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The extent of genetic variability in HBsAg C-terminus profoundly affects HBsAg levels in eAg-negative chronic HBV genotype D infection

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Background

➢ HBsAg levels are proposed as marker of intrahepatic HBV reservoir *(Volz et al 2007; Lu et al, 2008)*.

➢ A recent in-vitro study showed variation in HBsAg production in different HBV genotypes *(Sozzi et al, 2016)*.

➢ Limited information is available on:

  - HBsAg levels in patients infected with different HBV genotypes in HBeAg-negative chronic HBV infection

  - Virological factors underlying such differences.

Aim of the study

Our study aims to investigate HBsAg levels in patients infected with different HBV genotypes in HBeAg-negative chronic infection

Methods

This study includes **301 patients**
with HBeAg-negative chronic HBV infection, drug-naïve
and monitored for >1 year

The following groups were identified:

GROUP A
126 patients
with persistent serum HBV-DNA
<2,000 IU/ml and normal
transaminases

GROUP B
175 patients
with persistent serum HBV-DNA
>2,000 IU/ml and normal/altered
transaminases

Methods

Genetic analysis

- HBV genotype was determined by phylogenetic analysis

- Mean genetic distance was used to estimate the extent of genetic variability in HBsAg by the Tajima-Nei model of MEGAv5.

Co-variation analysis

- Binomial correlation coefficient (phi) was calculated to assess the strength of co-variation among mutations in HBsAg

Structural analysis

- I-Tasser is used to predict three-dimensional HBsAg structures (aa:1-226) and their stability ($\triangle\triangle$G[wt-mutated]<0 indicating reduced stability in presence of mutation based on Quan, 2016).

Results

Patients' characteristics

|	Group A (N=126) (HBV-DNA ≤2000 IU/ml and normal ALT)	Group B (N=175) (HBV-DNA >2000 IU/ml)
Patients' characteristics:		
Male, N (%)	74 (58.7)	112 (64.0)
Median age, years (IQR)	40 (31-54)	39 (32-52)
Italian nationality, N (%)	47 (37.3)	37 (21.1)
Other countries, N (%):	79 (62.7)	138 (78.9)
East European Countries, N (%)	33 (26.2)	61 (34.9)
North European Countries, N (%)	17 (13.5)	44 (25.1)
Africa, N (%)	23 (18.3)	18 (10.3)
Asia, N (%)	6 (4.8)	15 (8.6)
HBV characteristics:		
Median (IQR) HBV-DNA, log IU/ml	2.7 (2.3-2.9)	● 4.0 (3.7-5.0)
Median (IQR) HBsAg, IU/mL	1475 (308-7547)	● 4695 (1695-10343)
Median (IQR) ALT, IU/L	27 (21-38)	34 (26-55)
Median (IQR) AST, IU/L	21 (17-27)	25 (18-36)
HBV Genotype:		
D, N (%)	● 91 (72.2)	● 137 (78.3)
A, N (%)	20 (15.9)	25 (14.3)
E, N (%)	15 (11.9)	13 (7.4)

In both group A and B, HBsAg levels are significantly lower in HBV genotype D than in genotype A and E
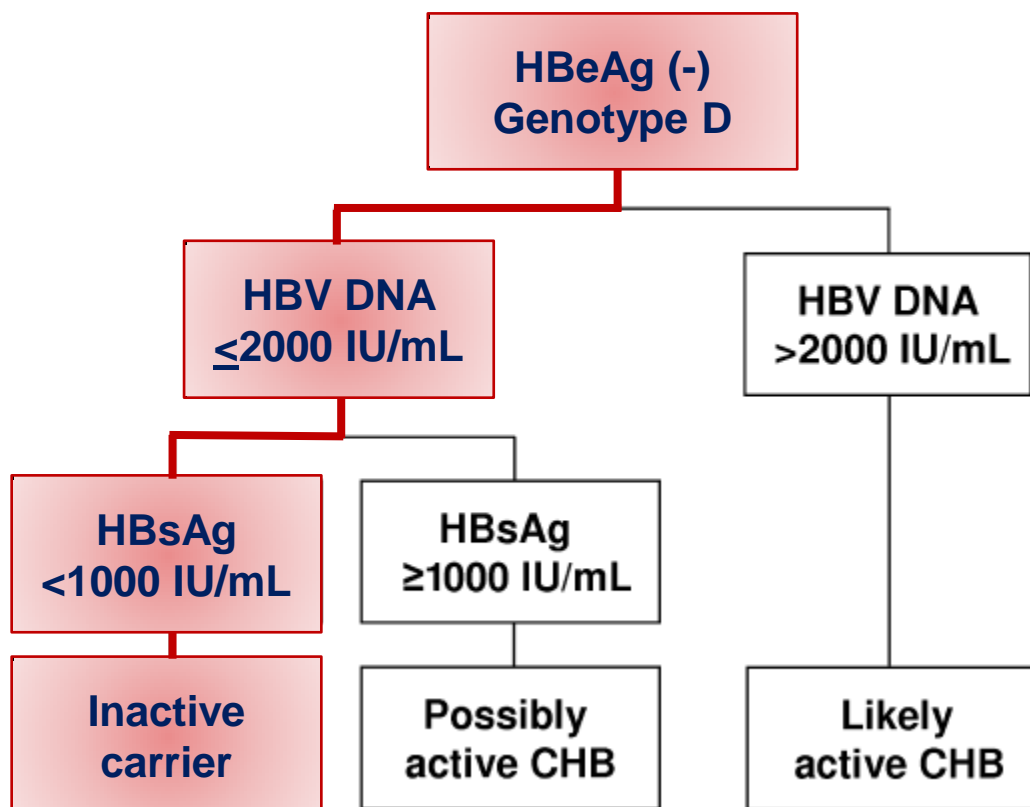
## Group A (low viremic)
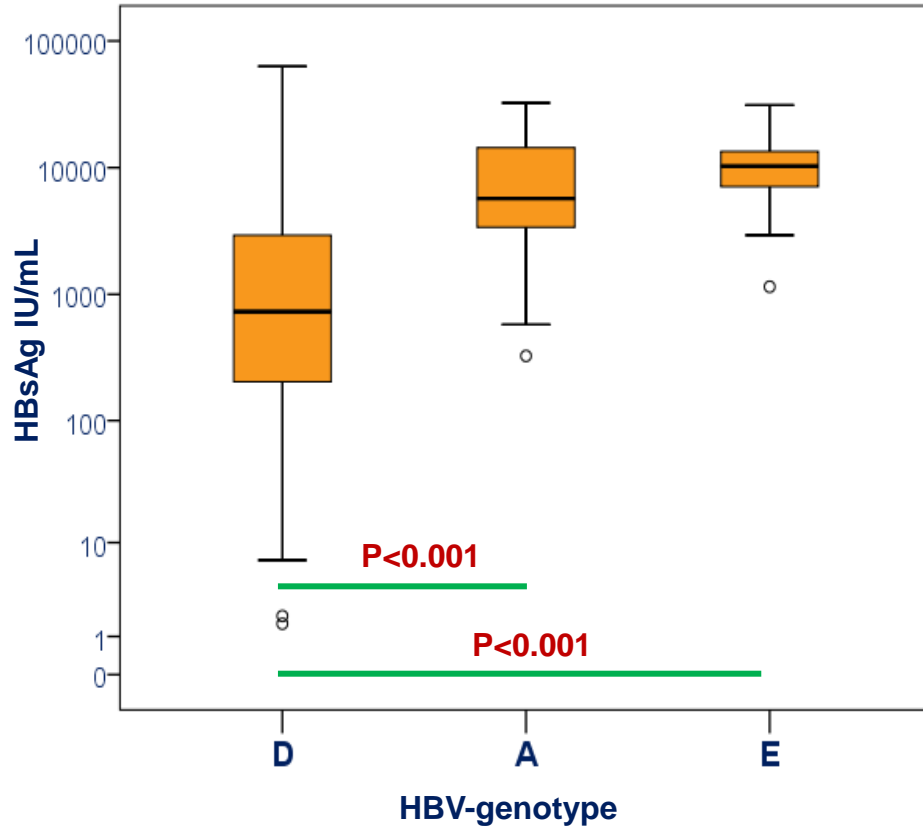
Group B (high viremic)
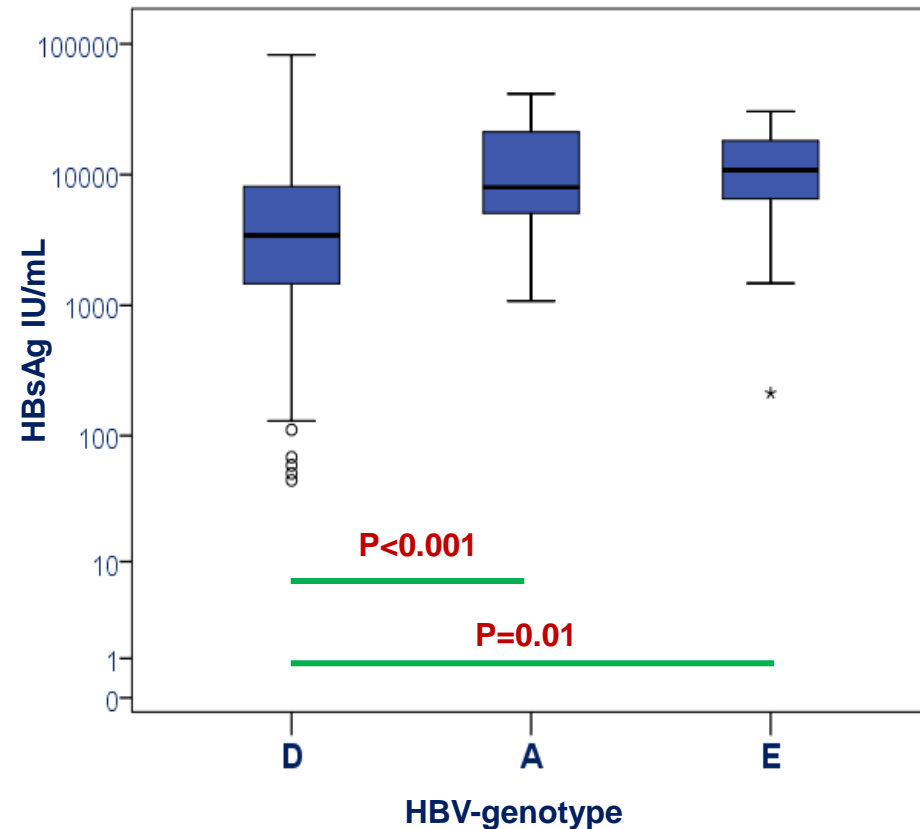
Box plots were used to report the distribution of HBsAg values (IU/mL) in group A and B. Box plots report median, 25th percentile, 75th percentile, lower and upper whiskers, minimum and maximum values. Statistically significant differences were assessed by Mann-Whitney Test.

In patients with HBV-DNA ≤ 2000 IU/ml, HBsAg <1000 IU/ml can help identifying true inactive carrier patients in clinical practice

Sonneveld et al., JVH 2011

Among group A (low viremic), the percentage of patients with HBsAg <1,000 IU/mL is significantly higher in genotype D than in genotype A and E
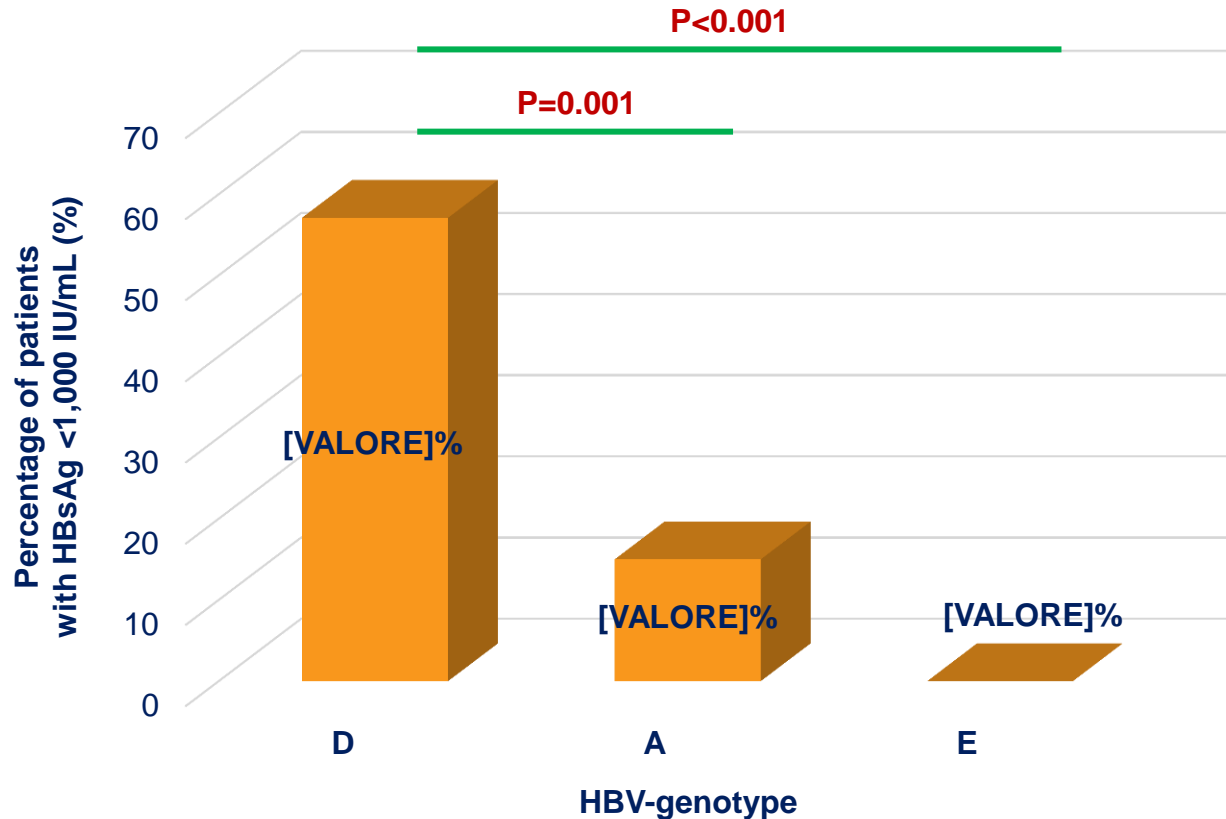

The histogram shows the percentage of group-A patients with HBsAg <1,000 IU/mL stratifying according to HBV genotypes (D, N=91 ; A, N=20 ; E, N=15). Statistically significant differences were assessed by Fischer Exact Test.

Overall results suggest that in eAg-negative chronic HBV infection, genotype D is characterized by lower HBsAg levels.

Can HBsAg genetic variability contribute to the low HBsAg levels in genotype D?

HBsAg C-terminus: a critical domain for HBsAg secretion
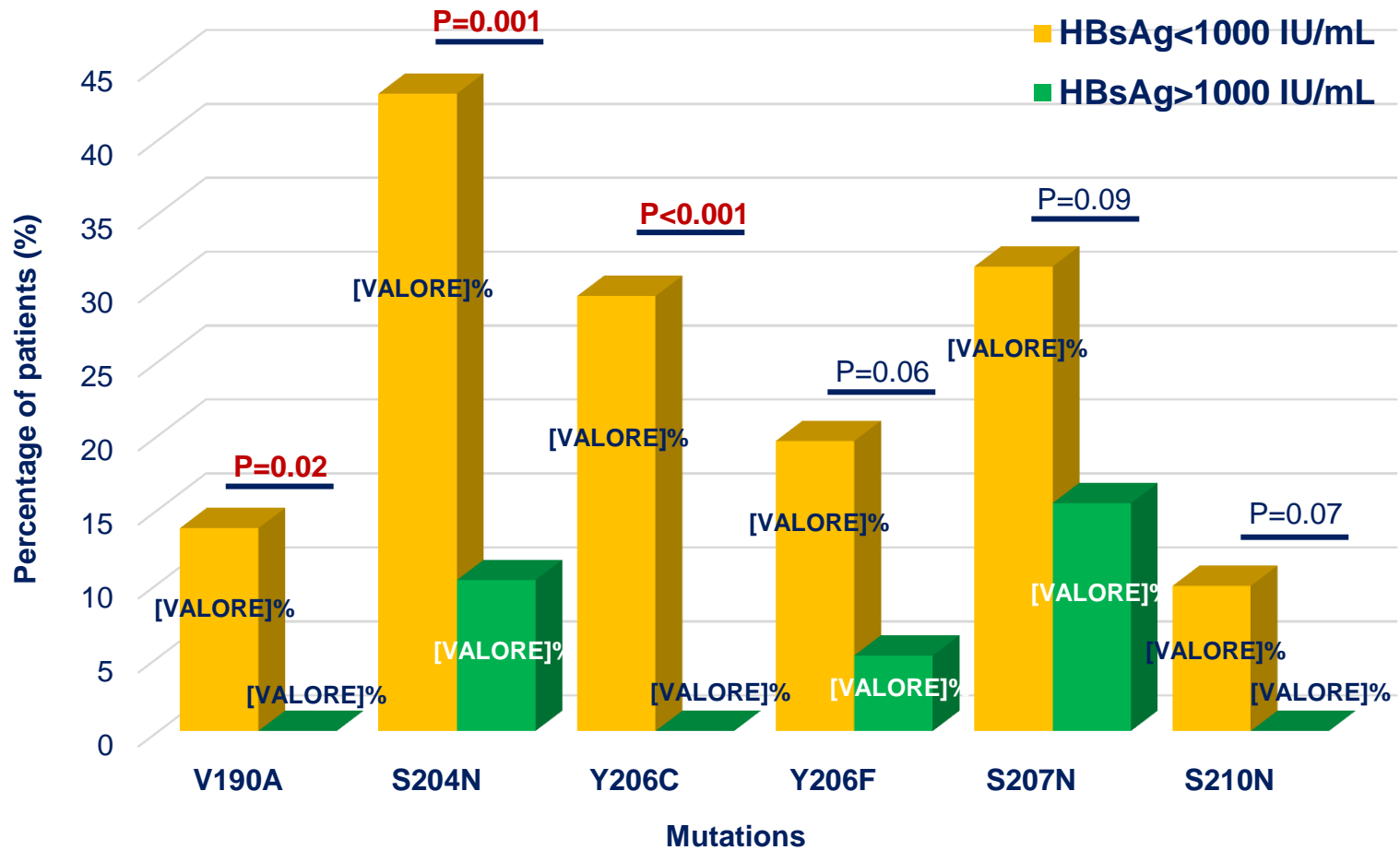

**HBsAg C-terminus domain
aa: 170-226**
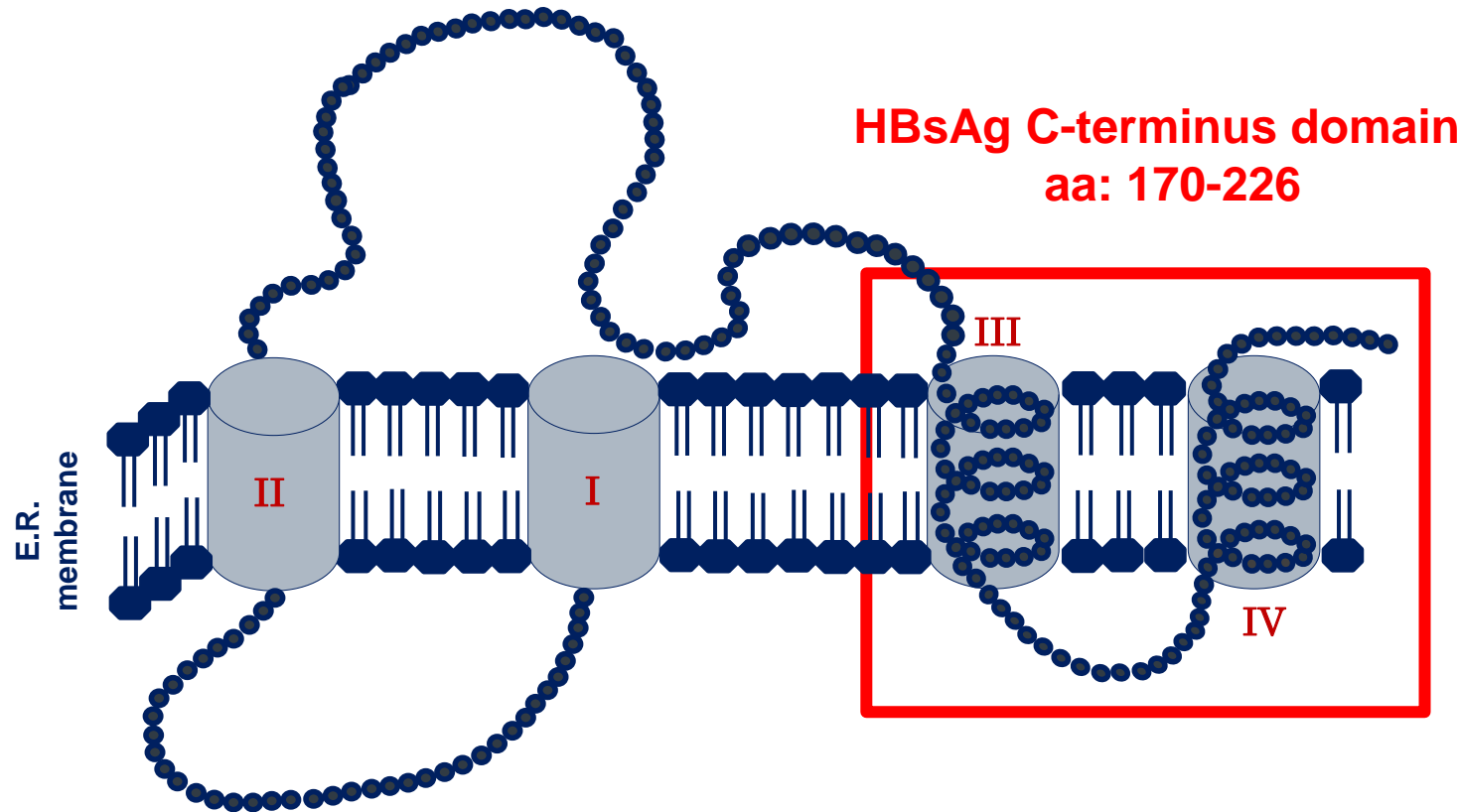
The figure reports the putative structure of the HBsAg by Stirk et al., 1992

In genotype D, low HBsAg levels correlate with a higher genetic variability in HBsAg C-terminus (⬇HBsAg ⬆genetic variability)
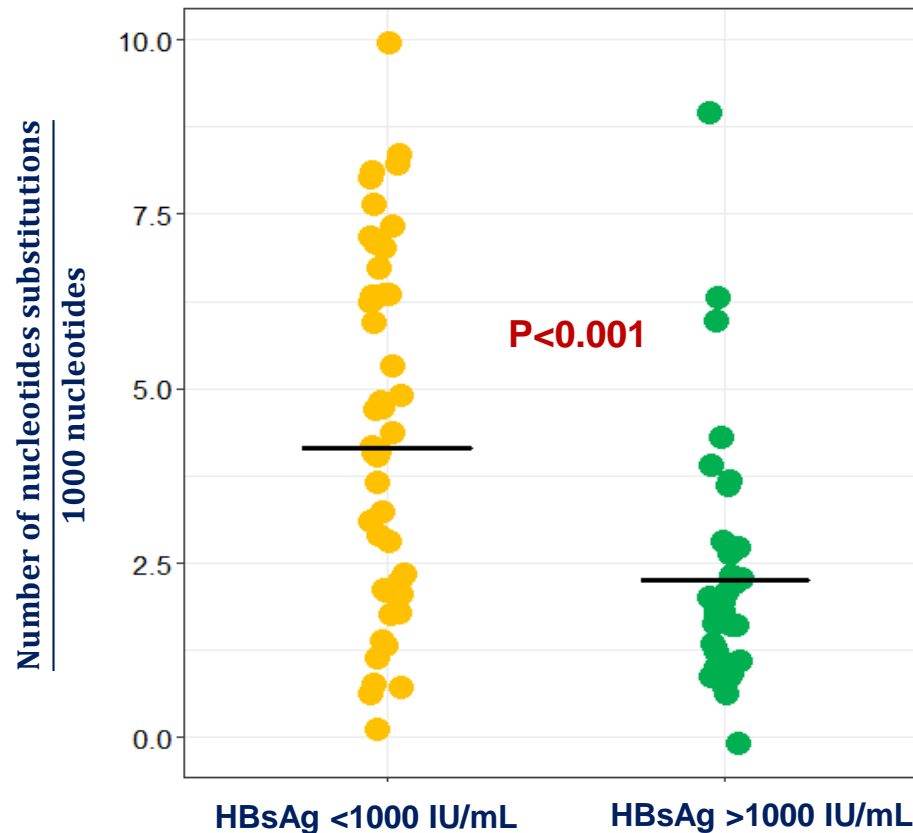

Scatter-plots report values of genetic distance (GD) for each patient. GD was used to define the extent of HBsAg C-terminus (aa: 170-226) variability in group-A patients infected with HBV genotype D stratifying according to HBsAg levels. Genetic distance was estimated as the extent of nucleotide substitutions per site determined by the Tajima-Nei model of MEGAv5. Statistically significant differences were assessed by Mann-Whitney Test.

Specific mutations in HBsAg C-terminus correlate with low HBsAg levels

The histogram shows the prevalence of mutations in HBsAg C-terminus, calculated in group A patients infected with genotype D, stratifying according to HbsAg <1,000 IU/mL or HBsAg >1,000 IU/mL. Statistically significant differences were assessed by Fischer Exact Test.

…..these mutations reside in III or IV HBsAg transmembrane domain
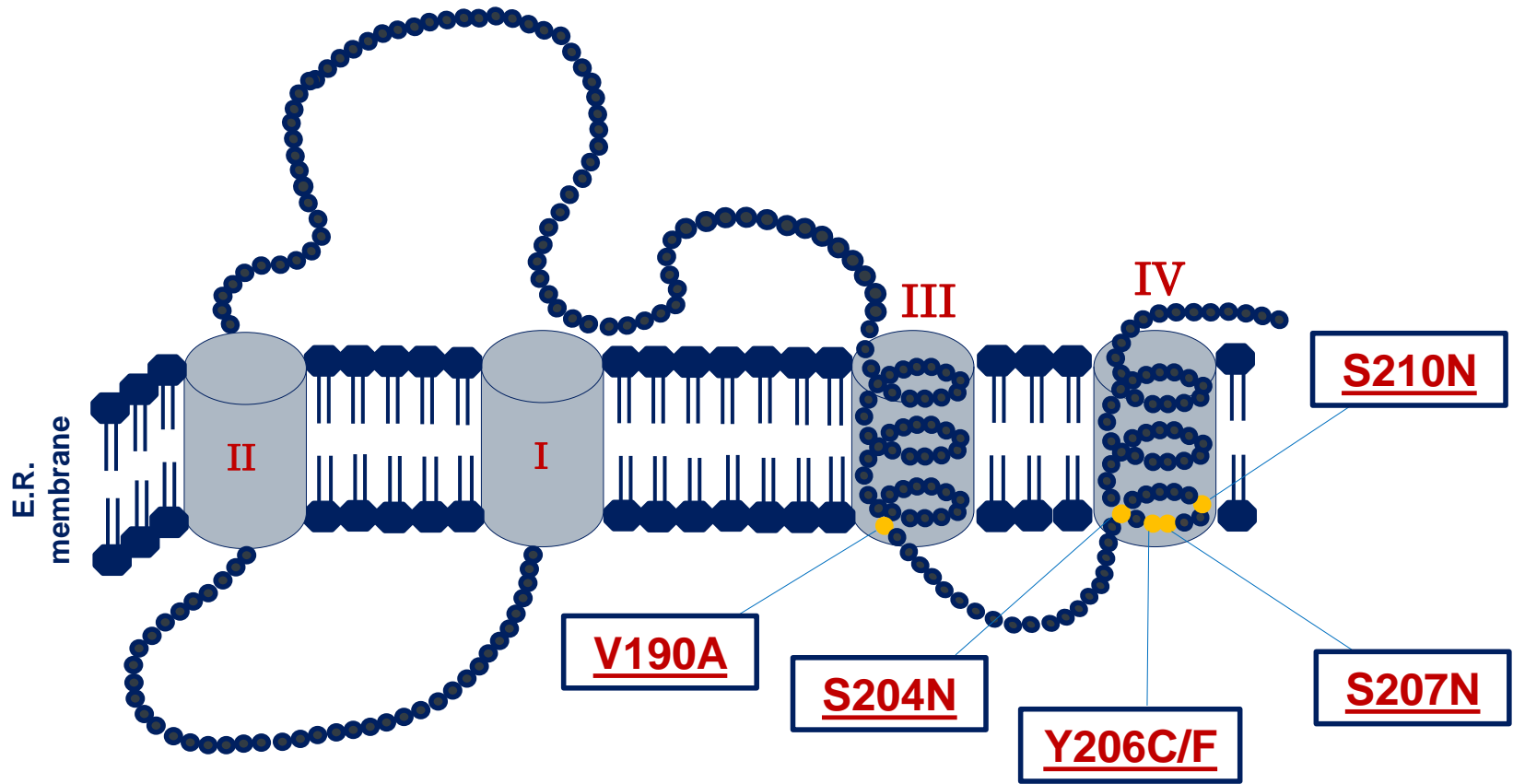
The figure reports the localization of 190, 204, 206, 207 and 210 positions in the putative structure of the HBsAg (Stirk et al., 1992)

- **Mutations associated with low HBsAg correlate with specific mutations in HBsAg C-terminus:**
 **- this suggests the existence of different mutational pathways underlying lower HBsAg levels**

Mutations associated with low HBsAg	Frequency[a], N(%)	Correlated mutations	Frequency[a], N(%)	Covariation Frequency[a], N(%)	Phi[b]	P-Value[b]
V190A	7 (7.7)	F220L	15 (16.6)	5 (5.5)	0.41	0.003
S204N	26 (28.8)	F220L	15 (16.6)	9 (10)	0.25	0.039
		L205P	5 (5.5)	5 (5.5)	0.36	0.005
Y206F	12 (13.3)	S210R	10 (11.1)	6 (6.6)	0.47	<0.001
S207N	22 (24.4)	Y200F	6 (6.6)	5 (5.5)	0.34	0.009
		P211R	4 (4.4)	4 (4.4)	0.36	0.007
S210N	5 (5.5)	F220L	15 (16.6)	4 (4.4)	0.40	0.006

Binomial-correlation coefficient (phi) was calculated to assess co-variation among mutations associated with with low HBsAg levels (< 1000 IU/mL). Statistically significant differences were assessed by Fischer Exact Test.
[a] Percentage were calculated on overall population of 90 HBV genotype D infected patients.
[b] Median (IQR) HBsAg was calculated in patients with each statistically significant pair of mutations.

Mutations associated with low HBsAg determine a decreased stability of HBsAg C-terminus and……
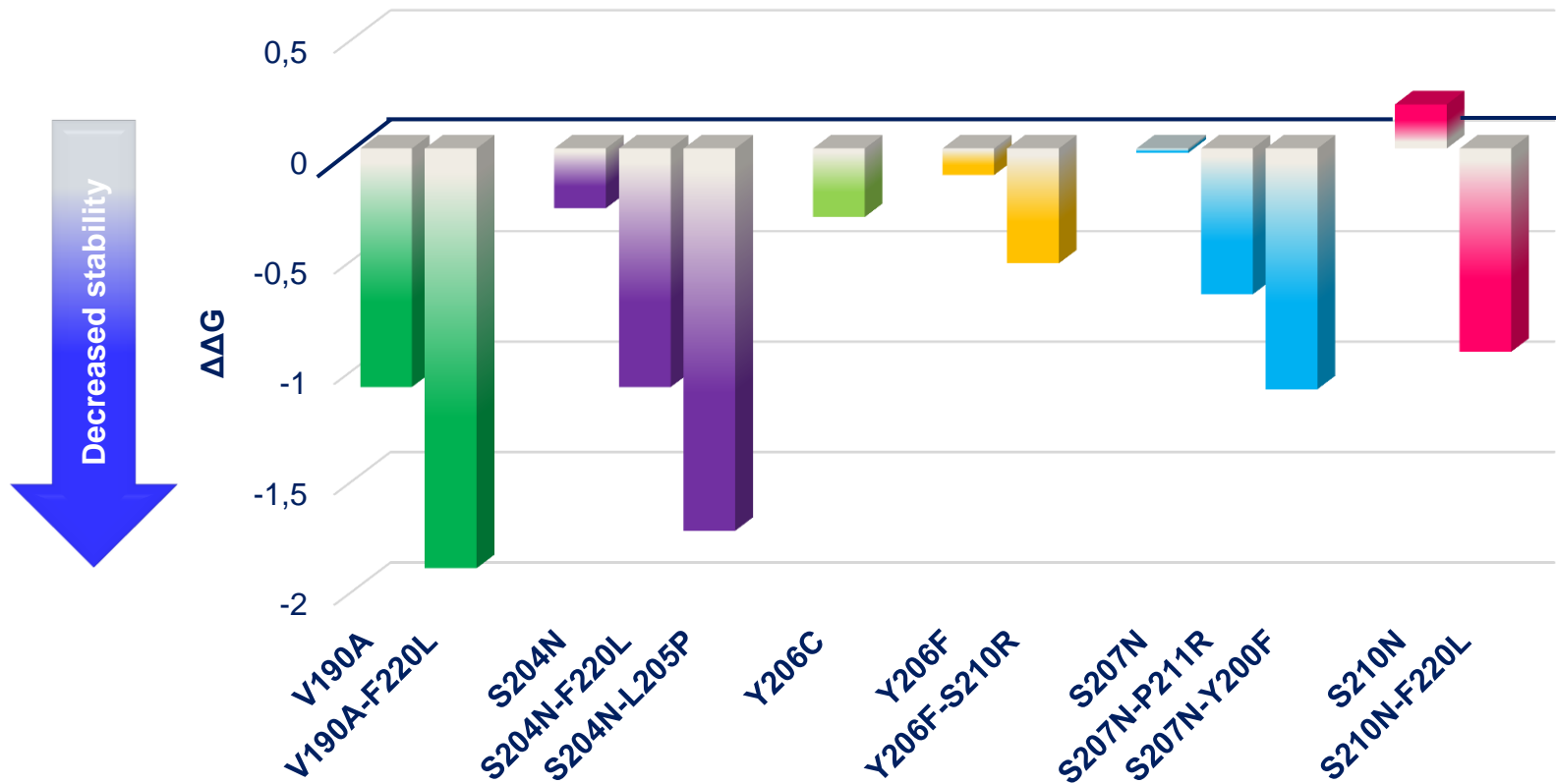
The histogram shows the variation of HBsAg C-terminus stability in presence of mutations (single or in pair) associated with low HBsAg. The HBsAg C-terminus stability was measured by calculating $\Delta\Delta G$ (mutated - WT). $\Delta\Delta G$ was calculated by STRUM (Quan et al., Bioinformatics, 2016).

………reduce the length of IV transmembrane α-helix

Mutations	IV α-helix (aa:START-STOP)	IV α-helix (LENGTH, n. of residues)
Wild Type HBsAg	L205-Y225	21
V190A	L209-Y225	18
V190A + F220L	L205-V224	20
S204N	L209-Y225	18
S204N + F220L	L209-Y225	18
S204N + L205P	L209-Y225	18
Y206F	F212-Y225	14
Y206F + S210R	L205-V224	20
S207N	L209-Y225	18
S207N + Y200F	L209-Y225	18
S207N + P211R	Y206-Y225	20
S210N	G202-V224	23
S210N + F220L	S204-Y225	22

The table reports the structural modifications in the length of IV α-helix in HBsAg C-terminus determined by I_TASSER.

Overall these data suggest a role of these mutations in altering the proper conformation of HBsAg C-terminus in ER membrane

Conclusions

➤ **HBsAg levels in HBV genotype-D are significantly lower than in genotype-A and -E** in different phases of HBeAg-negative chronic HBV-infection including inactive-carrier status.

➤ In **genotype-D infected patients**, specific **HBsAg C-terminus mutations significantly correlate with lower HBsAg-levels**, and profoundly **affect the conformation of this domain**. This can explain the lower HBsAg levels observed in genotype-D.

➤ In this setting, this supports **HBV-genotyping to better characterize patients with HBeAg-negative chronic HBV-infection**.

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