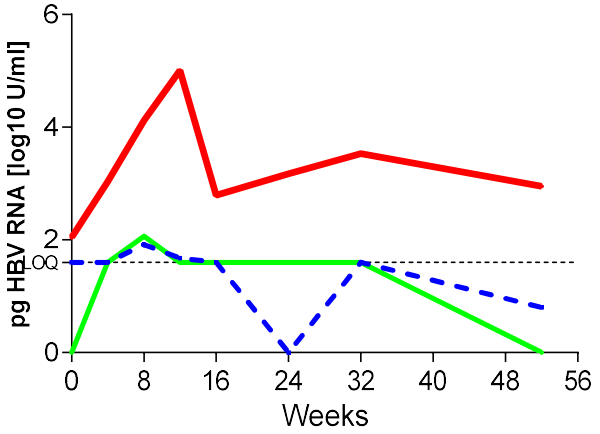




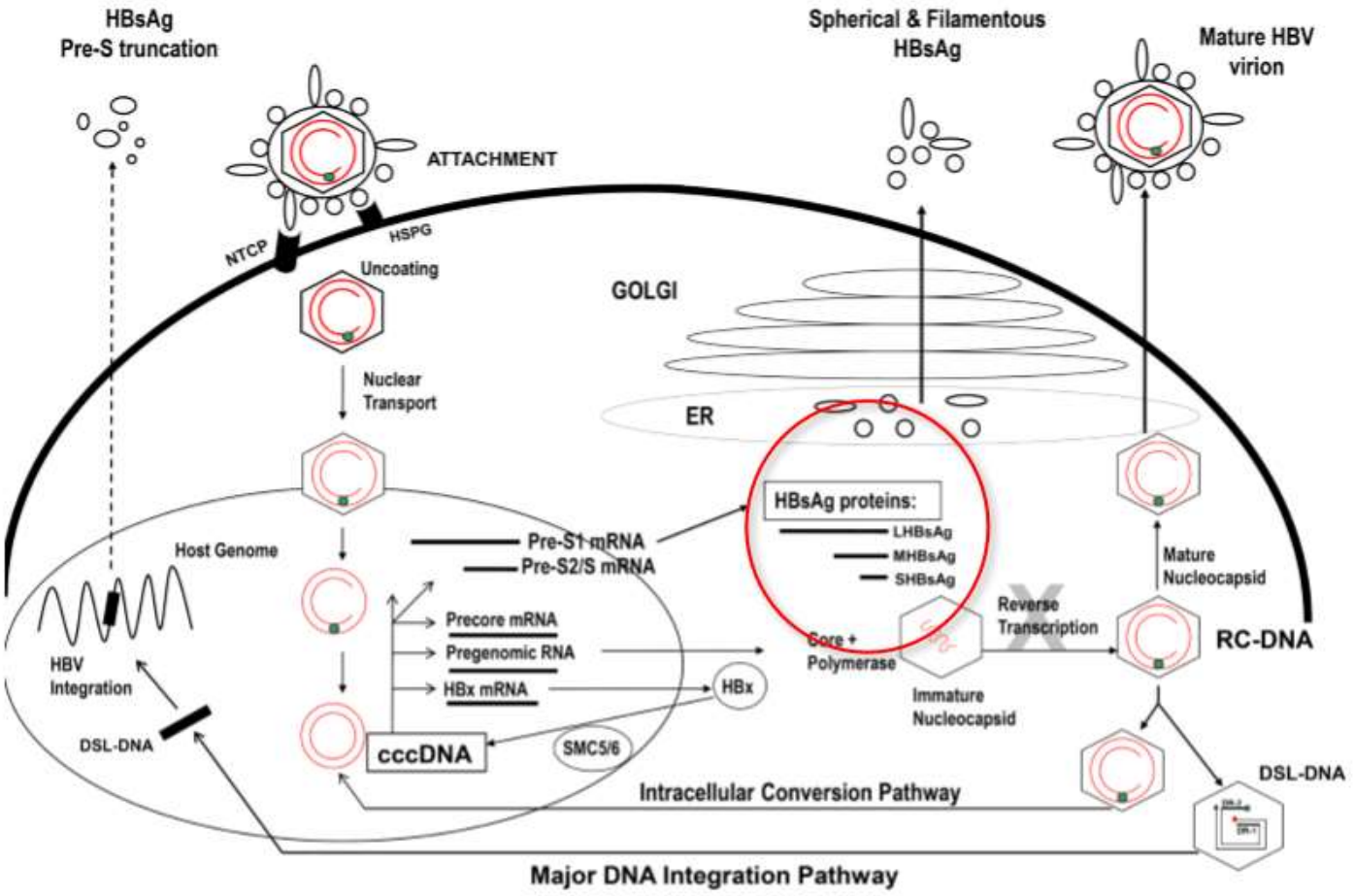








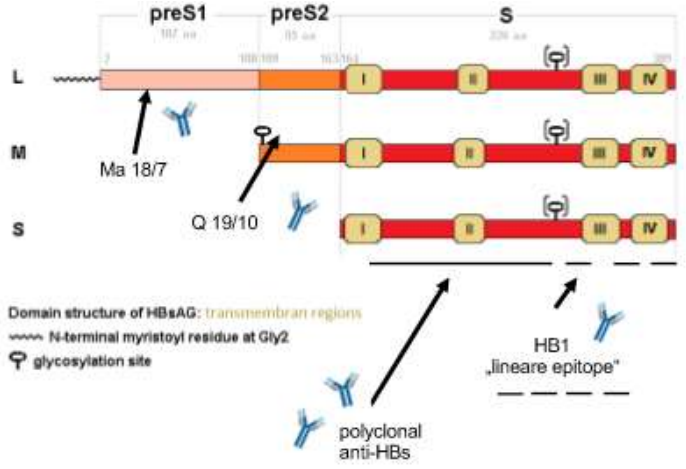
HBsAg proteins composition



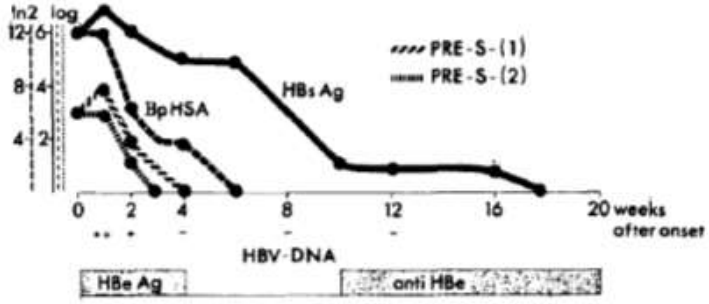
HBsAg proteins composition – HBsAg fragments

- **Stibbe and Gerlich (Virology 1982) first described variable protein composition of HBsAg in different HBV infected patients**

- **Gerken et al. (*Gastroenterology 1987*) showed a strong decrease of PreS1 (LHBs) and PreS2 (MHBs) during acute HBV infections and already described a prognostic value measuring the HBsAg components**

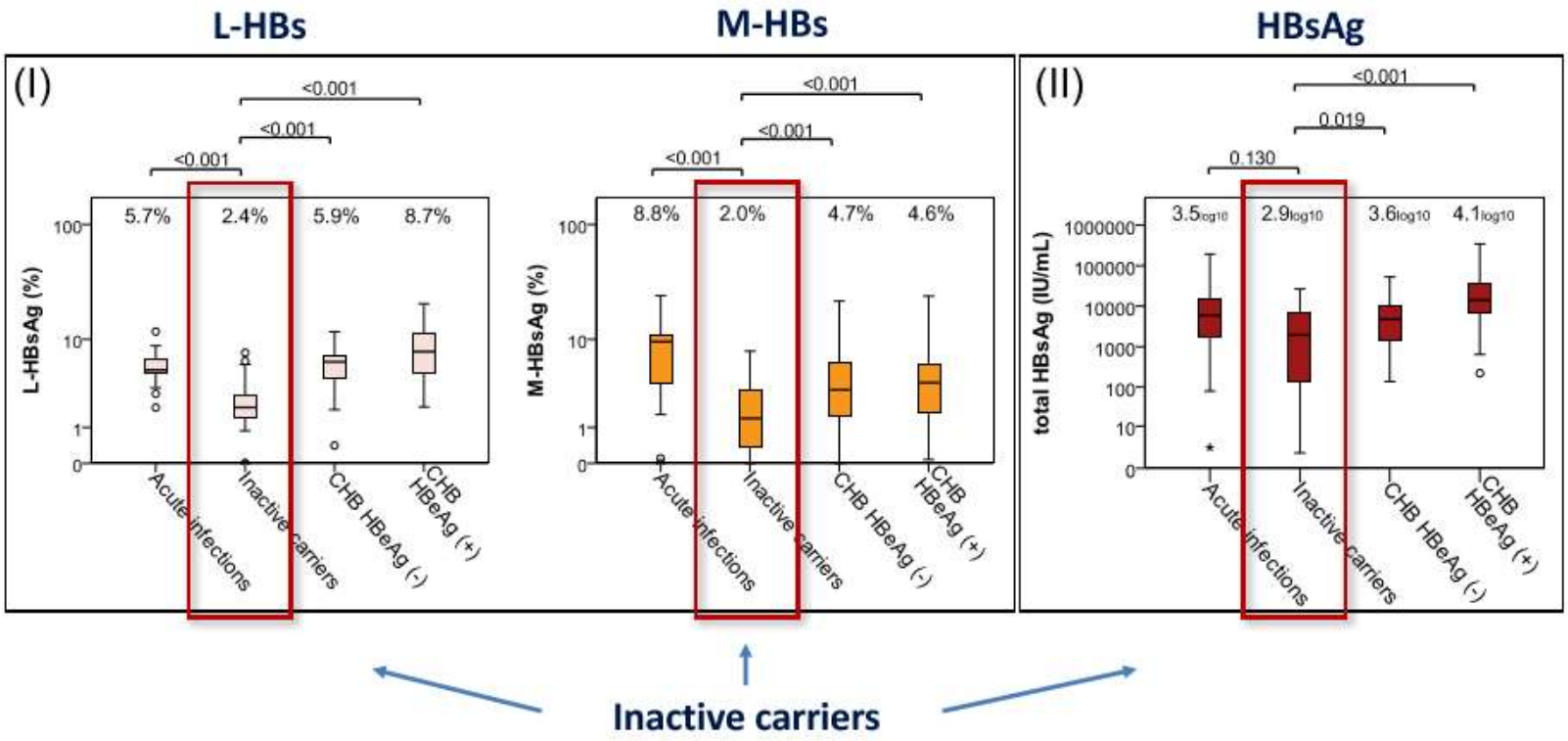


Modified from http://www.klinikum.uni-heidelberg.de/1-Morphology-Genome-Organization. 04932.0.html.



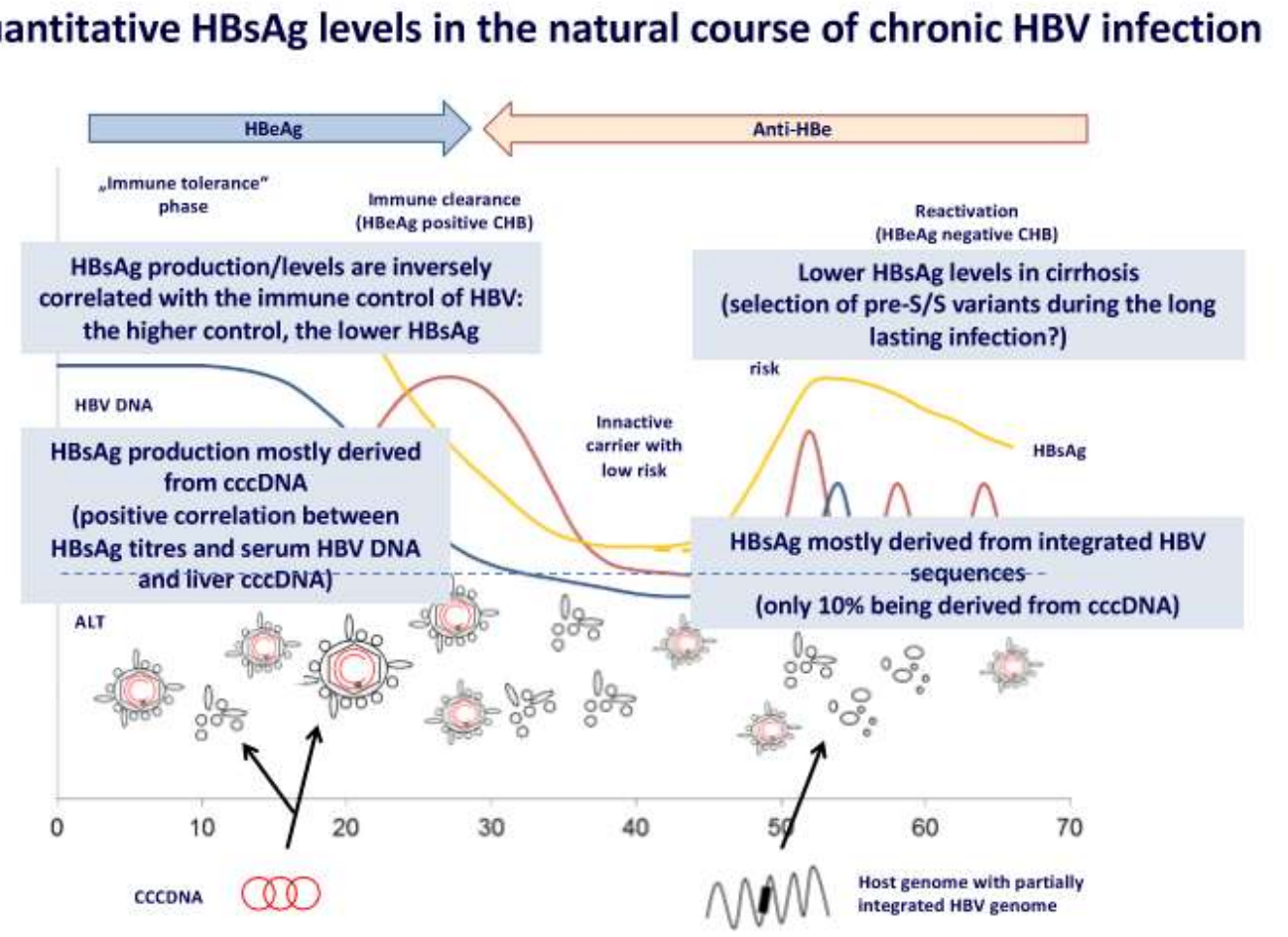
Gerken et al. (1987) Pre-S encoded surface proteins in relation to the major viral surface antigen in acute hepatitis B virus infection. *Gastroenterology*.

HBsAg proteins composition



Pffefercorn Gut 2018

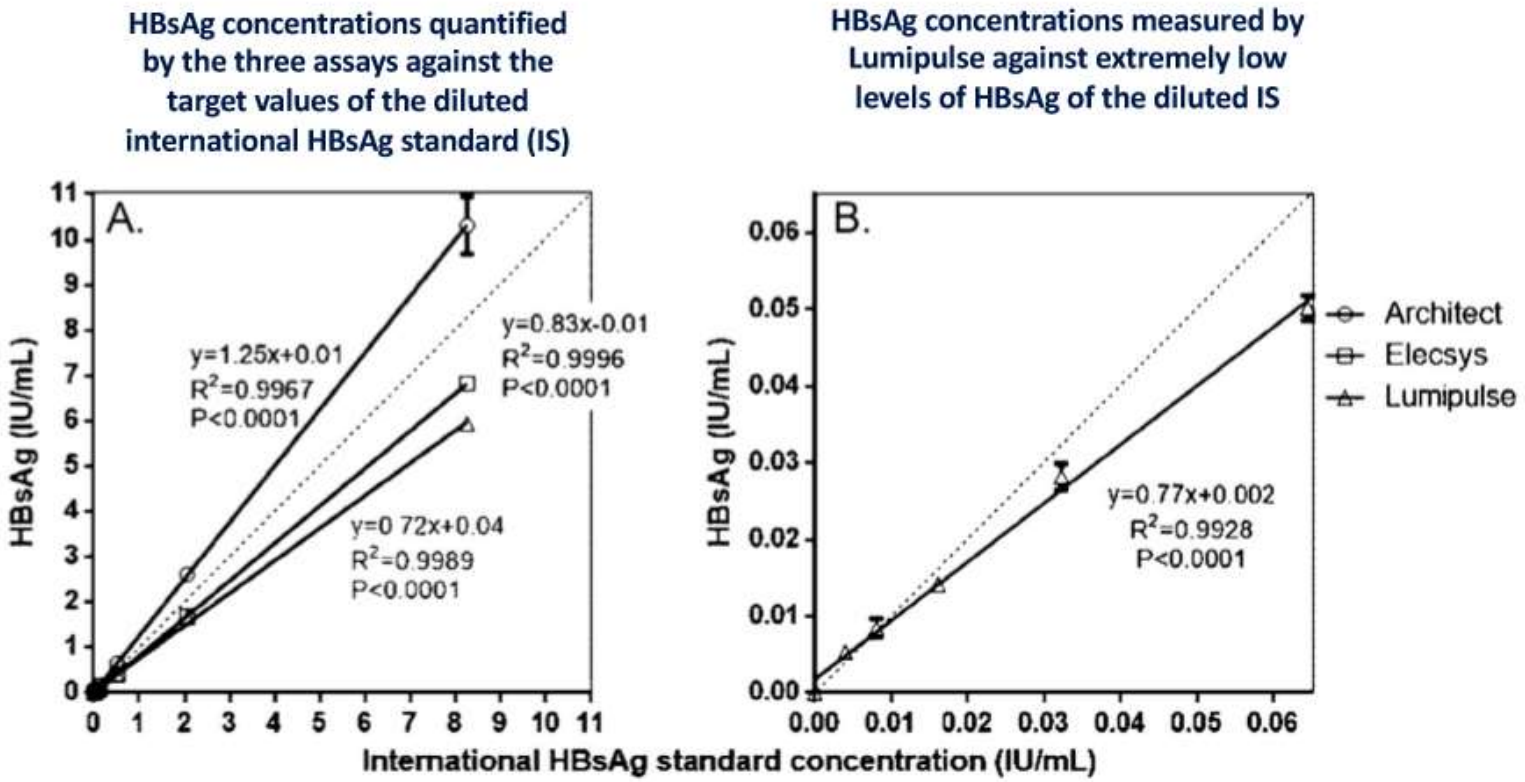
HBsAg loss is endpoint for functional cure



Modified according to Lok A et al. *Nat Rev Gastroenterol Hepatol. 2011* and Cornberg M et al. J Hepatol 2016, in press

The need for high resolution HBsAg quantification?

HBsAg levels Lumipulse vs. others assay



Yang R et al. J Viral Methods 2016; 228: 39

HBsAg levels Lumipulse vs. others assay

HBsAg concentrations quantified by the three assays against the target values of the diluted international HBsAg standard (IS)

HBsAg concentrations measured by Lumipulse against extremely low levels of HBsAg of the diluted IS

- **10 times higher sensitivity (0.005 IU/mL)**
- **Quantification of extremely low HBsAg levels (0.004 IU/mL) with CV < 4%**
- **Identified 1% (20/2043) clinical samples with trace amounts of HBsAg**
  - **six of them with positive HBV DNA 32- 600 IU/mL;**
  - **new interpretation of occult HBV infection?**

Each level of the diluted standard were tested in triplicate (mean ± SD IU/mL)

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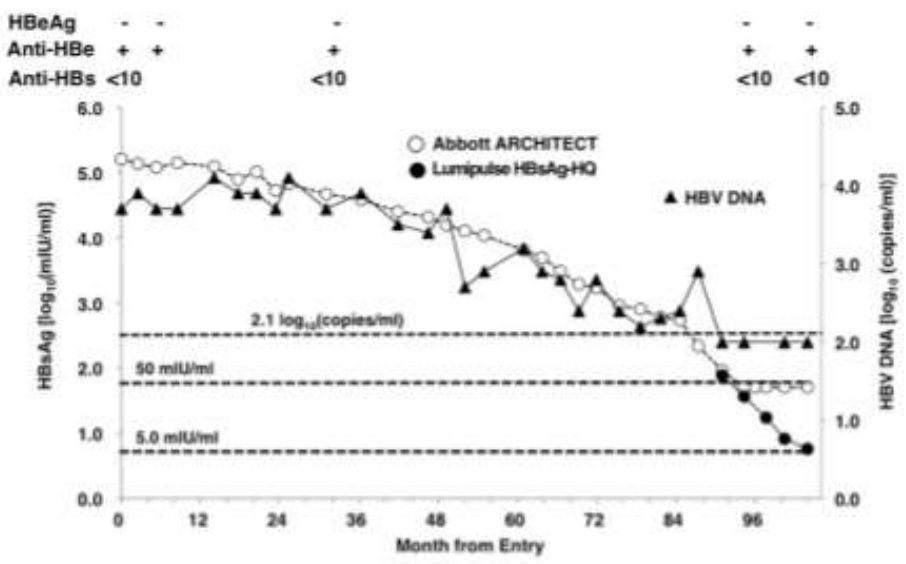
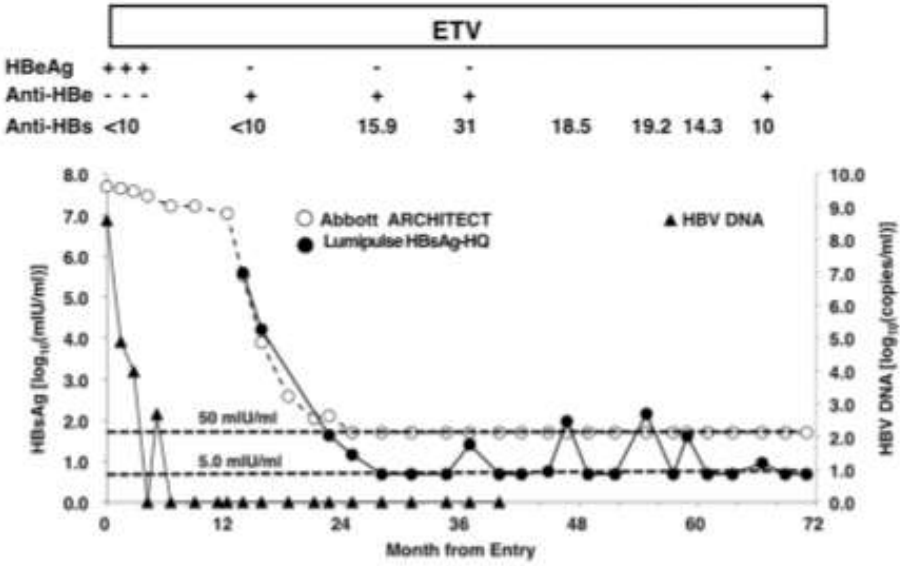
HBsAg levels Lumipulse vs. others assay

Spontaneous HBsAg clearance

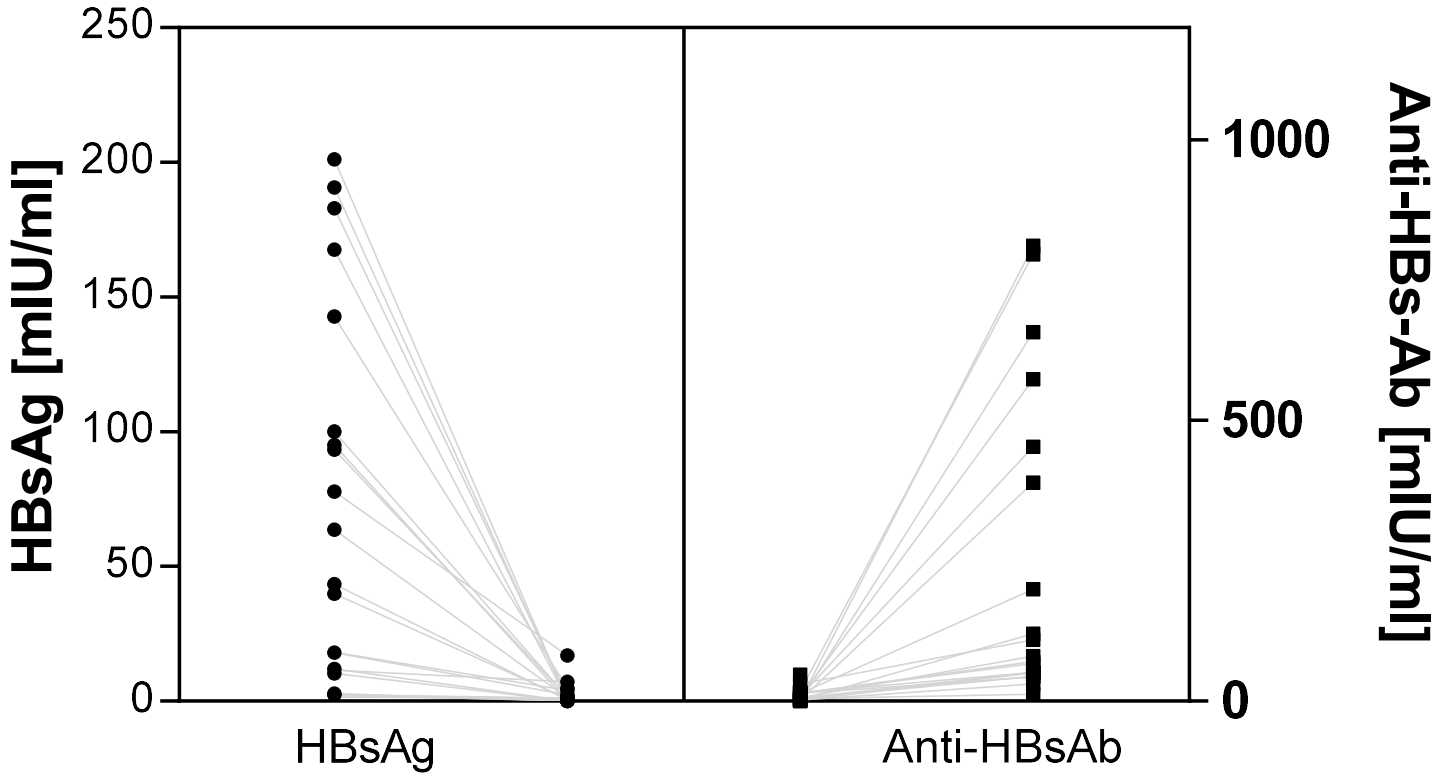


Treatment-associated HBsAg clearance



King’s experience: HBsAg negative with low anti-HBs (<10 mIU/ml) known chronic HBV

23 chronic hepatitis B patients (known HBsAg+ in past) had sequential samples from anti-HBs loss: ultrasensitive HBsAg and anti-HBs Ab concentrations were compared between consecutive follow up visit (median 18 months)



Summary – new HBV bio-markers

- Helpful markers, but more information needed
- The need for standardisation and defining cut offs
  - WHO standards for HBsAg fragments, HBcrAg and HBV RNA
- Increasing sensitivity and dynamic range
  - Important for HBeAg negative infection or exposed patients
- Performance of the assays across different subgroups:
  - Different phases of infection
  - HBV genotype/ subgenotypes
  - Viral variants

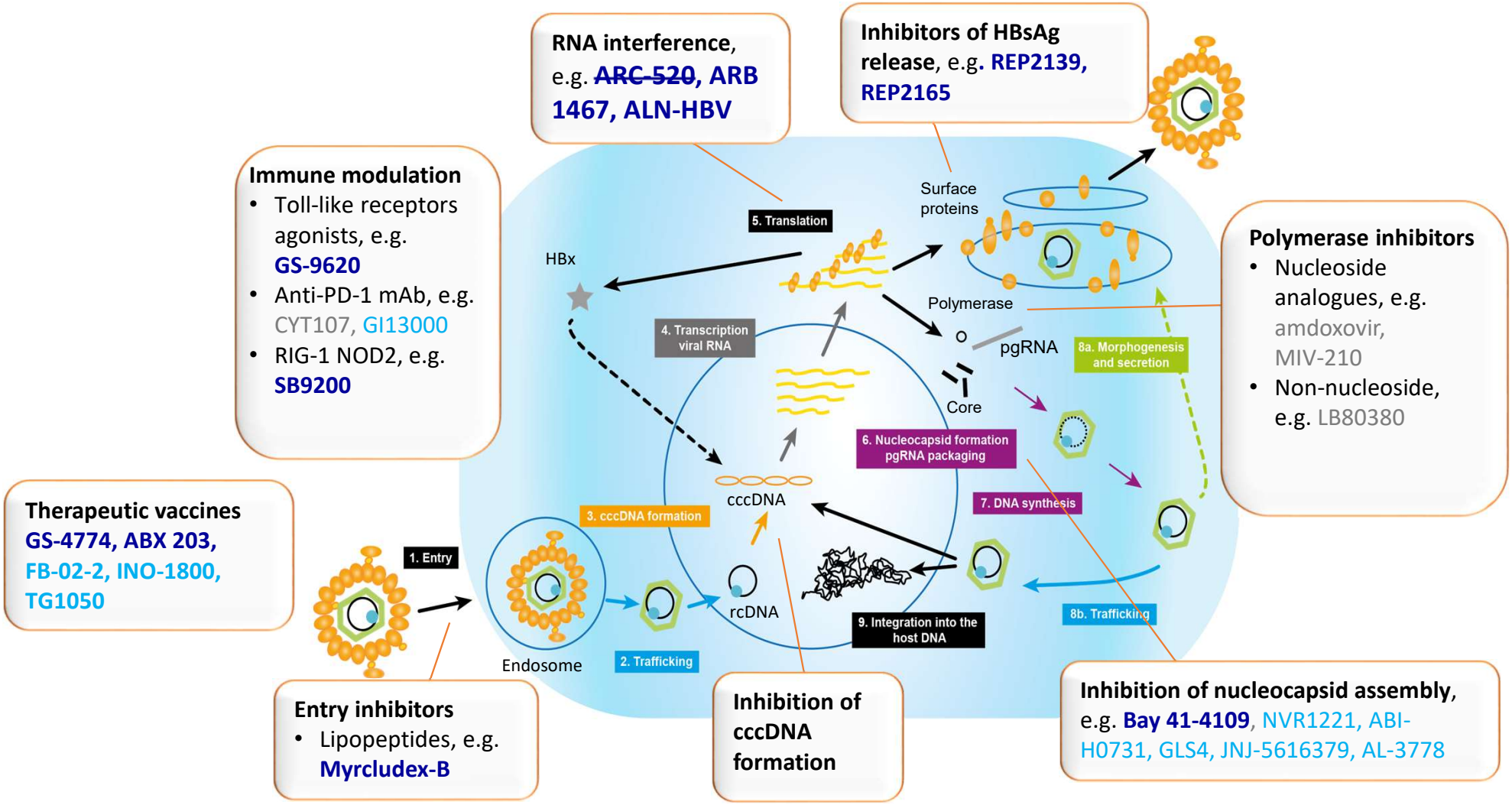
Unresolved issues – unmet needs for assays

- How to distinguish HBsAg derived from cccDNA vs. integrated DNA
- How to detect and quantify integrated HBV DNA
- Specific markers according to different treatment strategies
  - Direct antivirals
  - Immune mediated
  - Combination
- How to determine transcriptional activity of cccDNA

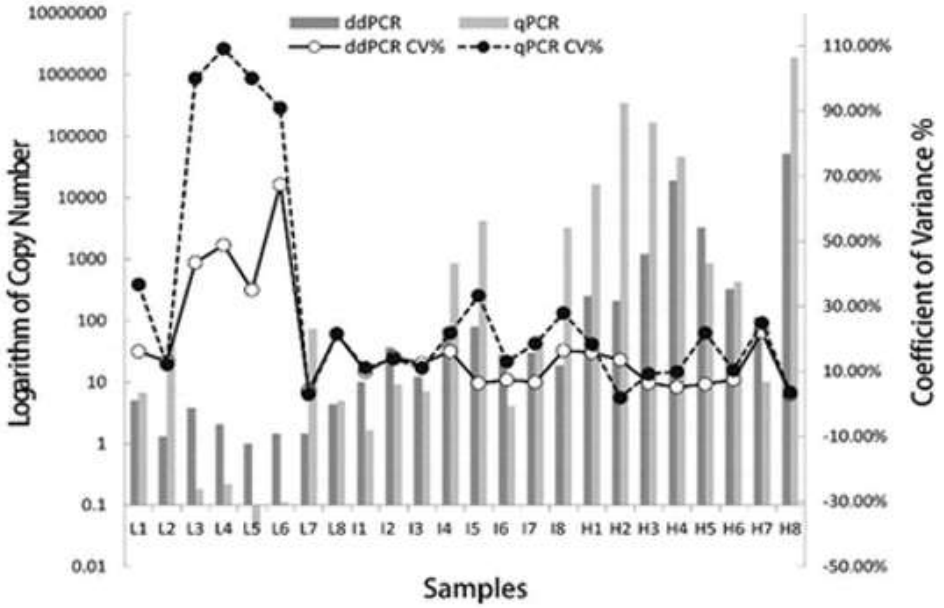
Summary – new non-invasive HBV bio-markers

- Allow to test simultaneously different replicative transcriptional and translational activity of HBV and help to understand in detail different stages of chronic HBV
- **Serum HBV RNA** is an interesting marker to study cccDNA transcriptional activity but may also reflect (in NUC treated) the ongoing production of pgRNA virions
- It should be further explored whether HBsAg positive patients with not detected HBV DNA and HBcrAg and pgRNA bellow limit of detection off therapy might be considered as the closest point to functional cure – '**non-replicative' phase**

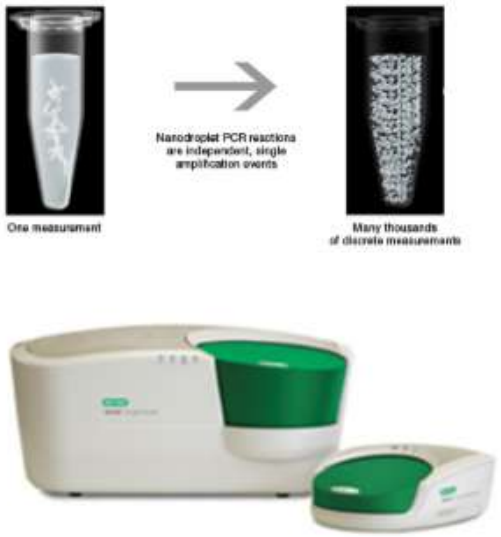
HBV replication cycle and drug targets



New technologies – droplet digital PCR



The ddPCR assay exhibited superior repeatable quantitative results over the qPCR assay, especially in settings with low copy number samples.



QX200 Droplet Digital PCR System. QX200 Droplet Reader (left) and the QX200 Droplet Generator (right) (*BIO RAD*)

Tang H et al. Bioscience, Biotechnology, and Biochemistry 2016; epub