HBsAg immune-escape Mutations and Stop-Codons, Circulating among European HBV-chronically infected patients can impact on HBV transmission and disease progression

Luna Colagrossi
Department of Experimental Medicine and Surgery
University of Rome Tor Vergata
Combined Analysis of the Prevalence of Drug-Resistant Hepatitis B Virus in Antiviral Therapy–Experienced Patients in Europe (CAPRE)

Lucas Etienne Hermans,1,2,* Valentina Svičer,3,* Suzan Diepstraten Pas,2 Romina Salpini,3 Marta Alvarez,4 Ziv Ben Ari,5 Greit Boland,1 Bianca Bruzzone,6 Nicola Coppola,7 Carole Seguin-Devaux,8 Tomasz Dyda,9 Federico García,4 Rolf Kaiser,10 Sukran Köse,11 Henrik Krarup,12 Ivana Lazarevic,13 Maja M. Lunar,14 Sarah Maylin,15 Valeria Michel,16 Orna Mor,17 Simona Paraschiv,18 Dimitrios Paraskevis,19 Mario Poljak,14 Elisabeth Puchhammer-Stöckl,20 François Simon,16 Maja Stanoević,21 Kathrine Stene-Johansen,22 Nijaz Tihic,22 Pascale Trimoulet,23 Jens Verheyen,24 Adriana Vince,25 Nina Weis,26 Tülay Yalcinkaya,27 Snjezana Zidovec Lepej,25 Carlo Perno,3 Charles A. B. Boucher,2 and Annemarie M. J. Wensing1; on behalf of the HEPVIR working group of the European Society for Translational Antiviral Research (ESAR)

Background. European guidelines recommend treatment of chronic hepatitis B virus infection (CHB) with the nucleos(t)ide analogs (NAs) entecavir or tenofovir. However, many European CHB patients have been exposed to other NAs, which are associated with therapy failure and resistance. The CAPRE study was performed to gain insight in prevalence and characteristics of NA resistance in Europe.

Methods. A survey was performed on genotypic resistance testing results acquired during routine monitoring of CHB patients with detectable serum hepatitis B virus DNA in European tertiary referral centers.

Results. Data from 1568 patients were included. The majority (73.8%) were exposed to lamivudine monotherapy. Drug-resistant strains were detected in 52.7%. The most frequently encountered primary mutation was M204V/I (48.7%), followed by A181T/V (3.8%) and N236T (2.6%). In patients exposed to entecavir (n = 102), full resistance was present in 35.3%. Independent risk factors for resistance were age, viral load, and lamivudine exposure (P < .001).

Conclusions. These findings support resistance testing in cases of apparent NA therapy failure. This survey highlights the impact of exposure to lamivudine and adefovir on development of drug resistance and cross-resistance. Continued use of these NAs needs to be reconsidered at a pan-European level.

Keywords. antiviral drug resistance; genotypic resistance testing; hepatitis B virus; nucleos(t)ide analogs.
In HBsAg, immune-escape mutations hamper HBsAg recognition from antibodies, and stop-codons can increase HBV oncogenic potential.

No information is available on the circulation of these mutations in patients with chronic HBV-infection (CHB) exposed to nucleos(t)ide analogues (NA) in Europe.
To gain insight in prevalence and characteristics of immune-associated escape mutations (NA-induced or not), vaccine-escape mutations and stop-codons in HBsAg in Europe
A dataset of 935 patients was collected in the framework of the European Society for translational Antiviral Research (ESAR).

Dataset was submitted from 18 European countries from 1997 to 2012:

**Patients per region:**
- Western Europe 213 (22.8%)
- Northern Europe 43 (4.6%)
- Eastern Europe 134 (14.3%)
- Southern Europe 545 (58.3%)

**Inclusion criteria:**
- CHB with detectable HBV-DNA
- exposure to ≥1 NA
- availability of a HBsAg sequence
- age ≥18 years
Phylogenetic analyses of HBsAg sequences identified eight distinct HBV genotypes...
Among 935 patients with CHB, the attention was focused on 828 patients infected with HBV genotype A (N=255) and D (N=573).
We analyzed the presence of **23 immune-associated escape mutations** retrieved from http://hbv.geno2pheno.org, among them **7 are vaccine-escape mutations**.

The figure reports the localization of immune-associated mutations (in **red**) and vaccine-escape mutations (in **dark red**) in the putative structure of the HBsAg (Stirk et al., 1992). Mutations analyzed are those resulting from an amino acid substitution according to the reference sequence of genotype A or D (GenBank: AB076679.1 for genotype-A and GenBank: HM358338.1 for genotype-D).
We analyzed the presence of **NA-induced immune-escape** mutations I195M, I196S, and E164D (resulting from drug-resistance mutation M204V, M204I, and V173L) (Torresi, 2002).

The figure reports the localization NA-induced immune-escape mutations (in yellow) in the putative structure of the HBsAg (Stirk et al., 1992). Mutations analyzed are those resulting from an amino acid substitution according to the reference sequence of genotype A or D (GenBank: AB076679.1 for genotype-A and GenBank: HM358338.1 for genotype-D).
### Patients’ characteristics

<table>
<thead>
<tr>
<th></th>
<th>Overall (N=828)</th>
<th>Genotype-A (N=255)</th>
<th>Genotype-D (N=573)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median Age (IQR), years</td>
<td>45 (38.0-58.8)</td>
<td>45 (33.5-56.5)</td>
<td>49 (40-59)</td>
<td>0.001</td>
</tr>
<tr>
<td>Male, N(%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>584 (70.5)</td>
<td>183 (74.4)</td>
<td>401 (73.6)</td>
<td>0.810</td>
</tr>
<tr>
<td><strong>HBV-status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median HBV-DNA, log IU/ml (IQR)</td>
<td>4.4 (3.2-6.4)</td>
<td>4.7(3.3-6.9)</td>
<td>4.4 (3.2-6.3)</td>
<td>0.079</td>
</tr>
<tr>
<td>Median ALT, IU/L (IQR)</td>
<td>46.5 (32.0-78.0)</td>
<td>46 (30-80)</td>
<td>48 (32-78)</td>
<td>0.473</td>
</tr>
<tr>
<td>HBeAg positive, N(%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>183 (44.1)</td>
<td>71 (59.7)</td>
<td>112 (38)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Geographical origin, N(%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Europe</td>
<td>213 (22.8)</td>
<td>67 (26.3)</td>
<td>75 (13.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Northern Europe</td>
<td>43 (4.6)</td>
<td>10 (3.9)</td>
<td>16 (2.8)</td>
<td>0.519</td>
</tr>
<tr>
<td>Eastern Europe</td>
<td>134 (14.3)</td>
<td>99 (38.8)</td>
<td>32 (5.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Southern Europe</td>
<td>545 (58.3)</td>
<td>79 (31)</td>
<td>450 (78.5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentages are calculated on 791 patients with the datum available, 246 patients for gen-A and 545 for gen-D;  
<sup>b</sup> Percentages are calculated on 415 patients with the datum available, 119 patients for gen-A and 295 for gen-D.  
Statistically significant difference was assessed by Chi Square Test based on a 2x2 contingency table.
Most patients were exposed to LAM (85%): 86.1% infected with genotype A and 84.3% with genotype D.

The histogram reports the percentage of patients treated with Lamivudine (+/- Adefovir) versus those treated with Entecavir, Adefovir and/or Tenofovir, in both genotypes. Treatment information is available for 638 patients (N=230 for gen-A and 408 for gen-D).
We analyzed the presence of immune-associated escape and vaccine-escape mutations...
1 immune-associated escape mutation is revealed in 33% of patients with an increasing trend over time: **Genotype D** presents a higher number of patients with ≥1 immune-associated escape mutation.

Genotype D presents a higher number of patients with ≥1 immune-associated escape mutation (gen-A: 14.9% vs gen-D: 40.5%, P<0.001).

The overall percentage of patients with ≥1 immune-associated escape mutation ranges from 16.4% in 1997-2002 to 29.5% in 2009-2012, P=0.03.
Among them, T118A is present more frequently in gen-D than gen-A (19.7% vs 0.4%, P<0.001). This may be related to the fact that the Alanine (Ala, A) is the wild-type amino acid at position 118 for D2 subgenotype. Statistically significant differences were assessed by Chi Square Test based on a 2x2 contingency table.
>1 vaccine-escape mutation occurs in 14.9% of patients; among them, **P120S** is present more frequently in gen-D than A.

The histogram reports the percentage of patients with at least one vaccine-immune escape mutation. Percentages are calculated on 255 patients for genotype-A and 573 for genotype-D. Statistically significant differences were assessed by Chi Square Test based on a 2x2 contingency table.
This child became an HBsAg carrier, despite having anti-HBs levels considered to be protective (610 mIU/mL). → loss of proline can affects the antibody binding pattern

This mutation were not detected in the mothers by direct sequencing → They emerged de novo or they were present originally as a minor quasi species in maternal serum ??
We analyzed the presence of NA-induced immune-escape mutations....
>1 NA-induced immune-escape mutation occurs in 29% of patients (genotype A: 39.6% vs genotype D: 23.7%, P<0.001), with a stable temporal trend. The overall percentage of patients with >1 NA-induced immune-escape mutation remains stable over time from 38.4% in 1997-2002 to 30.0% in 2009-2012 (P=0.163).
The histogram reports the percentage of patients with at least one NA-induced immune-escape mutation. Statistically significant differences were assessed by Chi Square Test based on a 2x2 contingency table.

The vaccine-escape pattern I195M+E164D occurs more frequently in genotype A than D (7.1% vs 3.7%, P=0.03).
The vaccine-escape pattern $\text{I195M+E164D}$ in the HBsAg correspond to lamivudine resistance mutations $\text{M204V+L180M+V173L}$ in RT, that strongly reduce the binding affinity with neutralizing antibodies including those induced by the vaccine (Torresi et al. Virology 2002)
The profile of mutational clusters associated with lamivudine resistance can be constrained by HBV genotypes

Valentina Svicher¹,†, Caterina Gori²,†, Maria Trignetti¹, Michela Visca³, Valeria Micheli⁴, Martina Bernassola³, Romina Salpini¹, Guido Gubertini⁴, Roberta Longo³, Fosca Niero⁴, Francesca Ceccherini-Silberstein¹,², Giuseppe Maria De Sanctis⁵, Alberto Spanò³, Giuseppina Cappiello³, Carlo Federico Perno¹,²,*


…genotype A is more prone to develop rtM204V (corresponding to I195M in HBsAg) than genotype D at LAM failure.
We also analyzed stop codons in HBsAg....
Stop codons in the HBsAg can induce an oxidative stress thus favoring the neoplastic transformation of the hepatocytes.

Modified by Pollicino et al., Hepatology 2014
Stop-codons are observed in 8.5% of patients with a similar prevalence in genotype A and D.

The histogram reports the prevalence of stop codons on 828 patients: 255 patients for genotype-A and 573 for genotype-D.
They occur at 20 HBsAg positions, including 172 (corresponding to drug-resistance mutation rtA181T) and 182, known to increase HBV oncogenic potential.

Yeh CT et al., Hepatology 2000; Warner N. et al., Hepatology 2008
Lee SA. et al., J. Hepatology 2012; Jiang SS. et al., Biochim Biophys Acta. 2014

The figure reports the localization of stop codons (in green) in the putative structure of the HBsAg.
Stop codons at HBsAg positions 182 and 199 occur with a significantly higher frequency in genotype A, while stop codon at position 172 in genotype D.

The histogram reports the percentage of patients with at least one stop codon at HBsAg positions **172**, **182** and **199**. Percentages are calculated on 255 for genotype-A and 573 for genotype-D.
CONCLUSIONS

- Immune-escape mutations and stop-codons circulate in a relevant proportion of patients exposed to nucleos(t)ide analogues in Europe.

- Genetic backbone of genotypes and use of specific drugs can influence their emergence.

- These mutations could favor HBV transmission (potentially including vaccinated persons with inadequate anti-HBs titer) and predispose to a faster progression to liver cancer.
ACKNOWLEDGEMENTS

University of Rome Tor Vergata, Italy
Perno C.F.
Svicher V.
Salpini R.

Erasmus Medical Centre, The Netherlands
Hermans L.E.
Pas S.D.
Boucher C.A.B.

Hospital San Cecilio, Granada, Spain
Alvarez M.
Garcia F.

Sheba Medical Centre, Ramat Gan, Israel
Ben Ari Z.

University Medical Centre Utrecht, The Netherlands
Boland G.
Wensing A.M.J.

IRCCS AOU San Martino - IST, Genoa, Italy
Bruzzone B.

Seconda Università degli studi di Napoli, Italy
Coppola N.

CRP-Santé, Luxembourg, Luxembourg
Seguin-Devaux C.

Hospital of Infectious Diseases, Warsaw, Poland
Dyda T.

University of Cologne, Cologne, Germany
Kaiser R.
L. Sacco” Hospital, Milan, Italy
Micheli V.

Ministry of Health, Ramat Gan, Israel
Mor O.

Clinic of Infectious Diseases and Clinical Microbiology, Izmir, Turkey
Köse S.

Aalborg University Hospital, Aalborg, Denmark
Krarup H.

University of Belgrade, Belgrade, Serbia
Lazarevic I.

Slovenia
Stanojevic M.

University of Ljubljana, Ljubljana, Slovenia
Lunar M.M.

Hospital Saint Louis, Paris, France
Poljak M.

National Institute for Infectious Diseases “Matei Bals”, Bucharest, Romania
Hirschel B.

National and Kapodistrian University of Athens, Athens, Greece
Paraskevis D.

Medical University of Vienna, Vienna, Austria
Puchhammer-Stöckl E.

Norwegian Institute of Public Health, Oslo, Norway
Stene-Johansen K.

University Clinical Centre Tuzla, Tuzla, Bosnia and Herzegovina
Tihic N.

Centre Hospitalier Régional et Université "Victor Segalen", Bordeaux, France
Trimoulet P.

University Duisburg-Essen, Essen, Germany
Verheyen J.

University Hospital for Infectious Diseases, Zagreb, Croatia
Vince A.

Zidovec Lepej S.

Copenhagen University Hospital, Copenhagen, Denmark
Weis N.

Refik Saydam National Public Health Agency, Ankara, Turkey
Yalcinkaya T.