Analysis of genetic and viral determinants of HBsAg levels in patients with chronic HBV infection

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SUMMARY

Single nucleotide polymorphisms (SNPs) in the interleukin 28B (IL28B) gene can influence the course of treated and untreated HBV infection. However, the correlation between different IL28B-SNPs and HBVDNA and quantitative HBsAg (qHBsAg) in chronic HBV infection remains to be fully elucidated. Patients with chronic HBV infection were analysed for qHBsAg, HBVDNA, HBV genotype and six IL28B-SNPs (rs12980275, rs8105790, rs8099917, rs7248668, rs12979860, rs10853728). Seventy patients were recruited: 80% Caucasian, 56% genotype D, 44% treated with nucleos(t)ide analogues, 11% cirrhotic, 37% inactive carriers (IC). Median (IQR) qHBsAg and HBVDNA were 3.2 log10 IU/ml (2.2-3.9) and 2.2 log10 IU/ml (0.3-3.3), respectively. Lower levels of qHBsAg were associated in the whole study population with rs12979860 CC *vs.* CT (p=0.05), rs12980275 AA *vs.* AG (p=0.04), rs8105790 TT *vs.* CT (p=0.05) and genotype D *vs.* A+E (p=0.01). rs8105790 TT was present in 81% of IC *vs.* 46% non-IC (p=0.005). These data were also confirmed in the untreated patients' subgroup. In multivariate analysis, IL28B-SNP haplogroups were associated with lower qHBsAg: CC/AA at rs12979860/rs12980275 (-0.70 log IU/mL, 95% CI -1.26;-0.14; p=0.01), CC/TT at rs12979860/rs8105790 (-0.78 log IU/mL, 95% CI -1.33;-0.23; p=0.006) and AA/TT at rs12980275/rs8105790 (-0.71 log IU/mL, 95% CI -1.27;-0.17; p=0.01) both in the whole population and in the untreated subgroup. Specific IL28-SNP haplogroups might be associated with lower qHBsAg.

Keywords: HBV, HBV DNA, Il28B, single nucleotide polymorphisms.

INTRODUCTION

One third of the world population shows serological evidence of previous or current Hepatitis B Virus (HBV) infection and approximately 257 million people are estimated to be chronic carriers of hepatitis B surface antigen (HBsAg) [1].
Different Chronic Hepatitis B (CHB) stages of variable duration are defined and there may be a spontaneous HBsAg clearance with loss or decrease to very low levels of serum HBV DNA; intrahepatic replication is usually persisting in these patients. The spontaneous clearance of HBsAg and the seroconversion to anti-HBs may occur in 1-3% of cases after several years of undetectability of plasma HBV DNA levels. Such patients retain a risk of reactivation during immunosuppression and some may still develop hepatocellular carcinoma (HCC) if liver disease progresses to cirrhosis [2, 3].
Serum HBsAg titer (qHBsAg) has been recognized as a biomarker for monitoring the natural history of infection and predictor of response to

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interferon (IFN)-based treatment. qHBsAg and the amount of covalently closed circular HBV DNA (cccDNA) in hepatocyte nuclei show a significant correlation to each other and maintain independent prognostic value in treatment-naïve individuals with CHB [4-7].

The decline of HBsAg is a surrogate marker predictive of Sustained Virologic Response (SVR) in HBeAg positive patients treated with pegylated-IFN: a rapid reduction of qHBsAg after HBV DNA suppression can identify those who will eventually clear HBsAg [4, 6, 8-11]. By contrast, during treatment with nucleos(t)ide analogues (NAs), HBsAg decline is much less pronounced, despite a marked suppression of serum HBV DNA.

In addition to other recognized factors influencing the natural evolution of CHB, host genetic factors with an impact on the natural history of HBV infection have been identified. Genome wide association studies (GWAS) have proven to be very useful in uncovering the host genetic factors that influence the clinical outcomes of HBV infection. For example, both class I and class II human leukocyte antigen (HLA) genes were implicated in persistence of HBV infection, suggesting a critical role for the surface antigen in HBV pathogenesis [12]. Similar to what previously shown for hepatitis C virus (HCV) infection, some single nucleotide polymorphisms (SNPs) in the gene coding for interleukin 28B (IL28B, also known as interferon lambda 3) on chromosome 19, also play a role for HBV [13-16]. Indeed, it has been recognized that specific IL28B SNPs are significantly associated with clearance of HBsAg, both spontaneous or induced by interferon treatment, as well as with seroconversion to anti-HBe induced by interferon treatment and with liver disease progression [17-20]. In particular, the CC genotype at SNP rs12979860 [21-27] in patients with CHB, is associated with a better virological response and higher levels of interferon lambda 3, suggesting that the presence of the C allele may have an inhibitory effect on viral replication by an improved production of endogenous interferon [26]. The objective of this study was to analyse the role of 6 different IL28B SNPs in a population with CHB, by evaluating their distribution and their association with serum levels of HBV DNA and qHBsAg.

PATIENTS AND METHODS

Patients

Between January 2014 and January 2015, 70 adult CHB patients (31 on NA treatment; 39 drug-naïve, of whom 26 were inactive carriers) were consecutively recruited at the Infectious Diseases University Unit, University Hospital of Siena, Italy. CHB

Table 1 - Patient characteristics (n=70).

Parameter	
Age, median years (IQR)	45.4 (CI 33.2-58)
Male gender, n (%)	46 (65.7)
Caucasian ethnicity, n (%)	56 (80)
Not Caucasian, n (%)	n. 9 (16.1) Sub-Saharan African n. 4 (7.1) Asian n. 1 (1.8) Afro-American
BMI (Kg/m^2), median (IQR)	23.8 (21.7-28.4)
Time from diagnosis of HBV infection, median (IQR)	5.3 (2.2-12.5)
Coinfections, n (%)	3 (4.3) HIV; 2 (2.9) HDV; 1 (1.4) HCV
Viral genotype, n (%)	9 (12.9) A; 1(1.4) B, 2(2.9%) C, 39 (55.7) D; 3(4.3) E; 16 (22.9) unknown
HBeAg carriers, n (%)	9 (12.9)
Inactive Carriers n (%)	26 (37)
On treatment with NAs, n (%)	31 (44.3) 23 (74.2) with ETV 7 (22.6) with TDF 1 (3.2) with LAM
HBV DNA <10 IU/mL, n (%)	23 (32.9)
qHBsAg $\log_{10}$ IU/mL, median (IQR)	3.2 (2.2-3.9)
HBV DNA, $\log_{10}$ IU/mL, median (IQR)	2.2 (0-3.3)
ALT, IU/mL, median (IQR)	26(16.7-34.2)
AST, IU/mL, median (IQR)	23 (19-30.2)
Alpha-fetoprotein, ng/dL, median (IQR)	1.9 (1.3-4.9)
Liver cirrhosis, n. (%)	8 (11.4)

Abbreviations: BMI (body mass index); HBeAg (HBV e antigen); qHBsAg (quantitative HBV s antigen), ALT (alanine aminotransferase); AST (aspartate aminotransferase); NAs (nucleos(t)ide analogues); ETV (Entecavir); TDF (Tenofovir disoproxil fumarate); LAM (Lamivudine).

was identified by persistent positivity of HBsAg for more than 6 months.
The stage of fibrosis was investigated before treatment by transient liver elastography (Fibroscan®. A clinical definition of cirrhosis, in cases without available liver elastography, was based on presence of ascites or encephalopathy, on laboratory abnormalities (coagulation markers deficit, hypoalbuminemia) or on abdominal ultrasound findings. Most patients had normal cytolysis enzymes levels and low level of HBV DNA replication as shown in Table 1. Among treated patients, samples were obtained after a median of 1.2 years (IQR 0.6-3.2) of NAs treatment. Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (7th revision, 2013) as reflected in a priori approval by the institutional human ethics committee.

Virological exams and SNPs genotyping
qHBsAg and HBV DNA were determined at baseline in all study enrolled subjects. Plasma HBV DNA was quantified using the Real Time PCR Abbott kit (Abbott Diagnostics Inc.). Quantification of serum HBsAg was performed using the Abbott Architect HBsAg kit (Abbott Diagnostics Inc.). HBsAg, HBeAg, anti-HBs, anti-HBe were detected by using the Elecsys system (Roche Diagnostics, Italy).
Whole blood and plasma samples were tested for human SNPs and HBV genotype, respectively. Total DNA was extracted automatically from 400 µL of peripheral whole blood and from 400 µL of plasma using the EZ1 DSP Virus Kit (Qiagen, Manchester, UK) according to the manufacturer's protocols. HBV genotyping was performed by sequencing a fragment of 956 bp in the viral polymerase region. Primers P478Fwd (5'-GCCTCATTTTGTGGGTCACC-3', coordinates 2805-2821 on the HBV reference strain AY123041.1) and P479Rev (5'-CAAAGCCCAAAAGACCCACAA-3', 998-1018) were used in the first 30-cycle PCR round and primers P480Fwd (5'-TGCTGGTGGCTC-

Table 2 - Primers for detection of IL28B polymorphisms.

Primer	*Sequence*	*From**	*To**	*SNP detected*	*Use*
P720_Fwd	CATTATGTCAGTGAAATAAGCCAGTCT	39241050	39241076	rs12980275 rs8105790	PCR and sequencing
P721_Rev	GTTGTCCACCTGGACCCAATGTC	39241928	39241950	rs12980275 rs8105790	PCR and sequencing
P591_Fwd	TTGTGCATATGTTTTCTGACTACC	39252349	39252372	rs8099917 rs7248668 rs10853728	PCR and sequencing
P723_Rev	GAATGATGTTACGCCCGACCTTCG	39254589	39254612	rs8099917 rs7248668 rs10853728	PCR and sequencing
P592_Rev	ATCCTAAATTGACGGGCCAT	39252653	39252672	rs8099917 rs7248668 rs10853728	Sequencing
P722_Fwd	CTGATTGGTAGGGTGGCAGGT	39253070	39253090	rs8099917 rs7248668 rs10853728	Sequencing
P724_Rev	ACAAAGTGAGACTGCATCTCTGG	39253331	39253353	rs8099917 rs7248668 rs10853728	Sequencing
P725_Fwd	AGTGACCCAAGCTACTTCTGCG	39254267	39254293	rs8099917 rs7248668 rs10853728	Sequencing
P581_Fwd	TCTTCCTCCTGCGGGACAAG	39247988	39248007	rs12979860	PCR and sequencing
P582_Rev	ACAATTCCCACCACGAGACCC	39248282	39248302	rs12979860	PCR and sequencing

*Homo sapiens chromosome 19, GRCh38.p12 Primary Assembly.

CAGTT-3', 58-74) and P481Rev (5'-CCCAAAA-GACCCACAATTCGTTG-3', 991-1013) were used in the second 35-cycle PCR round.

Six SNPs (rs12980275, rs8105790, rs8099917, rs7248668, rs12979860, rs10853728) located in the IL28B gene on human chromosome 19 were analysed by PCR amplification and sequencing of the region containing the site of interest. Specific primers for each SNP were designed using the Primer Blast tool (www.ncbi.nlm.nih.gov/tools/primer-blast) and the SNPs database (www.ncbi.nlm.nih.gov/projects/SNP/). Primer pairs were designed to generate three amplicons, each comprising 1 to 3 SNPs as shown in Table 2.

All PCR reactions were performed using 300 ng of genomic DNA, Go Taq Flexi Green Buffer 1x (Promega, Italia), 2.5 mM $MgCl_2$, 80 µM each dNTP, 5 pmol of each primer and 1U of GoTaq® Hot Start DNA polymerase (Promega, Italia) in a final volume of 30 µl. PCR conditions were 3' initial denaturation at 95°C followed by 35 cycles of 60°C for 30'', 72°C for 1', 95°C for 45'' and 7' of final extension at 72°C for rs12980275 and rs8105790; 3' initial denaturation at 95°C followed by 35 cycles of 62°C for 30'', 72°C for 30'', 95°C for 30'' and 7' of final extension at 72°C for rs12979860; 3' initial denaturation at 95°C followed by 35 cycles of 61°C for 30'', 72°C for 2' 40'', 95°C for 45'' and 7' of final extension at 72°C for rs8099917, rs7248668 and rs10853728.

The DNA fragments were analysed by bidirectional sequencing using the same primers used for PCR and additional internal primers where required, and the BigDye Terminator v1.1 Cycle Sequencing Kit according to the manufacturer's instructions (Applied Biosystems, USA). Sequencing products were then purified using the Big Dye X-Terminator® Purification kit (Applied Biosystems, USA) and analysed in an ABI 3130 XL Genetic Analyzer (Applied Biosystems, USA). HBV genotype was determined by submitting HBV sequences to http://hbv.bioinf.mpi-inf.mpg.de/.

Statistical analyses

ANOVA was used to investigate correlations between SNPs and qHBsAg or HBV DNA levels. Univariate and multivariate linear regression was used to identify predictors of qHBsAg and HBV DNA plasma levels. Linkage disequilibrium between allele pairs in the patient population was computed by chi-square analysis of cross-tabulations and compared with data obtained for the same set of SNPs by using the online tool LDlink available at https://ldlink.nci.nih.gov on the reference set made of five European populations.

RESULTS

The demographic and clinical characteristics of the whole study group of 70 patients are summarized in Table 1. The frequency of the six SNPs is shown in Table 3. As expected, there was a significant linkage between several SNP pairs, particularly any combination of rs8105790, rs8099917 and rs7248668 as well as the rs12980275/rs12979860 pair, which was also confirmed on the European population sets available through the LDlink online tool (Supplementary Tables 1 and 2).

Overall, we observed a significant association between lower qHBsAg and the CC *vs* CT allele at rs12979860 (p=0.05), the TT *vs* CT allele at rs8105790 (p=0.05) and the AA *vs* GG (p=0.05) or AG (p=0.04) allele at rs12980275 (Figure 1). The favourable allele TT at rs8105790 was also more frequent in the group of inactive carriers *vs* active carriers (21/26 [81%] *vs.* 20/44 [46%], p=0.005). Among those with known HBV genotype (n=39, 56%), the viral genotype D was significantly associated with lower qHBsAg *vs* genotype A or E, (mean difference -0.34 $\log_{10}$ IU/mL; 95% CI -1.55; -0.19; p=0.013) (Figure 2).

At univariate analysis, predictors of qHBsAg included: HBV DNA (+0.18 [95% CI 0.03; 0.33] per

Table 3 - Frequency of the six IL28B analysed.

SNP	*Allelic variant*		
rs12979860	CC	CT	TT
N (%)	26 (37.1)	30 (42.9)	14 (20)
rs8105790	TT	CT	CC
N (%)	41 (58.6)	27 (38.6)	2 (2.9)
rs12980275	GG	GA	AA
N (%)	13 (18.6)	32 (45.7)	25 (35.7)
rs8099917	TT	GT	GG
N (%)	43 (61.4)	25 (35.7)	2 (2.9)
rs7248668	GG	AG	AA
N (%)	44 (62.9)	24 (34.2)	2 (2.9)
rs10853728	CC	CG	GG
N (%)	7 (10.0)	29 (41.4)	34 (48.6)

Supplementary Table 1 - Distribution of cases with respect to SNP combinations in the 70 patients analysed.

rs12979860	*rs12980275*	*rs8105790*	*rs8099917*	*rs7248668*	*rs10853728*	*No. of cases*
CC	AA	TT	TT	GG	CC	6
CC	AA	TT	TT	GG	CG	10
CC	AA	TT	TT	GG	GG	8
CC	AG	CT	GT	AG	CG	1
CC	AG	TT	TT	GG	CG	1
CT	AA	TT	TT	GG	GG	1
CT	AG	CT	GT	AG	CG	9
CT	AG	CT	GT	AG	GG	8
CT	AG	CT	TT	GG	GG	1
CT	AG	TT	TT	GG	CG	5
CT	AG	TT	TT	GG	GG	6
TT	AG	TT	TT	GG	CG	1
TT	GG	CC	GG	AA	GG	2
TT	GG	CT	GT	AG	GG	6
TT	GG	CT	GT	GG	GG	1
TT	GG	CT	TT	GG	GG	1
TT	GG	TT	TT	GG	CG	2
TT	GG	TT	TT	GG	GG	1

Supplementary Table 2 - Association between SNP pairs within the group of 70 patients analysed and within the European data sets available at https://ldlink.nci.nih.gov (n = 1,006), as measured by chi-square associated P value of cross-tabulations.

SNP pair	*Chi-square and associated P value in the group of 70 patients analysed*	*Chi-square and associated P value in the reference European population*
rs12980275 and rs8105790	35.66, <0.0001	478.96, <0.0001
rs12980275 and rs8099917	32.35, <0.0001	435.67, <0.0001
rs12980275 and rs7248668	30.95, <0.0001	435.67, <0.0001
rs12980275 and rs12979860	118.94, <0.0001	852.49, <0.0001
rs12980275 and rs10853728	18.16, 0.001	152.31, <0.0001
rs8105790 and rs8099917	131.80, <0.0001	923.53, <0.0001
rs8105790 and rs7248668	127.98, <0.0001	923.53, <0.0001
rs8105790 and rs12979860	31.14, <0.0001	457.90, <0.0001
rs8105790 and rs10853728	8.33, 0.080	89.16, <0.0001
rs8099917 and rs7248668	135.67, <0.0001	1006.00, <0.0001
rs8099917 and rs12979860	28.09, <0.0001	430.90, <0.0001
rs8099917 and rs10853728	6.76, 0.149	88.79, <0.0001
rs7248668 and rs12979860	27.01, <0.0001	430.89, <0.0001
rs7248668 and rs10853728	6.15, 0.188	88.79, <0.0001
rs12979860 and rs10853728	16.12, 0.003	135.85, <0.0001

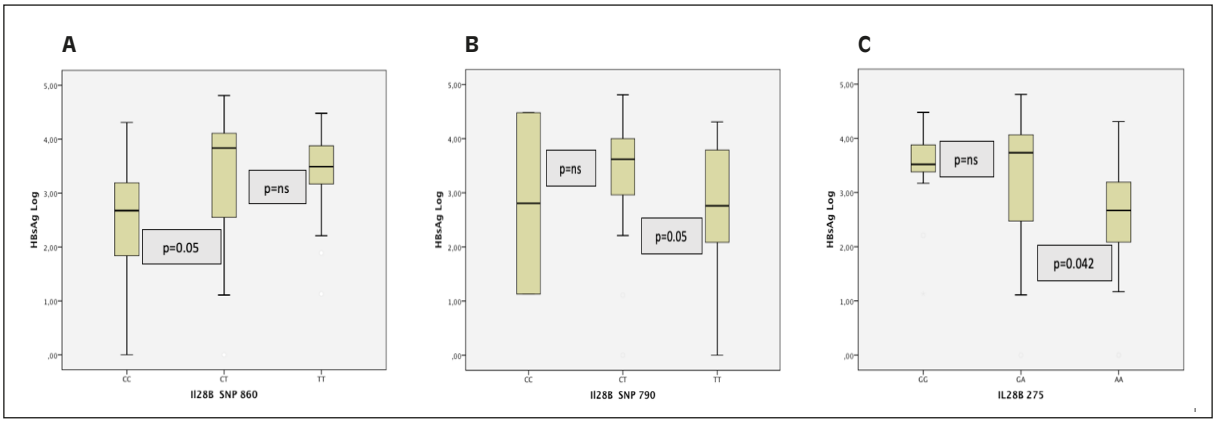

Figure 1 - a) Correlation between SNP rs12979860 and qHBsAg; b) Correlation between rs8105790 and qHBsAg; c) Correlation between rs12980275 and qHBsAg.

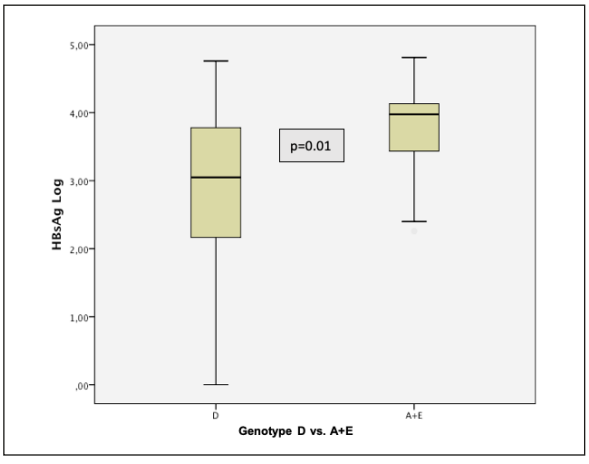

Figure 2 - Impact of viral genoype on qHbsAg.

1 $\log_{10}$ increase; p=0.023), viral suppression (-0.81 [95% CI 1.64; +0.009], p=0.052), higher ALT values (+0.13 per 10 IU/mL increase, p=0.04; [95% CI 0.004; 0.262]) and AST values (+0.20 per 10 IU/mL increase, p=0.06; [CI -0.006; +0.41]), not confirmed at multivariate analysis. At multivariate analysis, adjusting for viral genotype, genotype TT at rs8105790 was independently associated with lower qHBsAg (p=0.01) while genotype CC at rs12979860 and AA at rs12980275 showed a trend towards lower qHBsAg levels (p=0.08 and p=0.06, respectively). Combining the SNPs associated with lower levels of qHBsAg, we identified favourable haplogroups. In particular, the following favourable SNP genotype combinations associated with lower qHBsAg were identified: AA at

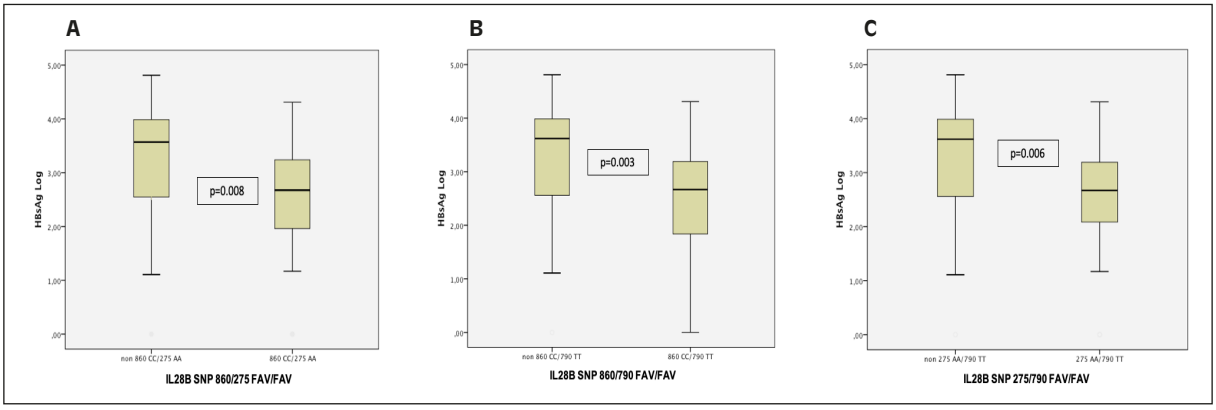

Figure 3 - Correlation between favourable haplogroups and qHBsAg. a) Correlation with CC/AA at rs12979860/rs12980275; b) Correlation with CC/TT at rs12979860/rs8105790; c) Correlation with AA/TT at rs12980275/rs8105790.

rs12980275 plus TT at rs8105790 (p=0.006), CC at rs12979860 plus AA at rs12980275 (p=0.008) and CC at rs12979860 plus TT at rs8105790 (p=0.003) (Figure 3). These haplogroups were independently associated to lower qHBsAg levels after adjusting for viral genotype at multivariate analysis. Namely, haplogroup CC/AA at rs12979860/rs12980275 was associated with mean adjusted difference of -0.70 $\log_{10}$ IU/mL (95% CI -1.26; -0.14, p=0.01), CC/TT at rs12979860/rs8105790 with -0.78 $\log_{10}$ IU/mL (95% CI -1.33; -0.23; p=0.006) and AA/TT at rs12980275/rs8105790 with -0.71 $\log_{10}$ IU/mL (95% CI -1.27; -0.17; p=0.01).

Among the 39 untreated patients (Table 4), we found a significant association between lower qHBsAg and CC *vs.* CT at rs12979860 (p=0.05), GG *vs.* AA at rs12980275 (p=0.048) and CG *vs.* CC (p=0.011) and GG *vs.* CC (p=0.022) at rs10853728 (Figure 4). Moreover, favourable SNPs genotype combinations associated with lower qHBsAg were confirmed also in this subgroup: AA at rs12980275 plus TT at rs8105790 (p=0.01), CC at rs12979860 plus AA at rs12980275 (p=0.02) and CC at rs12979860 plus TT at rs8105790 (p=0.02) (Figure 5). These haplogroups showed an independent association with lower qHBsAg at multivariate analysis: hap-

Table 4 - Characteristics of untreated patients (n=39).

Parameter	
Age (yy) (IQR)	40.1 (IQR 30.8-53.1)
Male gender, n (%)	17 (43.6)
Caucasian ethnicity, n (%)	33 (84.6)
BMI (Kg/m^2) (IQR)	24.6 (21.7-26.7)
Time from diagnosis of HBV infection (yy) (IQR)	4.1 (1.7-9.5)
Coinfections, n (%)	1 (2.6) HIV; 1 (2.6) HDV
Viral genotype, n (%)	27 (69.1) D; 7 (18) A; 1 (2.6) B; 1 (2.6) E; 3 (7.7) unknown
HBeAg carriers, n (%)	3 (7.7)
qHBsAg $\log_{10}$ IU/mL (IQR)	2.7 (2.1-3.9)
HBV DNA, $\log_{10}$ IU/mL (IQR)	2.9 (2.1-3.6)
ALT, IU/mL (IQR)	26 (17-34)
AST, IU/mL(IQR)	22 (19-28)
Alphafetoprotein, ng/dL(IQR)	2.1 (1.6-3.5)
Liver cirrhosis, n. (%)	1 (2.6)

Abbreviations: BMI (body mass index); HBeAg (HBV e antigen); qHBsAg (quantitative HBV s antigen), ALT (alanine aminotransferase); AST (aspartate aminotransferase); yy (years).

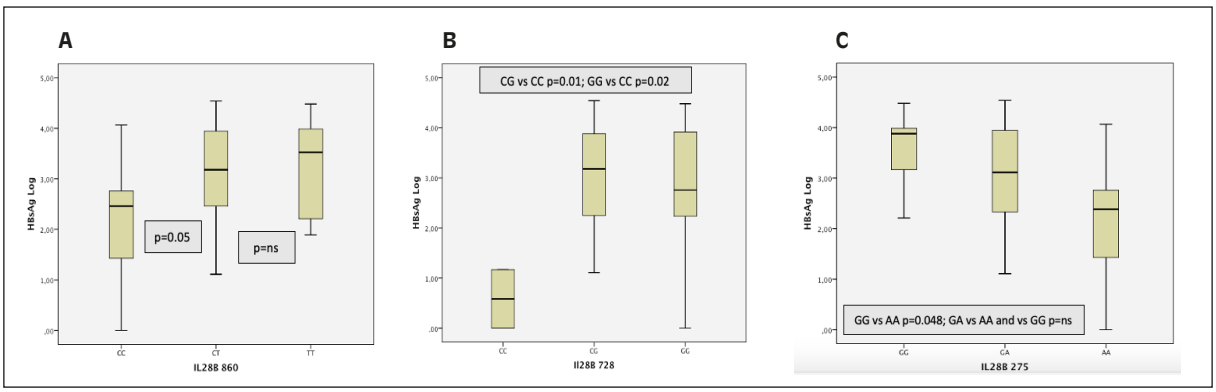

Figure 4 - a) Correlation between SNP rs12979860 and qHBsAg; b) Correlation between rs10853728 and qHBsAg; c. Correlation between rs12980275 and qHBsAg.

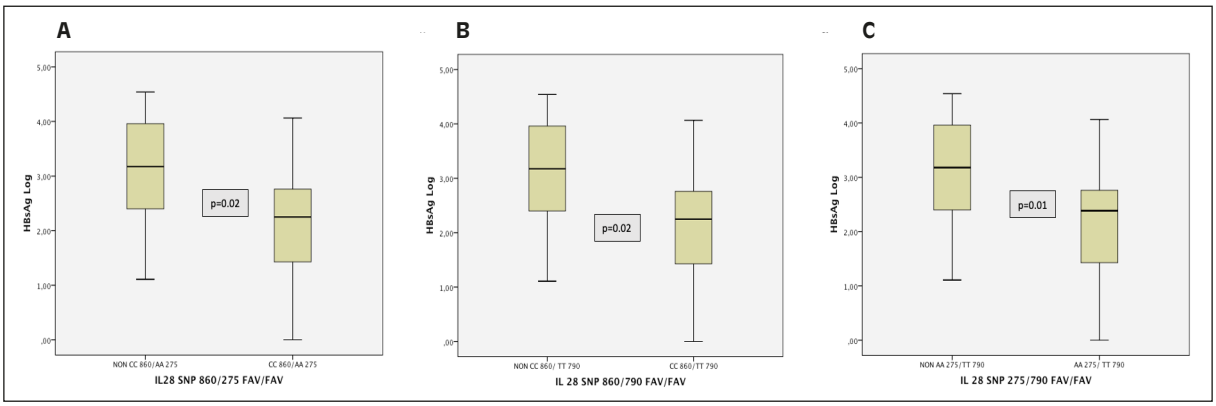

Figure 5 - Correlation between favourable haplogroups and qHBsAg. a) Correlations with CC/AA at rs12979860/rs12980275; b) Correlation with CC/TT at rs12979860/rs8105790; c) Correlation with AA/TT at rs12980275/rs8105790.

logroup CC/AA at rs12979860/rs12980275 was associated with mean adjusted difference of -1.01 $\log_{10}$ IU/mL (95% CI -1.74; -0.28; p=0.008), CC/TT at rs12979860/rs8105790 with -1.01 $\log_{10}$ IU/mL (95% CI -1.74; -0.28; p=0.008) and AA/TT at rs12980275/rs8105790 with -1.00 $\log_{10}$ IU/mL (95% CI -1.71; -0.28; p=0.007). No association was found between qHBsAg titres and SNPs among treated patients (data not shown). At univariate analysis, HBV genotype was associated with qHBsAg (Genotype A + E vs D; +0.86 [+0.04;1.68] $\log_{10}$ IU/mL), not confirmed at multivariate analysis.

Predictors of HBV DNA levels are shown in Table 5 for the whole patient population, and the same predictors were confirmed in the subgroup of untreated patients (data not shown). Significant correlations were not found between different SNPs and HBV DNA levels with the exception of genotype GA *vs.* GG at rs12980275 (p=0.03).

DISCUSSION

During recent years, GWAS have identified some genetic traits associated with host response to infections and with effectiveness of anti-infective drug therapies. Most of these studies analysed SNPs which represent 90% of all human genetic variation. In the present study, our aim was to analyse multiple SNPs and to investigate the correlation between some SNPs of the IL28B gene, namely rs12979860, rs8105790, rs12980275, rs8099917, rs7248668, rs10853728 and serum qHBsAg in patients with chronic HBV infection, irrespective of treatment.

It is well known that qHBsAg decline is very low with NA therapy and our treated patients had been on treatment for a median time of 1.2 years (IQR 0.66-3.25), much less than the estimated time of HbsAg loss, as indicated in previous literature [5,6,8]. The reason for the slow decline of HBsAg with NA treatment is probably attributable to NA inhibition of the reverse transcription of the pregenomic RNA without direct effect on the cccDNA [4].

Homozygous variants rs12979860 CC, rs8105790 TT and rs12980275 AA were associated to lower qHBsAg, after adjusting for viral genotype. These observations are in line with previous reports on SNP rs12979860 documenting the association of rs12979860 CC with higher levels of interferon λ3 and better virological response in CHB patients [21-26]. Similarly, recent studies showed that rs12979860 CC is significantly associated with spontaneous clearance of HBsAg [17, 24].

Shi et al. found increased levels of serum IL28B and enhanced expression of IL28A/B mRNA in carriers of rs12979860 CC compared to CT/TT and the same genotype was more frequent among inactive carriers and control groups as compared to subjects with active HBV hepatitis [25]. Regarding SNP rs8105790, we found a higher frequency (81%) of TT genotypes among inactive carriers as compared to the active carriers, which is in line with the lower qHBsAg associated with TT genotype. This finding is in contrast with a higher frequency of rs8105790 CC/CT among inactive carriers and of genotype CT among those recovering after acute HBV in-

Table 5 - Predictors of HBV DNA plasma levels.

	Univariate Analysis		*Multivariate Analysis*	
	Mean Difference in HBV DNA log_{10} cp/mL IU (95% CI)	*p*	*Mean Difference in HBV DNA log_{10} IU/mL (95% CI)*	*p*
NUCs Treatment	-0.37 (-2.71; -0.66)	0.002	-2.09 (-2.93; -1.25)	<0.0001
HBeAb	-3.05 (-4.52; -1.58)	<0.001	-3.14 (-4.49; -1.78)	<0.001
HBsAg log	0.46 (-0.009; +0.92)	0.054	0.59 (0.21; 0.978)	0.003
ALT per 10 IU/mL increase	0.02 (0.003; 0.044)	0.028	0.21 (0.03; 0.39)	0.018
AST per 10 IU/mL increase	0.05 (0.003; 0.09)	0.038	-0.43 (-0.80; -0.064)	0.022

Abbreviations: NUCs (Nucleos(t)ide analogues); HBeAb (HBV e antigen antibodies); qHBsAg (quantitative HBV s antigen); ALT (alanine aminotransferase, per 10 UI/ml increase); AST (aspartate aminotransferase, per 10 UI/ml increase).

fection as compared to those with chronic HBV infection in Turkish population, as reported from Karatyli et al. [26]. Such differences could be explained by the different ethnicity and the different prevalence of HBV genotypes in specific patient populations. While we found an association between genotype AA *vs.* GG or GA at rs12980275 and lower qHBsAg, a recent study suggested that the rs12980275 GG could have a protective role in HBV infection, as shown by its higher frequency among patients with spontaneous clearance of HBsAg [17]. Again, this discrepancy could be at least partially explained by interactions between host genetics and different HBV genotypes, as observed in a study of Sonneveld et al. [19]. Likewise, we were unable to find any correlation between the rs8099917 variants and qHBsAg while others found that specific variants at this SNP were associated with CHB without HCC compared with self-limited HBV infection and with virological response (OR=3.746, CI=1.235-11.355; p=0.020) [21, 23].

While several combinations of SNPs were shown to be in strong linkage disequilibrium, we detected haplogroups strongly associated with lower qHBsAg, including CC/AA at rs12979860/rs12980275, CC/TT at rs12979860/rs8105790 and AA/TT at rs12980275/rs8105790. These associations were confirmed at multivariate analysis after adjusting for viral genotype both in the overall population and in the subgroup of inactive carriers. The identification of specific haplogroups as determinants of lower qHBsAg in HBV chronic carriers could be helpful to select patients with a greater chance of viral elimination and therefore ideal candidates for future HBV cure research. Given the strong correlation among multiple SNPs, analysing a reduced SNP set appears to be feasible, saving time and cost while preserving relevant information. Moreover, these findings could stimulate investigating the pathophysiological mechanisms underlying improved virological control, likely to involve host innate immunity responses affected by IL28B SNPs.

Interestingly, we found only one weak association between a single IL28B SNP variant and lower HBV DNA levels. This finding could be due to the lack of sensitivity in our case series, due to the strong effect of NAs therapy in a relevant proportion of the patients.

Very recently, the combination of tenofovir with pegylated interferon has shown that HBsAg clearance may be achieved in a larger proportion of patients [29]. Whether IL28B SNP profiling can help identifying patients with a higher probability of HBsAg clearance by this treatment combination deserves additional investigation. There is still controversy regarding the true association between IL28B and chronic HBV infection and larger prospective studies are needed to clarify this open issue.

Limitations of this study include the relatively small sample size, the use of NA therapy in a proportion of patients, the inclusion of predominantly Caucasian ethnicity and the high predominance of HBV genotype D.

Thus, we speculate with caution against the generalization of the association of specific IL28B SNP variants with a better control of serum HBsAg levels, a marker of cccDNA replication and liver disease prognosis. The analysis indeed deserves replication on a larger sample size including different ethnicities and different HBV genotypes, to select human genetics traits to be integrated in initial evaluation of chronic HBV patients.

Conflict of interests
ADL received research grants for his institution from ViiV Healthcare, Gilead Sciences and Merck Sharp and Dohme. He has been consultant for ViiV Healthcare, Gilead Sciences, Bristol-Myers Squibb, Janssen, Merck Sharp and Dohme, Abbvie and Roche. MZ received research grants from ViiV Healthcare and Gilead Sciences, and honoraria and consultancy fees from ViiV Healthcare, Gilead Sciences and Janssen-Cilag.

Ethics approval
Informed consent was obtained from each patient included in the study, the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (7th revision, 2013) as reflected in a priori approval by the institutional human ethics committee.

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Authors' contributions
each author participated sufficiently in the work giving substantial contributions to realization; IV, GM, GL performed all virological and genetic tests; BR, AV, MZ, ADL made contributions to conception and design, acquisition, analysis and interpretation of data, they also have been involved in drafting and revising the manuscript; all authors contributed to the intellectual content and gave their final approval of the submitted manuscript.

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