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Clinical course of chronic hepatitis B patients receiving nucleos(t)ide analogues after virological breakthrough during monotherapy with lamivudine

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SUMMARY

Little is known about the optimal management of patients with chronic hepatitis B (CHB) who develop drug resistance. The aim of this study was to investigate the effectiveness of different drug regimens in chronically HBV-infected patients. HBV viral load was determined using a bDNA assay and the substitutions in HBV-DNA were studied by polymerase sequencing test. The study involved 38 patients who experienced a therapeutic failure to lamivudine (LAM). The sequential treatments used were: LAM + adefovir (ADV), LAM + tenofovir (TDF), entecavir (ETV) monotherapy, ADV monotherapy and TDF monotherapy. Similar activity against HBV replication was observed with all drug regimens. Of the patients treated with LAM, 44% developed resistance mutations. The rt M204I mutation was observed more frequently. Sequential ADV add-on LAM and TDF therapy induced the appearance of resistance in 3/18 (16.6%) and in 1/8 (5.5%) treated patients, respectively. Genotype D was the most prevalent (78.9%), followed by genotype A (13%), genotype E (5.2%) and genotype C (2.6%).
Our study showed that baseline serum HBV DNA is an important predictor of virologic response and that virologic breakthrough is significantly associated with the insurgence of genotypic resistance.

KEY WORDS: Resistance, Mutations, Antiviral treatments.

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INTRODUCTION

Chronic hepatitis B (CHB) infection is estimated to affect about 350 million persons worldwide (Dienstag, 2008). They are exposed to a progressive disease that may lead to liver cirrhosis and hepatocellular carcinoma (HCC) (Wiengand *et al.,* 2010). Several nucleos(t)ide analogues (NAs) have been developed over the past decade in addition to interferon α (IFN-α). They include lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir (TDF). The ultimate goals of antiviral therapy are clinical and histological improvement, including a decrease in the necroinflammatory grade, which consequently reduces the risk of cirrhosis and HCC. Therefore, the following surrogate markers are used to measure the achievement of these objectives: reduced HBV replication (measured by suppression of HBV DNA to undetectable levels), anti-HBe seroconversion and normalization of alanine aminotransferase (ALT) levels (Bhattacharya *et al.,* 2010). In the clinical setting, all these drugs have both advantages and disadvantages. IFN-α has the dual role of antiviral activity and immune modulation, leading to high rates of hepatitis B antigen (HBeAg) seroconversion. Entecavir and tenofovir confer strong viral suppression and low drug resistance, but are very

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expensive. Telbivudine is capable of strong viral suppression and the seroconversion rate is high, but its disadvantages include drug resistance and the risk of rhabdomyolysis. Lamivudine is safe and strongly suppresses viral activity, but has high rates of drug resistance. Mutations in the reverse transcriptase (rt) domain of the HBV polymerase gene, particularly in the YMDD motif at rt204 (rtM204V/I), allow the virus to become resistant to LAM and LdT treatments. The occurrence of this mutation in combination with another substitution in the rt domain (rt184, rt202, or rt250) causes resistance to ETV (Enomoto *et al.*, 2007). Because primary LAM resistance mutations rtM204V/I can compromise viral replication fitness, compensatory mutations in the rt domain (rtL180M, rtV173L, rtL80I/V) that partially restore replication efficiency are often co-selected in HBV rt204 mutants (Yuen *et al.*, 2009). LAM has different complications, including a high incidence of virological relapse when treatment is discontinued and a drug resistance rate that progressively increases over the course of the treatment and is found in 80% of patients after 48 months of administration (Lai *et al.*, 2003; Zoulim *et al.*, 2009). Available data indicate that the best treatment for patients with LAM resistance is to continue LAM and add on ADV or TDF (Zoulim *et al.*, 2009). ETV is not an optimal treatment for LAM-resistant HBV infection due to cross-resistance between these drugs. If ETV is chosen as a rescue therapy, LAM treatment should be discontinued. As the effectiveness of antiviral treatments against lamivudine-resistant patients with chronic hepatitis B in our region has been poorly studied, the purposes of this study were to evaluate the overall efficiency of monotherapy and combinations of nucleos(t)ide analogues in reducing DNA levels over a follow-up of two years, the frequency of drug-resistant HBV, and to establish an association of the type of mutation with the HBV genotypes circulating in Brescia, Italy.

MATERIALS AND METHODS

Study population

A retrospective study was performed in September 2012 to identify patients chronically infected with HBV and with a history of virological breakthrough induced by lamivudine therapy. Chronic infection was defined as persistence of serum HBsAg for more than 6 months. We identified 38 patients with these inclusion criteria. All patients were referred to the University Division of Infectious and Tropical Diseases at the Spedali Civili Hospital in Brescia, Italy. All of the patients were treated with different therapeutic strategies during distinct periods of time. The different strategies were as follows:

a) LAM (100mg/day) + ADV (10mg/day),
b) LAM (100mg/day) +TDF (300mg/day),
c) ETV (1mg/day) monotherapy,
d) ADV (10mg/day) monotherapy,
e) TDF (300mg/day) monotherapy.

Failures of previous NA therapies included suboptimal viral suppression (serum HBV DNA level >2000 IU/mL) or the development of resistance.

Medical records were collected to obtain data on the epidemiological, serological and clinical characteristics of the patients and analyzed retrospectively. The parameters considered were: sex, age, HBsAg and HbeAg reactivity, HBV viral load, alanine aminotransferase (ALT) levels before and after the first line treatment, and polymerase and HbsAg gene mutations in patients who were viraemic despite the antiviral treatment.

Laboratory assays

Serologies for HBV (HBsAg, anti-HBs,HBeAg, anti HBe, and total immunoglobulinG against HBV core antigen (anti HBc) were assayed with the use of commercial kits (Abbott Laboratories).

In all samples viral load was evaluated by Versant HBV-DNA 3.0-bDNA (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA), a signal amplification procedure with a reportable range of results from 2.0×10^3 to 1.0×10^8 HBV DNA copies/ml. Extraction was performed manually with commercial kit QIAmp DNA mini kit (QIAGEN Gmbh, Hilden,Germany) and virus sequences were obtained by the Trugene HBV Genotyping kit (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA), according to the manufacturer's recommendations. This method yields a bi-directional sequence from the overlapping surface antigen and do-

mains B through E of the reverse transcriptase region of the hepatitis B virus.
Siemens' OpenGene DNA Sequencing System was used to obtain sequence data in real time, and each pair of forward and reverse sequences were combined and aligned with stored reference sequences (wild type). The software module contains sequences that correspond to the surface antigen and polymerase regions for genotypes A through H and a universal mutation reporting reference sequence for comparison, showing the published mutations from key scientific journal articles.

Definition of treatment response
The mean reduction of HBV DNA levels was assessed during treatment. Complete virologic response was defined as a decrease in serum HBV DNA level below 2000 copies/ml after treatment. The primary non response was defined as a decrease in serum HBV DNA level of less than 2 log copies /ml after 24 weeks of therapy. Viral breakthrough (VB) was defined as an increase of 1 log copies/ml from nadir during different treatments. HBeAg clearance included HBeAg loss.

Statistical analysis
For a comparison of antiviral efficacy, and the frequency of the occurrence of viral breakthrough and genotypic resistance between each treatment group during the study period, one-way analysis of variance (ANOVA) for continuous data and Fisher's exact test for categorical data were carried out. The efficacy parameters included serum HBV-DNA and ALT levels. $P \leq 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics
A total of 38 patients with chronic HBV who had known virologic and genotypic resistance to lamivudine were included in this study. Baseline characteristics are presented in Table 1. The median age was 58 years (range 19 to 79 years) and 92% were male. Eighteen patients (47.3%) were HBeAg positive and the mean baseline serum HBV DNA level was 6.4±1.96 $\log_{10}$ copies/ml. Eighteen patients (47.3%) had been exposed to LAM+ADV. TDF treatment alone was used in 11 subjects (28.9%) and with LAM in 5 subjects (13.1%). ETV and ADV monotherapies were used as final rescue therapy in 3 (7.8%) and 1 (2.6%) patients, respectively. In fact, in some patients (18.4%) the rescue therapy used (ADV, 2 patients; LAM+ADV, 3 patients; and ETV, 2 patients) failed and they were treated with a different NA. More detailed information on nucleoside analogue therapy history and genotypic resistance profiles is provided in Table 2. All patients had been treated with LAM at first, and then developed resistance; the median prior LAM monotherapy duration was 19 (range 3-48) months. In LAM and ADV experienced patients, ADV was used as a sequential or add-on therapy in an attempt to suppress LAM resistant strains. HBV genotypes were determined for all subjects. Genotype D was the most prevalent (78.9%), followed by genotype A (13%), genotype E (5.2%) and genotype C (2.6%).

TABLE 1 - *Baseline clinical characteristics of patients (n = 38).*

Characteristics	
Age, yr, median (range)	58 (19-79)
Gender (Male), n (%)	35 (92)
HBeAg positive, n (%)	18 (47.3)
Serum HBV DNA (log10copies/ml), mean ± SD	6.4±1.96
Serum ALT, U/L, median (range)	84 (17-604)
Prior LAM treatment, mo, median (range)	19 (3-48)
Genotype, n, (%)	
D	30 (78.9)
E	2 (5.2)
A	5 (13.1)
C	1 (2.6)

HbeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; LAM: lamivudine.

Virologic response
The mean changes in serum HBV DNA concentrations over 24 months in the different groups are shown in Figure 1 and Table 3. The difference in mean decline in serum HBV DNA concentrations over 24 months of treatment did not differ between the groups, except for the mean reduction of HBV DNA concentration in TDF group *vs* the other groups at 3 months.

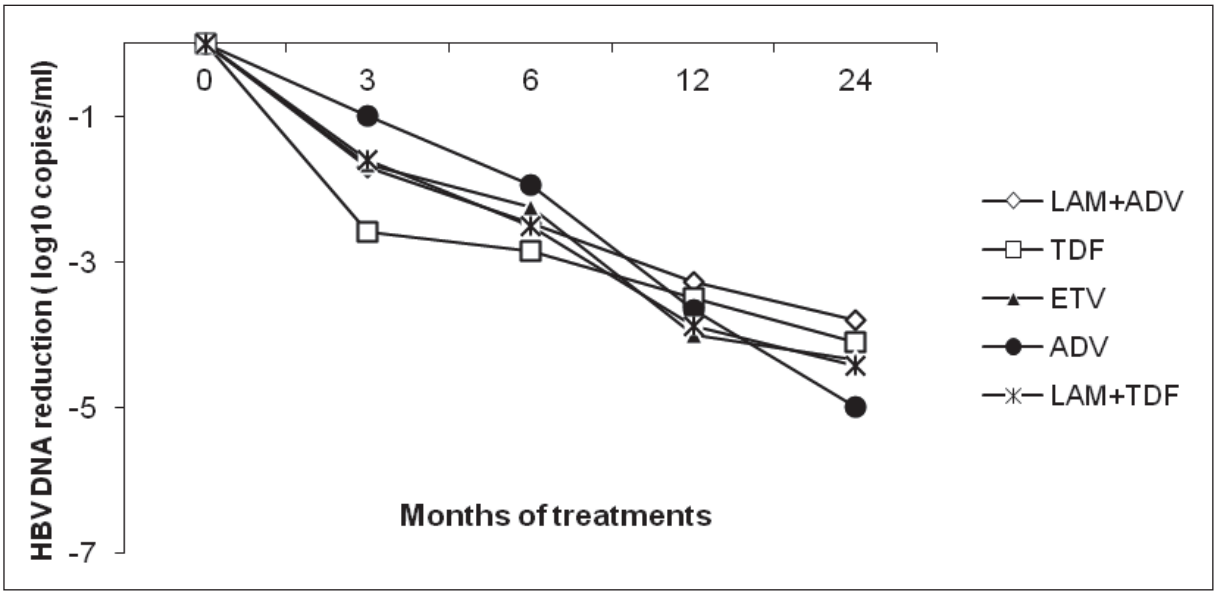

FIGURE 1 - *Mean changes in serum HBV DNA levels according to treatment groups*

Virologic response defined with respect to residual HBV DNA concentration as a complete virologic response (<2,000 copies/ml) was obtained in 10/18 (55%) of LAM + ADV-treated patients, in 5/11 (45%) of TDF-treated patients, in 3/5 (60%) of LAM + TDF-treated patients, in 1/3 (33%) of ETV-treated patients and in the one patient treated with ADV.

The rate of HBV DNA undetectability (<2,000 copies/ml) at 6 months after the initiation of ADV on-LAM therapy and TDF was 17% and 27%, respectively. At 12 months after the initiation of each rescue therapy, the rate of HBV DNA undetectability was 17% for LAM+ADV, 18% and 40% for TDF and LAM+TDF, respectively, 33% for ETV. At 24 months, 22% of LAM+ADV and 20% of LAM + TDF-treated patients and the ADV-treated patient had HBV DNA under 2000 copies/ml. Five (27%), 2 (18%) and 2 (66%) patients in the LAM+ADV, TDF and ETV groups, respectively showed a biochemical response.

The rates of HBe Ag seroconversion were 33% in the LAM+ADV group, 18% in TDF group and 33% in the ETV group. The three patients with virologic response and treated with ETV and LAM+TDF rescue therapy, respectively, had both HBeAg seroconversion. The efficacy of the different treatments are summarized and compared in Table 3.

Virologic breakthrough and genotypic resistance

Virologic breakthrough occurred in 2/38 (5.2%), 2/38 (5.2%) and 1/38 (2.6%) patients in the LAM+ADV, TDF and LAM+TDF groups, respectively, during the 24 months of treatments (Table 2).

Genotypic resistance analysis was performed for all patients and demonstrated mutations in all five patients with virologic breakthrough.

The main mutations observed were associated with LAM resistance: rtM204V/I alone (7/17, 41%) or in association with its compensatory mutations, rtL180M as double mutation (3/17, 17.6%) and rt V173 as triple mutation (2/17, 11.7%).

Mutations associated to ADV resistance were: Q215S, A181V, N236T and V214E. Single mutations occurred in 9 patients, double mutations in 5 patients and triple mutations in 3 patients. A194T associated with tenofovir resistance was detected in one patient.

Factors predictive of virologic response

Virologic response was defined as HBV DNA level <2000 copies/ml and included 20 patients, 13 patients showed no virologic response and five patients had a virologic breakthrough. Potential baseline predictive factors included in univariate analyses were age, gender, serum

TABLE 2 - *Summary of prior nucleotide analogue treatment regimens and genotypic resistance analysis*

Treatment history	*LAM resistance*	*ADV resistance*	*ETV resistance*	*TDF resistance*	*Unknown[a] (n°)*	*Outcome*
LAM → LAM+ADV	L180M+T184S +M204V	None			4	VR
	L180+M204I/V	None			2	No VR
	M204I	Q215S			8	VB
	None	None			7	No VR
	M204I	None			4	VB
	None	None			5	VR
	None	None			0	VR
	L180M+M204V	None			3	VR
	None	A181V + N236T			1	VR
	None	None			3	No VR
	None	None			5	No VR
	Q215S	None			6	VR
	M204I	None			3	VR
	None	None			0	VR
	None	V214E			1	VR
	M204I	None			4	No VR
	M204I	None			2	VR
	None	None			5	No VR
LAM → TDF	None			None	5	No VR
	V173L+L180M+ M204V			None	5	VB
	None			None	10	VR
	None			None	1	VR
	V173L+L180M+ M204V			None	4	VR
	L180M+M204I			None	0	VB
	None			None	0	VR
LAM → ADV	None	None			0	VR
LAM → ETV	None		None		4	No VR
	None		None		3	VR
	Q215S		None		2	No VR
LAM → ADV → TDF	None	None		A194T	6	No VR
	L180M+M204V	None		None	12	No VR
LAM → LAM+ADV → LAM+TDF	M204I	None		None	2	VR
	None	None		None	1	No VR
	None	None		None	4	VR
LAM → LAM+TDF	M204I			None	3	VR
	L180M+M204V			None	2	VB
LAM → ETV → TDF	None		None	None	3	No VR
	None		None	None	6	VR

ADV: Adefovir dipivoxil; VB: Viral Breakthrough; ETV: Entecavir; LAM: Lamivudine; VR: Virologic response; TDF: tenofovir; [aa]Patients with virologic breakthrough during continued lamivudine treatment without detection of any known genotypic resistance mutation to lamivudine (rtM204V/I or rtL180M).

HBV DNA level, treatment, genotypic resistance and the type of genotype (Table 4).
A lower baseline of HBV DNA levels was correlated with a virologic response ($p<0.05$).
A higher baseline of HBV DNA level was correlated with no virologic response, whereas genotypic resistance was significantly associated with virologic breakthrough ($p<0.01$).

DISCUSSION

Treatment of chronic hepatitis B necessitates long-term therapy with different nucleoside analogues to control viral reactivation and the progression of liver disease. However, drug resistance and costing are leading limitations in long-term antiviral therapy. Drugs with a high

TABLE 3 - *Virologic, biochemical and serological response.*

	LAM+ ADV (n=18)	*TDF (n=11)*	*LAM+TDF (n=5)*	*ETV (n=3)*	*ADV (n=1)*
Serum HBV DNA baseline (log_{10}copies/ml)[a]	6.2±1.8	5.5±2.1	5.45±2.1	7.5±1.0	8.5
HBV DNA Reduction (log_{10}copies/ml)[a]					
Month 3	-1.7±0.75	-2.6±1.5	-1.6±0.8	-1.66±0.07	-0.99
Month 6	-2.46±1.76	-2.86±1.46	-2.5±1.08	-2.24±1.26	-1.94
Month 12	-3.28±1.26	-3.5±1.8	-3.89±0.5	-4.01±1.43	-3.66
Month 24	-3.8±1.37	-4.1±1.22	-4.42±0.79	-4.34±1.63	-5
Normalization of ALT					
Month 6	1 (5.5%)	2 (18%)	0	1 (33.3%)	0
Month 12	4 (22%)	0	0	1 (33.3%)	0
HBeAg loss at month 24	6 (33 %)	2 (18%)	2 (40%)	1 (33.3%)	1 (100%)
HBV DNA undetectability (<2000 copies/ml)	*LAM+ ADV*	*TDF*	*LAM+TDF*	*ETV*	*ADV*
Month 6	3 (17%)	3 (27 %)	0	0	0
Month 12	3 (17%)	2 (18 %)	2 (40%)	1 (33%)	0
Month 24	4 (22 %)	0	1 (20%)	0	1 (100%)

The alanine aminotransferase (ALT) reference range was ≤40 IU/L. [a]Mean ± SD

TABLE 4 - *Univariate analysis of different parameter with virologic response.*

Univariate analysis Parameter	*VR[a]*	*No VR*	*VB[b]*	*P-value*
Age, years	58 (32-77)	61 (19-79)	48 (19-62)	ns
Gender				
Male	20/35 (57%)	11/35 (31%)	4/35 (11%)	ns
Female	0	2/3 (67%)	1/3 (33%)	ns
HBV DNAlog_{10}copies/ml	4.55 (3-8.5)	5.82 (3.3-8.5)	6.25(3.4-8.6)	p<0.05
Type of therapy				
Monotherapy	7/15 (47%)	6/15 (40%)	2/15 (13%)	ns
Combinations	13/23 (57%)	7/23 (30%)	3/23 (13%)	ns
Genotypic resistance	9/20 (45%)	4/13 (30.7%)	5/5 (100%)	p<0.01
Genotype				
D	15/30 (50%)	12/30 (40%)	3/30 (10%)	ns
E	1/2 (50%)	1/2 (50%)	0	ns
A	3/5 (60%)	0	2/5 (40%)	ns
C	1/1 (100%)	0	0	ns

Values are given as the median (range) or n (%); [a]VR, virologic response; [b]VB, virologic breakthrough; ns, not significant

genetic barrier and/or low resistance such as entecavir and tenofovir are important in the prevention of drug resistance. However, entecavir and tenofovir are very expensive and no more efficacious than lamivudine in terms of HBeAg seroconversion (Yuen *et al.,* 2009b), thereby limiting its widescale use.

Another strategy to prevent or delay HBV drug resistance involves combination pharmacotherapy where two or more antiviral drugs are co-administered. This concept emerged from studies of patients with lamivudine-resistant HBV who were managed by adding adefovir to ongoing LAM (Keefe *et al.*, 2008; Lok *et al.*, 2007). In those studies, the rates of virological breakthrough and genotypic resistance to adefovir were significantly higher in the group switched to adefovir than in the add-on group, thus emphasizing the benefit of combination therapy in preventing the development of multidrug resistance.

This study provides a comparison of the antiviral efficacy of different therapies for treatment of chronic hepatitis B patients who have failed to respond to LAM monotherapy. It is known that prolonged LAM therapy is associated with virological failure because of the frequent emergence of mutations in HBV genome. Nearly half of the patients (17/38, 44%) undergoing LAM monotherapy had LAM resistant isolates. In accordance with previous studies (Damerow *et al.*, 2010), our results indicated that the rt M204I mutation preferentially appears alone and it was the pattern found in the majority of resistant isolates (7/17, 41%). The association of M204V plus L180M mutations was found in three isolates. Triple polymerase mutation (M204V+ L180M+V173L) was present in 2 patients. This association has been described in different studies and seems to enhance HBV replication compared to M204V+L180M alone (Bottecchia *et al.*, 2008; Iacomi *et al.*, 2009; Mendes-Correa *et al.*, 2010). A different triple polymerase mutation was found in this study (L180M+T184S+M204V) in one patient (1/17, 5.8%). The rt Q215S mutation was also found in 2 resistant isolates (2/17, 11.7%).

Because of the overlap between the RT and HBsAg genes, changes in the polymerase gene often produce mutations also in the HBsAg (Torresi *et al.*, 2002; Villet *et al.*, 2006; Sheldon *et al.*, 2008). We frequently found an aminoacid change (sI195M) in the overlapping surface gene (6/38, 15.7%). This mutation strongly reduces the binding affinity with anti-HBs to levels similar to the vaccine escape mutant G145R (Zanetti *et al.*, 1988) and might determine structural and functional alterations in reverse transcriptase with lower efficacy of antiviral molecules (Colson *et al.*, 2007). Other mutations associated with immune escape were detected in this study: P120T (4/38, 10.5%), G145R (3/38, 7.8%), E164D (1/38, 2.6%) and M133I (1/38, 2.6%). Infection with an immune escape virus implies a lower control over viral replication and a major likelihood of new mutations opening a serious epidemiology issue if these isolates begin to circulate in the environment.

The distribution of HBV genotypes confirmed previous studies reporting a higher prevalence of genotype D in Italy (Palumbo *et al.*, 2007). The other genotypes found, genotype A followed by E and C, are probably due to the migratory flow in our country.

In the present study, the different therapies used had no significant differences in displaying activity against HBV replication. However this study found that in 3 months TDF monotherapy produced faster and greater suppression of HBV DNA compared with the other groups. The rate of HBV DNA undetectability showed no significant differences between the groups analyzed. However, at 6 months TDF therapy showed that a higher percentage of patients (27%) was able to achieve a complete virologic response. In our study, HBeAg seroconversion at month 24 was 33% in the LAM +ADV group and there were no significant differences with the other groups.

Our study showed that baseline serum HBV DNA is an important predictor of virologic response and that virologic breakthrough is significantly associated with the insurgence of genotypic resistance.

Combination therapy, an emerging concept for the management of antiviral resistance, has been the subject of several reports (Rapti *et al.*, 2007; Lampertico *et al.*, 2007; Inoue *et al.*, 2011). However, in this study combination therapies showed an efficacy similar to monotherapies. In patients with LAM-resistant chronic hepatitis B who had been treated with sequen-

tial ADV plus LAM, ADV resistance was found in 3 subjects (3/18, 16.6%) and in patients who were treated with sequential TDF, we found only one TDF resistant isolate (1/8, 12.5%). No resistance was found for the patients treated with sequential ETV. In this study, 7 patients failed multiple nucleoside analogue treatments including LAM, ADV, TDF, ETV LAM plus ADV and LAM plus TDF. Among them, only 3 (3/7, 42%) had virologic response.

This study has some limitations. It was a retrospective observational study of a relatively heterogeneous patient population, representing the outcome of rescue therapy in atients who failed to respond to LAM treatment; furthermore, the number of subjects included was not large enough (n=38). Nevertheless, the patients in this study represent the most difficult-to-treat population in the management of chronic hepatitis B. So far, further studies with larger sample sizes are necessary to overcome these shortcomings and to elucidate the long-term outcome of different NA treatments.

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