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Not uncommon: HBV genotype G co-infections among healthy European HBV carriers with genotype A and E infection

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Abstract

Background & Aims: HBV genotype G (HBV/G) is mainly found in co-infections with other HBV genotypes and was identified as an independent risk factor for liver fibrosis. This study aimed to analyse the prevalence of HBV/G co-infections in healthy European HBV carriers and to characterize the crosstalk of HBV/G with other genotypes.

Methods: A total of 560 European HBV carriers were tested via HBV/G-specific PCR for HBV/G co-infections. Quasispecies distribution was analysed via deep sequencing, and the clinical phenotype was characterized regarding qHBsAg-/HBV-DNA levels and frequent mutations. Replicative capacity and expression of HBsAg/core was studied in hepatoma cells co-expressing HBV/G with either HBV/A, HBV/D or HBV/E using bicistronic vectors.

Results: Although no HBV/G co-infection was found by routine genotyping PCR, HBV/G was detected by specific PCR in 4%-8% of patients infected with either HBV/A or HBV/E but only infrequently in other genotypes. In contrast to HBV/E, HBV/G was found as the quasispecies major variant in co-infections with HBV/A. No differences in the clinical phenotype were observed for HBV/G co-infections. In vitro RNA and DNA levels were comparable among all genotypes, but expression and release of HBsAg was reduced in co-expression of HBV/G with HBV/E. In co-expression with HBV/A and HBV/E expression of HBV/G-specific core was enhanced while core expression from the corresponding genotype was markedly diminished.

Conclusions: HBV/G co-infections are common in European inactive carriers with HBV/A and HBV/E infection, but sufficient detection depends strongly on the assay. HBV/G regulated core expression might play a critical role for survival of HBV/G in co-infections.

Abbreviations: BCP, basal core promoter; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, hepatitis B x protein; HCC, hepatocellular carcinoma; PC, precore; real-time PCR, real-time polymerase chain reaction; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; WB, Western blot.

*Michael Basic and Alica Kubesch contributed equally to this work.

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KEYWORDS

co-infection, core expression, genotype G, HBV, prevalence

Key points

- HBV genotype G was mainly found in co-infections with other HBV genotypes.
- HBV genotype G was identified as an independent risk factor for liver fibrosis.
- In this study coinfections with HBV genotype G were detected in 4-8% of healthy European HBV carriers infected with either HBV genotype A or E.
- Sufficient detection of HBV genotype G depends strongly on the genotyping assay.
- In vitro expression of HBV genotype G-specific core was enhanced when HBV genotype G was coexpressed with either HBV genotype A or E.

1 | INTRODUCTION

Chronic infection with Hepatitis B Virus (HBV) is one of the leading causes for progressive liver disease and development of hepatocellular carcinoma (HCC).[1] The individual course of disease is variable and depends on viral and host factors. To date, 10 different HBV genotypes (A-J) have been described based on at least 8% divergence in their nucleotide sequence. HBV genotypes have been found to be associated with varying risk of disease progression as well as differing response to interferon treatment.[2,3] HBV genotype G (HBV/G) was mainly found in co-infections with other genotypes, however, occasional mono-infections predominantly in blood donors were observed.[4,5] Co-infections with HBV/G were primarily reported in combination with HBV genotype A (HBV/A) and H from North and South America, Europe and in some cases from Asia.[6] These cases of co-infection were predominantly found among HIV co-infected men having sex with men.[7,8] Based on the similarity of a 30 base pair fragment in the preS region of HBV/G that is almost identical to HBV/E, a hypothesis on a plausible African geographic origin of HBV/G was proposed in 2005,[6,9] but co-infections or recombinants of HBV/G and HBV/E were not found in a host population until now. HBV/G exhibits several unique molecular characteristics such as a 36 nucleotide (nt) insertion in the core gene and two stopcodons in the precore region leading to HBeAg negativity in vitro and in mono-infections.[4,10] Recently, we observed an intracellular perinuclear accumulation of HBsAg-containing subviral particles (SVPs) in HBV/G expressing cells which is due to a HBV/G-specific sequence in the preS1 domain and explains the low HBsAg/HBV DNA ratio that was observed in HBV/G mono-infected patients.[4,10] In addition, the accumulation of HBsAg in the endoplasmic recticulum (ER) in combination with a diminished Nrf2 activation leads to higher levels of reactive oxygen intermediates (ROIs) in HBV/G when compared to HBV/A in vitro, which might impact pathogenesis and fibrogenesis. Indeed, several reports suggest HBV/G as an independent risk factor for establishment of liver fibrosis and disease progression in HBV/HIV co-infected patients.[11] However, how the presence of HBV/G varies among patients in specific phases of infection is mostly unknown. This study aimed to analyse the prevalence and virological characteristics of HBV/G infection in HBeAg negative patients without need for treatment, so called inactive carriers, as patients in this group are at risk for HBV reactivation and HBV/G (co-)infection might act as a potential risk factor for disease progression. In addition, the crosstalk of HBV/G with other genotypes was studied. For this purpose, hepatoma cell lines were transfected with bicistronic vectors co-expressing HBV/G with either HBV/A, HBV/D or HBV/E.

2 | METHODS

2.1 | Study populations

In total, 1192 inactive carriers were enrolled in the German Albatros trial (Clinical.Trial.gov: NCT01090531), of which 943 patients were included in the analysis based on their viral load with a set threshold of 10 IU/mL or more, to attempt determination of the genotype. Determination of the genotype was possible for a total of 560 patients (defined as 100% from this point on). For the main inclusion and exclusion criteria of the Albatros trial see the Supporting Information section. Serum or plasma of these patients was prospectively collected and stored at −80°C. Virologic parameters as HBV DNA viral load and qHBsAg were determined in clinical routine (n = 560; 100% and n = 525; 93.75%, respectively). The study was approved by local ethics committees, and written informed consent was obtained from all patients. The study was performed in accordance with the provisions of the *Declaration of Helsinki* and good clinical practice guidelines.

2.2 | HBV DNA extraction

Viral DNA was extracted from 200 μL of serum using the QIAamp DNA Blood Mini Kit (Qiagen) or the QIAamp UltraSens Virus Kit (using 1 mL of serum) (Qiagen) according to the manufacturer's protocol.

2.3 | Primers

Primers are described in the Supporting Information section.

2.4 | Genotyping

HBV genotyping was performed either in clinical routine or via direct sequencing of the polymerase region. A part of the *polymerase* gene was amplified by semi-nested PCR in two rounds using the specific primer HBV_381 (5′-TGCGGCGTTTTATCATCTTCCT-3′, nt381-402), HBV_840 (5′-GTTTAAATGTATACCCAAAGAC-3′, nt840-861) and HBV_801 (5′-CAGCGGCATAAAGGGACTCAAG-3′, nt801-822).[12] The amplification was carried out using a reaction volume of 20 μL following the manufacturer's protocol for the Fast Cycling PCR Kit (Qiagen). The PCR conditions are detailed in the Supporting Information section. The corresponding DNA was purified and subjected to sequencing PCR according to the manufacturer's protocol (BigDyeDeoxy Terminators; Applied Biosystems). The DNA was sequenced on a 3130xl Genetic Analyser (Applied Biosystems). Genotypes were determined by alignment to reference genomes (Accession numbers: HBV/A AY128092; HBV/B AB073858; HBV/C AB554021; HBV/D AY090453; HBV/E AB091256; HBV/G AP007264) and additional nucleotide BLAST (NCBI).

2.5 | HBV genotype G specific PCR

Detection of HBV genotype G was performed by semi-nested PCR as previously described by Kato et al (2001).[13] The 36 nucleotide insertion characteristics for genotype G were used to design a specific antisense primer and combined with two sense primer that bind upstream of the core region to create a hemi-nested amplification PCR targeting genotype G. For PCR conditions and primer see Supporting Information section. Amplified sequences were subsequently purified and used for direct sequencing to confirm the presence of HBV/G.

2.6 | Amplification and direct sequencing of the HBV BCP/precore and preS region

Parts of the region encoding the BCP and the precore gene (nt1600-2286) were amplified by nested PCR in two rounds. The preS region was amplified by seminested PCR in two rounds, respectively. The detailed conditions are given in the Supporting Information section. The corresponding DNA was subjected to sequence PCR according to the manufacturer's instructions (BigDyeDeoxy Terminators; Applied Biosystems). DNA was sequenced on a 3130xl Genetic Analyser (Applied Biosystems). A sensitivity level of about 15%-20% was assumed.

2.7 | Deep sequencing

A part of the BCP/precore, core and preS region was amplified by nested PCR in two rounds. PCR was done with the Fast Cycling PCR Kit (Qiagen). For detailed PCR conditions and primers used please see Supporting Information material. Samples were analysed by Illumina deep sequencing (Seq-It GmbH) as described.[14,15] A conservative 1% frequency cutoff (occurrence rate of the variant in percent of the quasispecies) was assumed for calling variants.

2.8 | Cell culture and cell treatment

The human hepatoma-derived cell line Huh 7.5 was cultivated as described.[16]

2.9 | Plasmids

To analyse the virological characteristics of genotype G in co-infection, in vitro bi-cistronic vectors containing either genotypes A, D or E in combination with G were created. For this purpose, a pIRES vector (Takara Bio) containing two multiple cloning sites (MCS) and an internal ribosome entry site (IRES) under the control of the CMV promoter was used. The first MCS holds the 1.2 × wildtype sequence of genotype G, while the second MCS contains either the 1.2 × wildtype sequence of genotype A, D or E using the IRES for translation initiation. GenScript performed cloning and sequencing. A 1.5× HBV/G, 1.5× HBV/A and a cDNA HBV/E genome served as control for size comparison of the surface proteins.

2.10 | Chemicals, antibodies and enzymes

Chemicals, antibodies and enzymes used for Western blotting and immunofluorescence microscopy are described in the Supporting Information section. HBsAg-ELISA (Enzygnost, Siemens) and HBcAg-ELISA (Cell Biolabs) were used according to the manufacturer's protocol.

2.11 | SDS-Page and Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page) and Western blot analysis was performed according to standard procedures.[17] The monoclonal SHBs-specific antibody (HB01, mouse) was kindly provided by Aurelia Zvirbliene (Lithuania).[18] The monoclonal MA18/7 LHBs-specific antibody was kindly provided by D. Glebe[18] (Giessen, Germany). Anti-β-actin for loading control in western blot analyses was ordered from Sigma-Aldrich and used in a 1:1000 dilution.

2.12 | Transfection of cells

For transient transfection, Huh 7.5 cells were transfected using a 4:1 ratio of X-tremeGene HP DNA Transfection Reagent (Roche) to DNA according to the manufacturer's instructions. Cells and supernatants were harvested 48 hours after transfection. For immunofluorescence microscopy, cells were transfected in 12-well plates and fixated 48 hours after transfection using 4% formaldehyde.

2.13 | Indirect immunofluorescence analysis

Immunofluorescence staining was performed as described[19] and analysed using a confocal laser scanning microscope and LAS X software (Leica Microsystems).

2.14 | Statistical analysis

χ^2 test or Fisher's exact test was used for group comparisons of categorical data and the Wilcoxon–Mann–Whitney U test was used for group comparisons of ordered data as appropriate. In vitro data were analysed using multiple *t* tests and corrected for multiple group comparisons using the Holm–Sidak method. Outliers were identified using the ROUT method ($Q = 1\%$). Statistical analysis was done with BiAS for Windows, version 11 (Epsilon) and GraphPad Prism 8 (GraphPad Software, Inc). *P* values $< .05$ were considered significant.

3 | RESULTS

3.1 | Genotype-dependent prevalence of HBV genotype G in inactive carriers

Overall, 1192 inactive carriers were enrolled into this European study cohort, of which 943 samples were used in an attempt to determine the viral genotype. Out of these patients, we determined the genotype for a total of 560 patients (defined as 100% from this point onwards). Our patients were predominantly infected with HBV genotype D (GTD: 52.7%; 295/560) and HBV genotype A (GTA: 28.8%; 161/560; subgenotype A1: 41/161, subgenotype A2: 83/161) (for detailed patient's demographics see Table 1).

In addition, HBV genotypes E (GTE: 8.5%; 48/560), B (GTB: 6.4%; 36/560) and C (GTC: 3.6%; 20/560) were identified infrequently (Table 1). No HBV genotype G infection was detected via genotyping by PCR of the polymerase region. By HBV/G-specific PCR of the core region and direct sequencing HBV/G was detected in 4% (7/161) and 8% (4/48) of patients infected with either HBV/A (subtype A1 2/7 and subtype A2 3/7) or HBV/E, respectively. In contrast, HBV/G was detected in only 0.3% (1/295) of patients infected with HBV/D and in none of the patients infected with HBV genotype B and C (Figure 1A). To determine the proportion of HBV/G variants in the viral quasispecies, we performed deep sequencing of the core region for an HBV/G-specific sequence (nt 6-41 in core). All samples that were tested positive for HBV/G via specific PCR were also positive for HBV-specific sequences in our deep sequencing analysis. Deep sequencing revealed that HBV/G was present as the major variant in the quasispecies of all patients infected with HBV/A, while it was found only in

TABLE 1 Demographics of patients with inactive carrier status

Parameter	Total, n (%)	GTA, n (%)	GTB, n (%)	GTC, n (%)	GTD, n (%)	GTE, n (%)
n[a]	560 (100)	161 (28.7)	36 (6.4)	20 (3.6)	295 (52.7)	48 (8.6)
Age (years, mean ± SD)	40.7 ± 11.9	45.0 ± 13.1	37.6 ± 9.7	39.0 ± 8.0	39.5 ± 11.4	37.5 ± 10.1
Male gender	228 (41.6)	62 (38.5)	13 (36.1)	3 (15.0)	131 (44.4)	19 (39.6)
Female gender	332 (58.4)	99 (61.5)	23 (63.9)	17 (85.0)	164 (55.6)	29 (60.4)
HBV DNA (mean log IU/mL ± SD)	2.8 ± 0.8	2.7 ± 0.8	3.0 ± 0.7	2.9 ± 0.5	2.9 ± 0.8	2.8 ± 1.0
qHBsAg (mean log IU/mL ± SD)[b]	3.2 ± 1.0	3.6 ± 0.8	2.3 ± 0.9	3.4 ± 0.9	3.0 ± 0.9	3.6 ± 0.7
ALT (mean U/L ± SD)	28.7 ± 14.0	29.3 ± 13.3	23.5 ± 11.7	25.9 ± 10.7	29.4 ± 14.8	27.5 ± 13.1
Ethnicity[c]						
White	404	130	2	1	268	3
Asian	84	9	34	19	21	1
African-American	60	14	0	0	6	40

Abbreviations: ALT, alanine transaminase; GTA/B/C/D/E, genotype A/B/C/D/E; HBV, hepatitis B virus; qHBsAg, quantitative hepatitis B surface antigen; SD, standard deviation.

[a]Patients where genotyping failed were excluded, yielding a total of 560/1192 patients which were further defined as 100%.

[b]qHBsAg was available from 525 patients.

[c]Ethnicity was available from 548 patients.

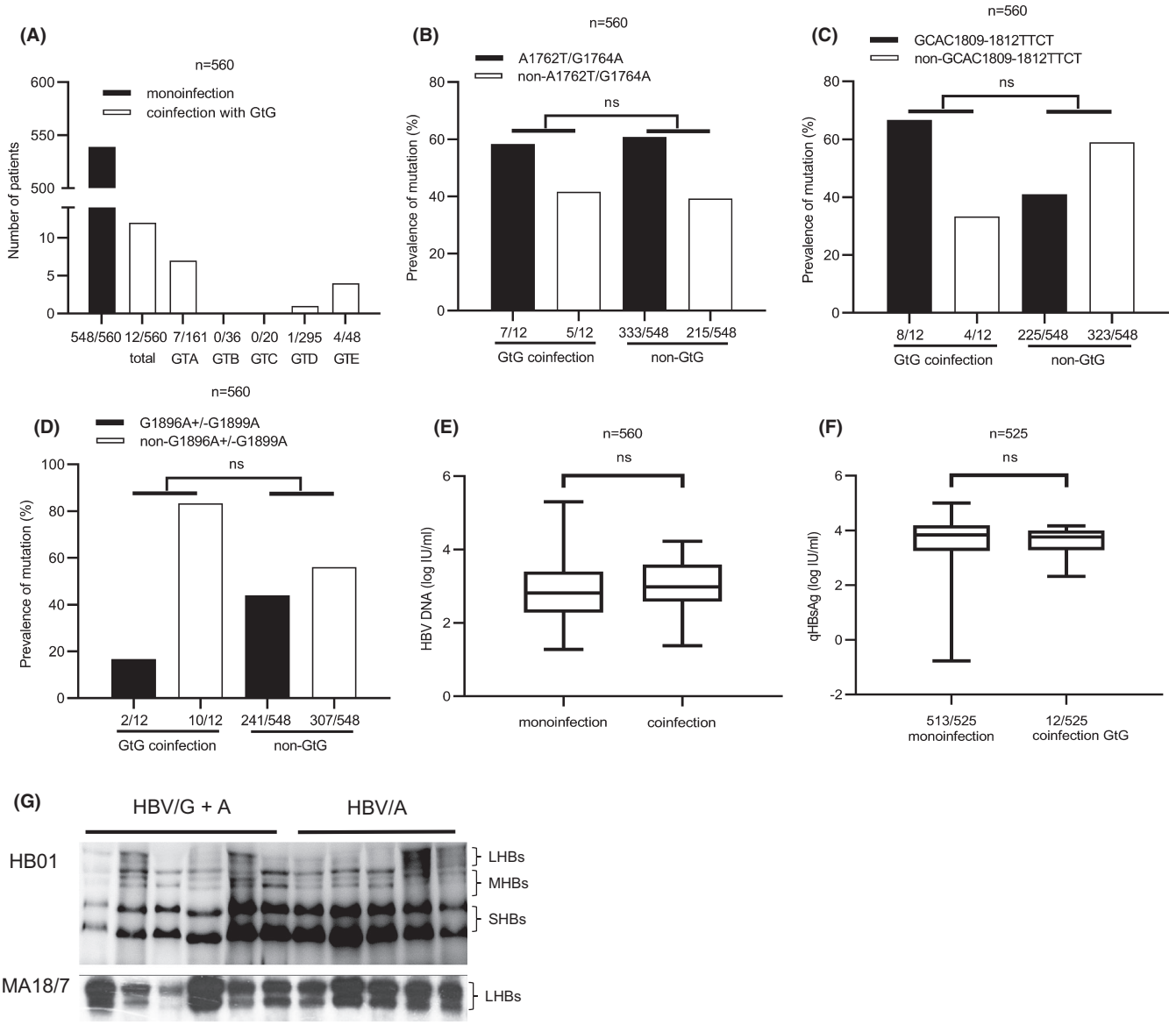

FIGURE 1 No significant association between HBV/G co-infection and common polymorphisms, HBV DNA levels or HBsAg levels (A-D) Prevalence of (A) HBV/G co-infection in total and per genotype, (B) BCP double mutation A1762T/G1764A, (C) quadruple mutation GCAC1809-1812TTCT and (D) PC mutation G1896A±G1899A among 560 patients with a chronic HBV infection (HBsAg carriers) with and without HBV/G co-infection from the Albatros cohort. (E, F) Association of HBV/G co-infection with (E) HBV DNA levels and (F) HBsAg levels in inactive carriers from the Albatros cohort. Data are shown as following: median (line inside the box), first and third quartiles (upper and lower limit of the box, respectively), and the highest and lowest values are represented by the top and bottom whiskers. A Fisher's exact test or Mann–Whitney U test was performed to determine statistical significance where appropriate, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. (G) Exemplary western blot using a HBsAg-specific antibody (HB01) and a LHBs-specific antibody (MA18/7) with the serum of patients with chronic HBV/A infection, either mono-infected or co-infected with HBV/G

low frequencies in three out of four patients co-infected with HBV/E (Table 2). In addition, the genotype G-specific nucleotide sequence in core was also detected by direct sequencing of the core region in all samples, in which HBV/G represents the major variant of the quasi-species. Of note, most likely due to low viral loads of our samples, co-infection with genotype G was only consistently detected when viral DNA was extracted via an extraction kit using a high input volume.

Taken together, these data indicate that although HBV/G co-infections are common with a frequency of 4%-8% in inactive carriers with HBV/A and HBV/E infection, genotyping PCR of the polymerase region failed to detect HBV/G in all cases. Interestingly, HBV/G was found to be the major variant in co-infection with HBV/A, while it represented only the minor variant in co-infection with HBV/E.

TABLE 2 Genotype G represents the major species in co-infection with genotype A

Patient no.	Co-infected genotype[a]	HBV-DNA (IU/mL)	HBsAg (IU/mL)	GTG detected by GTG-specific PCR and direct sequencing	GTG detected by PCR of BCP/PC/core and deep sequencing	Percentage of GtG in the viral quasi species
1	A2	16 900	6614	Yes	Yes	97
2	A	24	207	Yes	Yes	93
3	A1	494	2078	Yes	Yes	97
4	A	1740	1900	Yes	Yes	97
5	A1	6230	14 741	Yes	Yes	97
6	A2	34	10 040	Yes	Yes	71
7	A2	818	5774	Yes	Yes	95
8	D	4240	13 951	Yes	Yes	97
9	E	3090	5080	Yes	Yes	4
10	E	351	3.2	Yes	Yes	2
11	E	780	12 947	Yes	Yes	2
12	E	1126	18 144	Yes	Yes	77

[a]Subtype for HBV/A could only be determined for 5/7 patients.

3.2 | No significant association of HBV/G co-infection with common polymorphisms in basal core promoter, Precore and preS

The occurrence of HBV/G co-infection was correlated with the prevalence of common polymorphisms in basal core promotor, Precore and preS. Basal core promoter double mutation A1762T/G1764A was present in seven patients with HBV/G co-infection, which was comparable to the prevalence in mono-infected patients of our cohort (60.77%, n = 333, Figure 1B). The quadruple mutation GCAC1809-1812TTCT[20] was found in eight (66.67%) patients with HBV/G co-infection, which was tendentially higher compared to the prevalence of this polymorphism in mono-infected patients of our cohort (41.06%, n = 225, Figure 1C); however, this trend did not reach statistical significance. Precore (PC) mutation G1896A was found in 2 patients co-infected with HBV/G (16.67%) in comparison to 241 mono-infected patients (43.98%) in the cohort (Figure 1D). Again, this tendential lower prevalence of PC mutation G1896A did not reach statistical significance ($P = .07$). PreS1 mutations H51P and I84T, which were described to be characteristic for HBV/G[21] and contribute mechanistically to HBsAg retention in the endoplasmic reticulum,[10] were found only in 2 of the 12 samples (16.67%) with HBV/G co-infection by direct sequencing (Table 3). However, in these positive samples, this polymorphism was also confirmed as the major variant at this location in deep sequencing of the preS region (Table 3). In addition, by deep sequencing, these two polymorphisms were also observed in all other samples with HBV/G co-infection but in much lower frequencies.

3.3 | No impact of HBV/G co-infection on HBV DNA/HBsAg levels and molecular weight of secreted LHBs

As HBV/G is characterized by a decreased HBsAg/HBV DNA ratio in vitro and in mono-infected patients, HBsAg and HBV DNA levels in our HBV/G co-infected patients were analysed. However, no differences in HBV DNA level and quantitative HBsAg were observed among HBV/G co-infected patients and the overall cohort (Figure 1E,F). As a slightly elevated molecular weight of the specific signal for the large HBsAg (LHBs) in Western Blot (WB) analysis is characteristic for HBV/G derived LHBs,[10] WB analysis with a sHBsAg- and LHBs-specific antibody of patients sera was performed to analyse the origin of the LHBs detected in the sera (Figure 1G). In this analysis, no differences in the molecular weight of secreted LHBs were observed among HBV/A infected patients with and without HBV/G co-infection, which argues against an origin of the detected LHBs from HBV/G genomes.

3.4 | Higher HBsAg expression and release in cells co-expressing HBV genotype G in combination with A or D

To further investigate the impact of HBV/G-co-infection on molecular virology of other HBV genotypes and vice versa, we used

TABLE 3 Detection of genotype G specific polymorphisms in the preS region

Patient no.	Co-infected genotype[a]	HBV-DNA (IU/mL)	GTG detected in the preS region by direct sequencing	GTG detected in the preS region by deep sequencing	Percentage of the viral quasi species
1	A2	16 900	No	Yes	4
2	A	24	Yes	Yes	83
3	A1	494	No	Yes	6
4	A	1740	No	Yes	12
5	A1	6230	No	Yes	18
6	A2	34	No	Yes	14
7	A2	818	No	Yes	16
8	D	4240	Yes	Yes	74
9	E	3090	No	Yes	19
10	E	351	No	Yes	15
11	E	780	No	Yes	11
12	E	1126	No	Yes	1

[a]Subtype for HBV/A could only be determined for 5/7 patients.

bicistronic vectors, which harbour an HBV/G genome in combination with either HBV/A, HBV/D or HBV/E genomes. Therefore, we ensured the co-expression of both genotypes in each transfected cell. In WB analyses of lysates of transfected hepatoma cells using an HBs-specific antibody, we found that in case of all three constructs all HBs proteins (SHBs, MHBs and LHBs) were expressed (Figure 2A). Slightly higher HBsAg signals were observed via WB and HBsAg-specific ELISA in cells expressing HBV/G in combination with HBV/A in comparison to cells expressing HBV/G in combination with HBV/D (Figure 2A,C). Drastically lower amounts of HBsAg were detected in cells expressing HBV/G in combination with HBV/E in comparison with the two other genotypes. Similar results were observed for the relative amount of HBsAg in the supernatant (Figure 2B). Comparable levels of extracellular HBsAg for cells expressing HBV/G in combination with HBV/A and HBV/D were detected using an HBsAg-specific ELISA. However, co-expression with HBV/E did not show any detectable HBsAg in the supernatant and only a very weak signal in WB (Figure 2B,D). As the LHBs signal of HBV/G appears at a higher molecular weight in WB analysis than the LHBs of the other genotypes, expression of both genotypes can be confirmed using an LHBs-specific antibody.[10] For size comparison of the surface proteins an HBV/G, an HBV/A and an HBV/E genome were used. Here we observed that all bicistronic constructs produced both HBV/G LHBs and LHBs of the respectively co-expressed genotype (Figure 2E). However, the amount of HBV/G-LHBs was lower in comparison to the amount of HBV/A-LHBs and HBV/D-LHBs, respectively. As the size of HBV/D-LHBs differs with respect to the N-terminus of the PreS1 domain with a lack of 11/10 aa as compared to A or E, a shift in molecular weight was discernible when compared to the other genotypes.[22] The subcellular localization of LHBs in cells co-expressing HBV/G was analysed by confocal laser scanning microscopy using the LHBs-specific antibody MA18/7. As shown in Figure 3, the LHBs-specific signal in HBV/A expressing cells was distributed throughout the cytoplasm with visible dot-like accumulation in the perinuclear area, which is in accordance with previous publications.[10,23,24] In comparison, the observed pattern for HBV/D and HBV/E was a more uniform distribution in the cytoplasm with occasional dot-like accumulation for HBV/E, while cells expressing HBV/G displayed an exclusive perinuclear accumulation of LHBs.[10] Co-expression of HBV/G with HBV/A led to a change in the distribution pattern of LHBs towards an HBV/G-typical accumulation around the nucleus (Figure 3). A similar but less pronounced change in the distribution was observed when co-expressing HBV/G with HBV/D. Interestingly, the combination of HBV/G with HBV/E seemed to lead to a reduction in LHBs expression while leaving the distribution pattern of HBV/E unaffected.

3.5 | Predominant HBV/G-core detection in cells expressing HBV genotype G in combination with A or E

As HBV/G contains a 36 nt insert in core, the core-specific signal in WB analysis appears at a higher molecular weight than the core signal of the other genotypes and can therefore be identified as HBV/G-core. In lysates of cells expressing HBV/G and HBV/D from the bicistronic vector two core signals of both genotypes were observed in an almost comparable intensity in the same lane (Figure 4A). In contrast, in case of co-expression of HBV/G with either HBV/A or HBV/E from the bicistronic vectors a strong core signal was obtained, which corresponds to the molecular weight of HBV/G-core when compared to the control constructs. Moreover, in case of co-expression of HBV/G with HBV/A, only HBV/G-core and no HBV/A-core was detected. In case of co-expression with HBV/E, an additional weak signal was observed for HBV/E core. In WB analysis of supernatants core was barely detectable; however, a similar pattern was observed (Figure 4B). In case of co-expression of HBV/G with HBV/D, core protein of both genotypes was detected in

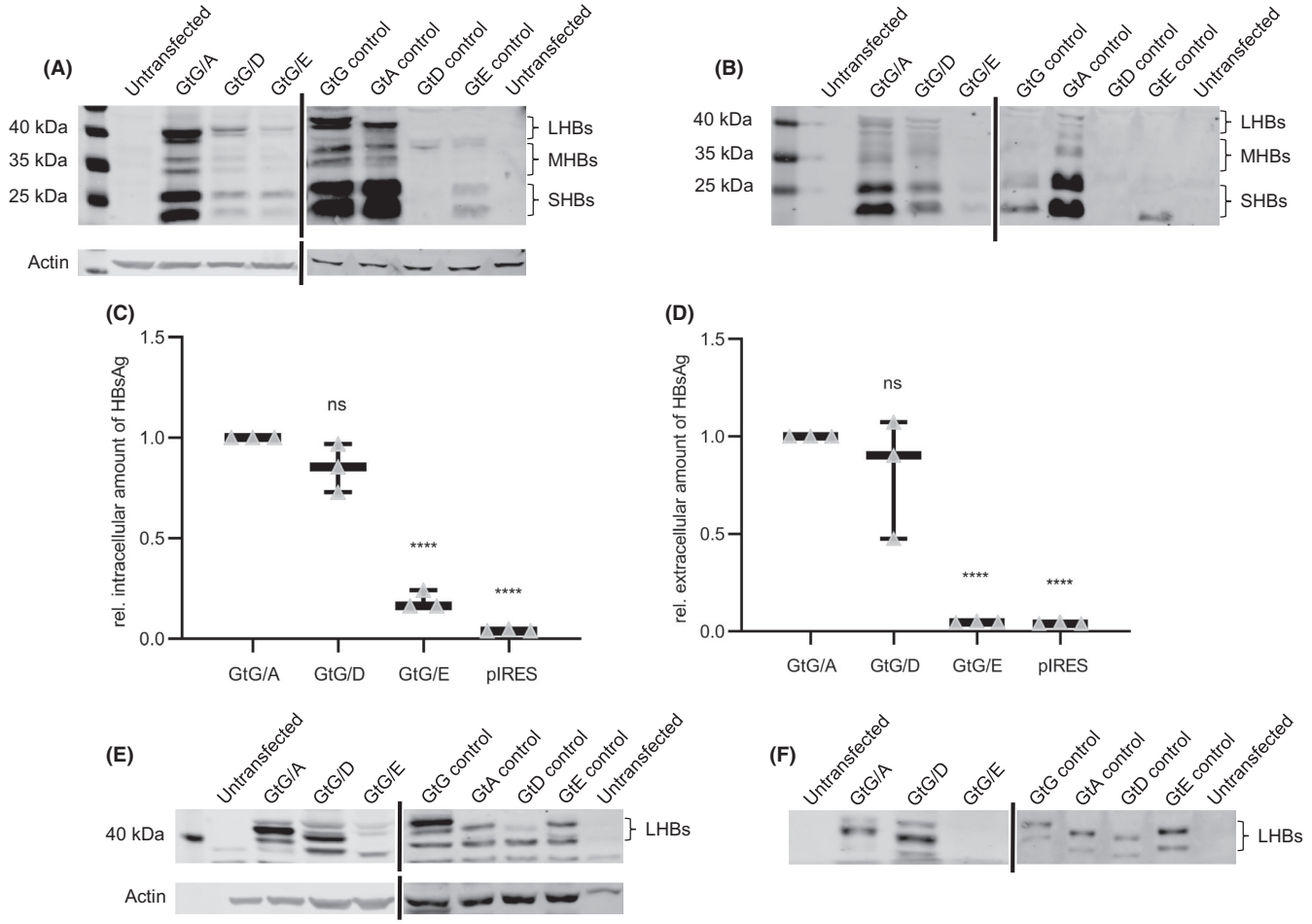

FIGURE 2 Higher levels of HBsAg in cells expressing HBV/G with either HBV/A or HBV/D. (A, B) Western blot analysis using an HBsAg-specific antibody (HB01) of (A) lysates and (B) supernatants. To better visualize protein size differences, constructs carrying the HBV/G, HBV/A, HBV/D and HBV/E genome were added as control. Mutations in the HBV/D control affect the epitope of the HB01 antibody preventing detection of HBsAg in western blot analysis. (C, D) HBsAg-specific ELISA of (C) lysates and (D) supernatants, n = 3. Data are shown as following: median (line inside the box), first and third quartiles (upper and lower limit of the box, respectively), and the highest and lowest values are represented by the top and bottom whiskers. Multiple *t* tests with the Holm–Sidak method were performed to correct for multiple group comparisons and to determine statistical significance, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$. (E, F) Western blot analysis using a LHBs-specific antibody (MA18/7) of (E) lysates and (F) supernatants

the supernatants as well. In contrast, in co-expression with HBV/A only HBV/G-core was observed. In co-expression with HBV/E predominantly HBV/G-core and in addition, small amounts of HBV/E-core were detected. Core-specific ELISA further confirmed the observed relative levels of core protein in the lysate and supernatant (Figure 4C,D). Co-expression with either HBV/A or HBV/E resulted in similar levels of intracellular core protein, while the combination with HBV/D yielded a 2.5-fold increase (Figure 4C). To further study the impact of HBV/G co-expression with the other genotypes on the overall replicative capacity, we measured intracellular HBV RNA levels of cell lysates (Figure 4E) and HBV DNA levels (Figure 4F) in the supernatants of co-expressing cells. However, no significant differences were detected in intracellular RNA and extracellular DNA levels among all constructs.

These data suggest that in HBV/A and HBV/G co-expressing cells HBV/G-core levels are enhanced, while core levels from the HBV/A genome are markedly diminished. A similar but less pronounced phenomenon was observed for co-expression with HBV/E. However, no impact of HBV/G in co-expression with the other genotypes on overall replicative capacity was observed.

4 | DISCUSSION

HBV genotype G is a mysterious genotype as infections with HBV/G were observed almost exclusively in co-infections with genotype A and H and HBV/G might be an independent risk factor for the establishment of liver fibrosis and disease progression.[6,11] However, also a few mono-infections that were detected, which were found mostly in blood donors, were HBeAg and (almost) HBsAg negative and did not lead to the establishment of a chronic HBV infection.[4,5] In our study, HBV/G co-infections were also detected in 12 of 560 patients with an HBeAg negative chronic HBV infection without the need for treatment. In line with other studies, we also observed HBV/A as the

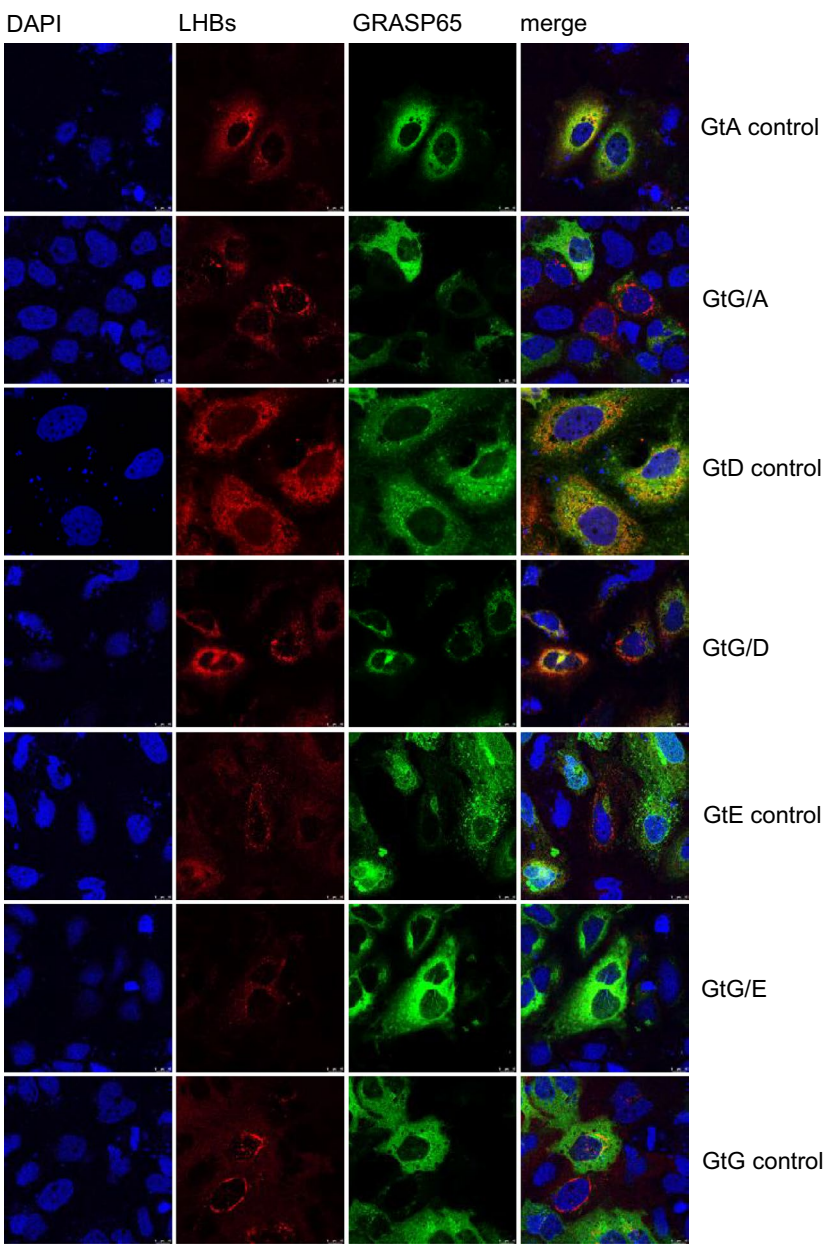

FIGURE 3 HBV/G typical LHBs distribution is more dominant in combination with HBV/A and HBV/D. CLSM analysis of cotransfected Huh 7.5 cells stained with the LHBs-specific antibody MA18/7 and labelled using a mouse-specific Alexa546 secondary antibody. Cells were transfected with the bicistronic constructs and HBV/A, HBV/D, HBV/E and HBV/G control constructs. Additionally, all cells were cotransfected using a construct encoding the golgi reassembly-stacking protein 65 (GRASP65) fused to GFP

main co-infection partner as 4% of all HBV/A infected patients were in addition co-infected with HBV/G. Interestingly, HBV/G was found with a substantial prevalence of 8% also in HBV/E infected patients, which is to our knowledge the first description of co-infections among these two genotypes. This might be explained on one hand by the relatively low percentage of HBV/E serum samples included in previous studies. On the other hand, our deep sequencing analysis showed that HBV/G was found in three out of four patients as the minor variant with a low percentage of only 2%-4% of the viral quasispecies, which might also impact the sensitivity for HBV/G detection in genotyping assays. In contrast, our deep sequencing analyses of patients coinfected with HBV/A and HBV/G revealed evidence that HBV/G represents the major variant with percentages of 71%-97% of the quasispecies in all seven patients co-infected with genotype A and G. This is in line with a study in which chimeric mice carrying human hepatocytes mono-infected with genotype G did not raise the detectable HBV DNA in serum. But when they were either superinfected or co-infected with genotype A, HBV DNA of genotype A developed, which was replaced almost completely by that of genotype G within a short period of time[25] leaving HBV/G as the major variant in the quasispecies of these mice. Despite the high percentage of HBV/G in the viral quasispecies in our co-infected patients, HBV/G was not detected by the genotyping PCR, which is used in the clinical routine of our hospital.[12] Indeed, the primers used for the nested PCR of this assay seem to be suboptimal for HBV/G detection in our low viral load samples. A possible solution to overcome the problem of missing HBV/G when using genotyping PCRs might be the use of different universal primer sets providing higher HBV/G sensitivity and specificity as, for example, suggested recently by Chook et al.[26] To further characterize the HBV/G co-infected samples, we analysed if HBV/G co-infection was associated with distinct patterns of frequent mutations in Precore (G1896A) and

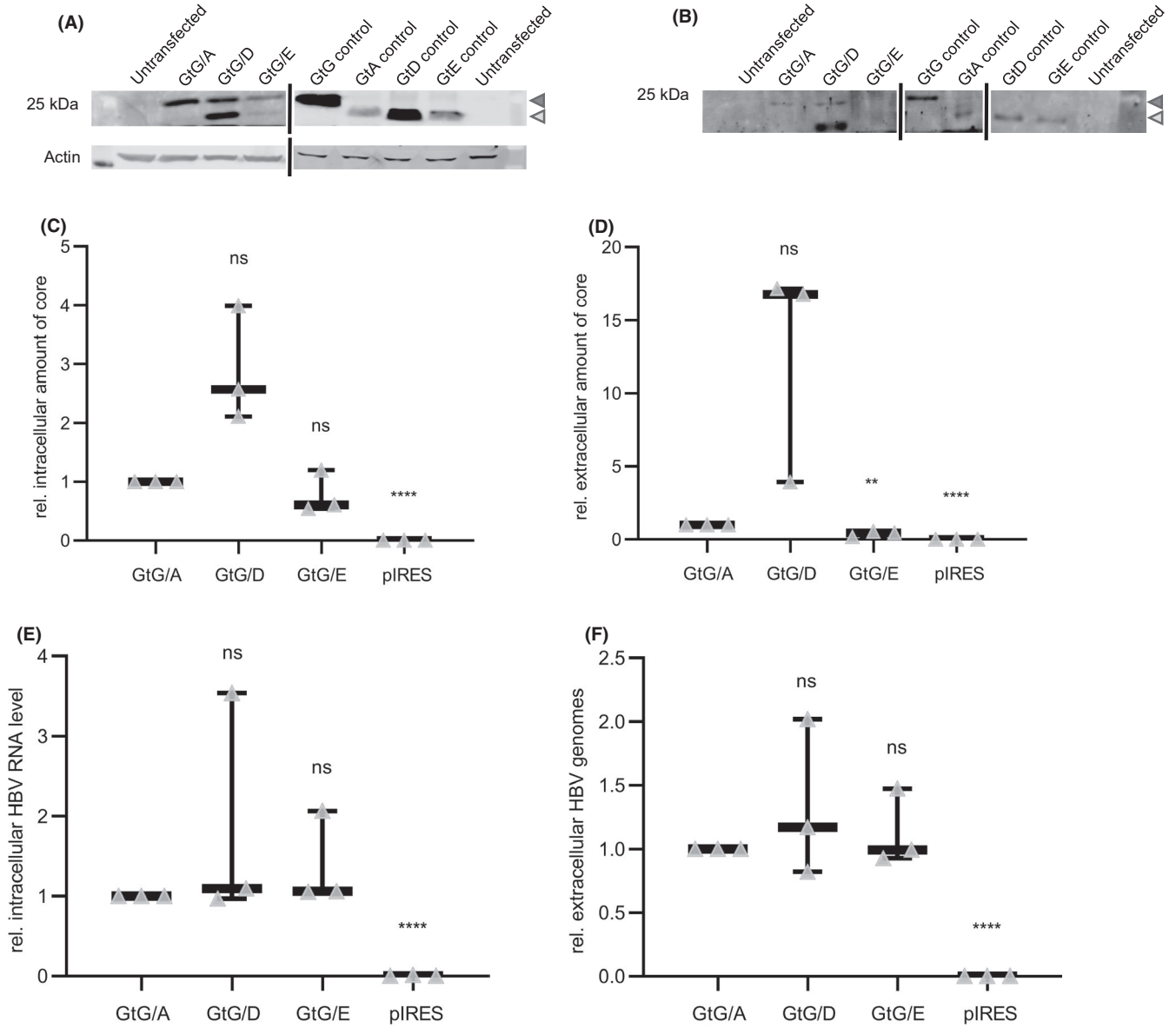

FIGURE 4 HBV/G-core is detected dominantly in combination with HBV/A and HBV/E. (A, B) Western blot analysis using a Core-specific antibody (K46) of (A) lysates and (B) supernatants of Huh 7.5 cells expressing the bicistronic constructs. To better visualize genotype-dependent core size differences, constructs carrying the HBV/G, HBV/A, HBV/D or HBV/E genome were added as control. The yellow arrowhead indicates the core protein size of genotypes A, D and E, while the red arrowhead indicates core protein translated by HBV/G. (C, D) Core-specific ELISA of (C) lysates, and (D) supernatants, n = 3. (E, F) Real-time PCR analyses of (E) intracellular HBV RNA and (F) extracellular HBV genomes, n = 3. Data are shown as following (C-F): median (line inside the box), first and third quartiles (upper and lower limits of the box, respectively), and the highest and lowest values are represented by the top and bottom whiskers. Multiple *t* test with the Holm–Sidak method was performed to correct for multiple group comparisons and to determine statistical significance. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

BCP (A1762T/G1764A), but no significant association was observed. However, this analysis was limited by the low overall prevalence of HBV/G co-infections among the overall cohort. Interestingly, the HBV/G-characteristic mutation in preS1, which is linked to HBsAg retention in the endoplasmic reticulum,[10] was found by direct sequencing only in 2 of the 12 samples with HBV/G co-infection. In contrast, by deep sequencing, this mutation was detected also in all other samples with HBV/G co-infection, but mostly as minor variants, which might indicate that in patients with long-lasting chronic HBV infection this HBV/G characteristic polymorphism might vanish over time.

Further, we observed that although HBV/G fails to secret significant amounts of HBsAg in vitro and in mono-infected patients, HBsAg levels were uncompromised in HBV/G co-infected patients even when HBV/G was found to be the major variant of the quasispecies. On one hand, this can be explained by the small percentage of HBV/G-co-infected patients in our cohort which makes it difficult to detect statistically significant differences. However, in

WB analysis of sera of HBV/A and HBV/G co-infected patients, only LHBs characteristic for HBV/A but no LHBs characteristic for HBV/G was detected. These observations indicate that while HBV/G is the major variant in the circulating quasispecies and represents, therefore, the main proportion of the cccDNA pool, at least serum LHBs must be derived from another genetic source, which is most probably integrated DNA of genotype A genome. This is in line with several recent reports, including one study from our cohort, indicating that integrated DNA has to be considered as a potent source for HBsAg expression in HBeAg negative patients.[27-31] As our in vivo analyses were restricted to inactive carriers, these observations cannot be generalized for patients at other stages of the disease.

In our in vitro analyses, we observed that in cells expressing HBV/G in combination with HBV/A and HBV/D all three HBs proteins are expressed and sufficiently released. This is in line with other in vitro studies showing that the HBV/G-characteristic incompetence of HBsAg release, which is due to the HBV/G-specific preS1-sequence, can be sufficiently compensated by co-transfection/-infection with HBV/A.[10,32] However, the HBV/G-characteristic perinuclear accumulation of HBsAg was still detectable in a fraction of co-expressing cells in our immunofluorescence analysis, which might favour ER-stress and genotype-specific pathogenesis.[10,11,25] In addition, we found that in HBV/G-HBV/E positive cells drastically lower amounts of HBsAg are expressed and released in comparison to cells positive for HBV/G in co-expression with either HBV/A or HBV/D. Lower transfection efficacy in case of HBV/G-HBV/E co-expression might be an explanation for this phenomenon, but comparable intracellular HBV RNA and comparable extracellular HBV DNA levels among all co-expressed genotypes argue against a lower transfection efficacy to be causative. Furthermore, an inhibitory impact of HBV/G on HBV/E-HBsAg expression or a genotype-characteristic lower expression level in case of HBV/E might be also possible, but further comparative in vitro studies are needed to sufficiently explain this phenomenon.

Very interestingly, we observed that in HBV/G and HBV/A co-expressing cells almost exclusively HBV/G originated core was detectable and released. In case of co-expression with HBV/E, we found a similar but less pronounced trend with a predominance of HBV/G core. However, in contrast to HBV/A also small amounts of HBV/E-core were detected. In sharp contrast, in HBV/G and HBV/D co-expressing cells core of both genotypes were detectable in comparable intracellular amounts and higher amounts of HBV/D-core were found extracellular. Therefore, our data suggest a regulating function of HBV/G favouring HBV/G core expression in case of co-expression with HBV/A and HBV/E but not in co-expression with HBV/D, which might favour HBV/G-dominant encapsidation and virus assembly. In other in vitro studies, it was observed that expression of core was enhanced in HBV/G due to the HBV/G-specific 32 nt insertion in the core gene.[33,34] Moreover, Li et al showed that the enhancing impact of the 32 nt insertion on core expression was mediated at the level of protein translation while no alteration on mRNA level was found[33] which suggests the HBV/G-specific 32 nt insertion as a regulating factor favouring HBV/G-core translation also in our study. However, our observation that core expression of HBV/G is favoured in case of co-expression with HBV/A and HBV/E but not in co-expression with HBV/D might help to explain why HBV/G was mostly found in co-infection with HBV/A and HBV/E in our cohort but not with HBV/D, which was seen also in several other studies.[8,35]

In conclusion, we found that although HBV/G co-infections are common with a frequency of 4%-8% in European inactive carriers with HBV/A and HBV/E infection, routine genotyping PCR of the polymerase region failed to detect HBV/G in all cases. While HBV/G was found to be the major variant in co-infection with HBV/A, HBV/G was predominantly found as the minor variant in co-infection with HBV/E. In our in vitro analysis, we observed that core expression from HBV/G is favoured when HBV/G is co-expressed with either HBV/A or HBV/E but not when it is co-expressed with HBV/D, which might help to explain why HBV/G was mostly found in co-infection with HBV/A and HBV/E.

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AUTHORS CONTRIBUTION

Guarantor of the article: Kai-Henrik Peiffer. Concept and design: KP, EH; acquisition of data: LK, MB, AK, JD and FF; analysis and interpretation of data: MB, AK, KP, EH, AB, MM, VM, CS, LK, FF, CG, JD and SC; drafting of the manuscript: KP and MB; critical revision of the manuscript for important intellectual content: EH, SC, SZ and CS; statistical analysis: MB.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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