Tenofovir disoproxil fumarate monotherapy maintains HBV suppression achieved by a "de novo" combination of lamivudine-adefovir: a pilot study

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SUMMARY

Chronic hepatitis B (CHB) treatment aims at long-term suppression of HBV replication and improvement in clinical outcomes. We describe the data of a pilot, non-profit study in which patients with CHB were treated with *de novo* combination lamivudine-adefovir (LAM-ADV) for at least four years with a view to HBV suppression and resistance prevention, and shifted to tenofovir (TDF) when new antiviral agents were available.

Fifty-one HBeAg negative patients were enrolled. Histology was available for 39 patients and data of liver stiffness (LS) for 24 patients at baseline. Serum quantification of HBsAg and HBVDNA was obtained regularly during the follow-up. In 10 and 7 patients, a paired histology and LS were available at the end of LAM-ADV treatment, respectively. The *de novo* LAM-ADV combination was able to obtain HBVDNA suppression and ALT normalization in one year in most of the patients and in the second year in the remaining. Histology improved in patients with paired biopsy, but tissue HBsAg was present in all but one patient after 48 months of therapy. TDF maintained biochemical and virological response throughout the follow-up. Renal impairment during LAM-ADV therapy improved on shifting to TDF; only in 4 cases was a second shift to entecavir needed. TDF was safe and effective in maintaining HBV DNA suppression achieved by a long-term course of LAM-ADV *de novo* combination for the treatment of HBeAg-negative CHB.

Keywords: chronic hepatitis B, treatment, tenofovir, lamivudine, adefovir.

INTRODUCTION

Infection by Hepatitis B Virus (HBV) is a global health problem. It is estimated that more than 240 million people are chronically infected worldwide and that about 600 000 people die each year due to the consequences of the disease [1]. The burden of HBV infection also depends on its potential to progress to end stage liver disease and hepatocellular carcinoma with high risk of death. HBeAg-negative Chronic Hepatits B (CHB) is by far the most prevalent form of the disease in the Mediterranean geographical area [2].

Antiviral therapy with oral agents is attractive as nucleos(t)ide analogues are much better tolerated than α-IFN, can be used to treat advanced disease as well, ensure effective, long-term suppression of

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HBV replication and improve clinical outcomes [3-5].
We started a study on the treatment of HBeAg-negative CHB with the *de novo* combination lamivudine (LAM) and adefovir (ADV) for four years in 2005, when LAM and ADV were the only oral agents licensed for treatment of CHB (THECLA study: Treatment Hepatitis Combination Lamivudine Adefovir). The main purpose of the study was to prevent HBV resistance to either drug and in turn to achieve long-term suppression of viral replication. The use of the upfront combination was supported by the knowledge that cross-resistance between LAM and ADV was limited and by the evidence that in the treatment of LAM-resistance HBV infection, the earlier ADV is added on LAM the greater the therapeutic benefit is [6, 7]. All patients we treated with the combination achieved a prolonged suppression of HBV replication, without appearance of viral resistance, as long as clinical improvement.
After 2006 two new oral drugs, entecavir (ETV) and tenofovir (TDF), became available and both proved to be highly active against HBV [8,9] and to have a high genetic barrier [10, 11]. Patients who had received LAM and ADV were then offered to be switched to TDF to ascertain whether TDF monotherapy was able to maintain the virological and clinical benefits achieved with the previous course of treatment. The use of one highly active drug was considered preferable to that of two also because it would improve compliance and reduce side effects.
The purpose of the present study was to report on a cohort of patients with HBeAg-negative CHB who were first treated with the combination LAM and ADV achieving clinical benefit and prolonged suppression of HBV replication and then switched to TDF. Efficacy and safety data on the initial treatment, LAM and ADV, prolonged for at least 4 years and on TDF monotherapy at four years are given.

PATIENTS AND METHODS

The study is a pilot, multicenter, non profit study on the treatment of HBeAg-negative CHB coordinated by the Internal Medicine and Hepatology Unit, Second University of Naples, Medical School. The first-phase treatment was the *de novo* combination LAM and ADV (THECLA study) given for at least four years. The second phase treatment was TDF monotherapy which was offered to all subjects who had completed the LAM and ADV course of therapy. The study includes data from 2005 to 2014.

Study patients
A total of 57 patients were screened, of whom 6 failed eligibility criteria and 51 were enrolled. Thirty-five of the 51 patients were from the coordinating centre.
Inclusion criteria were as follows: age 18-65 years, diagnosis of chronic hepatitis, compensated or decompensated cirrhosis by HBV, made on the basis of clinical, laboratory and virological data. Diagnosis was confirmed by liver biopsy taken during the preceding 12 months. Liver biopsy was not performed to patients who had:
1) a biopsy taken in the last 3 years;
2) cirrhosis at the last biopsy;
3) decompensated cirrhosis;
4) at least one contraindication.
A total of 39 patients had a pre-treatment liver biopsy. Other inclusion criteria were: presence of HBsAg, absence of HBeAg and presence of HBeAb in serum; serum level of HBV DNA higher than 2000 IU/ml and of ALT higher than 1.5 x Upper Normal Limit (UNL) for patients with CHB and HBV DNA higher than 200 IU/ml irrespective of serum ALT for patients with cirrhosis. Both naïve and patients previously treated with α-IFN, standard or pegylated, lamivudine, adefovir, alone or in any combination were eligible, but for experienced patients it was required a one year wash out period from the last therapy along with a negative serum test for HBV strains resistant to the preceding NA, *i.e.* LAM or ADV. Exclusion criteria were: end-stage liver disease refractory to medical treatment; co-infection by HDV, HCV, HIV; evidence of HCC; alcohol or drug abuse; co-morbidity clinically relevant; pregnancy and lactation; previous transplantation; any other reason of predictable non compliance.

Treatment
The initial treatment consisted of a *de novo* combination of lamivudine (100 mg/day) and adefovir (10 mg/day p.o.), taken in the morning after breakfast, for 48 months. Primary end points were: 1) stable undetectable HBV-DNA and 2)

prevention of HBV strains resistant to LAM and/or ADV. Laboratory tests (liver function tests, peripheral blood cells, renal function) and adverse events, with particular attention to renal function (creatinine, proteinuria, creatinine clearance, Na^+, K^+, Ca^{2+}, PO_4^{3-} in serum) and pancreatic enzymes, were monitored every three months and whenever indicated. At the end of treatment, a repeat biopsy was offered to compensated patients, with a baseline biopsy.
At the end of the 4-year combination TDF was offered.
All the procedures used in the study were in accordance with the international guidelines, with the standards on human experimentation of the Ethics Committee of the Second University of Naples and with the Helsinki Declaration of 1975 and revised in 1983. The patients signed their informed consent in accordance with the rules of the Ethics Committee of the Second University of Naples.

Methods
Serological determinations: Serum markers for HBV, HCV, HDV and HIV infection were tested in serum using commercially available immunoenzymatic assays (Abbott Laboratories, North Chicago, IL, and Ortho Diagnostic Systems, Raritan, NJ, USA).
HBV genotype, viral load and HBsAg quantification: The HBV genotype was determined using HBV genotype assay Lipa (Bayer, France) following the manufacturer's instructions.
HBV resistance were detected by reverse hybridization line probe assay (INNO-LIPA HBV DRv2, Innogentics N.V., Gent, Belgium), following the manufacturer's instructions.
HBV DNA viral load was done by PCR real-time using commercial kits (COBAS®AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 Roche).
Serum HBsAg was quantified by Elecsys HBsAg II quantitative assay (Roche Diagnostics GmbH Mannheim, Germany) according to the manufacturer's instructions.

Liver biopsy
All biopsies were blindly evaluated by two independent pathologists for necro-inflammatory activity, fibrosis and steatosis [12]. Baseline and end paired biopsies were available for 10 patients and were processed with immunoistochemical staining for cytoplasmic HBsAg as previously described [13].

Liver stiffness measurement
We performed liver stiffness measurement (LSM) by Fibroscan® (Echosens, Paris, France) with M-probe by an experienced operator (>500 exams) as previously described [14].

Statistical analysis
Data are expressed as media or median. Differences between groups were evaluated by t student's for parametric data and by Mann-Whitney U test for non-parametric data. Data were analysed by SPSS 13.5 and a $p<0.05$ was assumed to denote significance.

RESULTS

Treatment
Fifty-one Italian patients started the treatment. Table 1 shows the principal characteristics of patients at baseline. HBV genotype D was present in 72% of patients. Except for AST and ALT, other liver function tests were normal in all but one patients who had decompensated cirrhosis. This patient died after 6 months of treatment for larynx malignancy. Table 1 shows also the evolution of the essential parameters during treatment.
During the first year of therapy with LAM+ADV, transaminases were normalized in 90% of patients and HBV DNA clearance was achieved in 96% of patients (in most of them during the first six months). HBV DNA clearance was obtained in 100% of patients during the second year of treatment and maintained thereafter. AST and ALT were sometimes just upper normal limits in few patients and were correlated with drugs assumption (FANS, antibiotics) for undercurrent diseases, nutritional excess or metabolic disorders. Five patients had to stop treatment during the follow-up; 2 during the first year (one died for larynx malignancy, one had ALT elevation due to Facio-Scapulo-Humeral Dystrophy), 1 during the second year (hepatocellular carcinoma) and 2 during the third year (one died for colon cancer, one presented polyarthritis correlated to ADV).
After 4 years of treatment 36 patients shifted to TDF; the obtained biochemical and virological response was perfectly confirmed in all patients

Table 1 - Characteristics of patients at baseline, during treatment with LAM-ADV for 48 months and then switched to TDF monotherapy.

	LAM+ADV					*TDF*		
	T0	*T6m*	*T12m*	*T24m*	*T36m*	*T48m*	*T0 TDF*	*T48 m*
No of patients	51	49	49	48	46	46	36[a]	34[b]
Male, n (%)	29 (57)	27 (55)	27 (55)	27 (56)	26 (56)	26 (56)	20 (55)	22 (65)
Age,yrs (mean±SD)	50.78±8.54		51.73±8.3	52.75±8.39	53.67±8.56	54.67±8.56	54.3±8.65	59±8.26
AST, x UNL (mean±SD)	2.25±2.03[A]	1.07±0.25	1±0.02	<1±0	<1±0	1.01±0.04B	<1±0	<1±0
ALT, x UNL (mean±SD)	3.86±5.35[C]	1.09±0.3	1.04±0.16	1.04±0.15	1.04±0.13	1.01±0.07D	1.01±0.08	<1±0
HBV-DNA, UI/mL, median (range)	6.12*105 (8.46*10^2-1.1*10^8)[E]	0 (0-26400)	0 (0-60)	undetectable	undetectable	undetectable[F]	undetectable	undetectable
HAI score (mean±SD)	7.59±4.06	/	/	/	/	/	/	/
Fibrosis score (mean±SD)	3.38±1.58	/	/	/	/	/	/	/
Steatosis score (mean±SD)	1.25±0.91	/	/	/	/	/	/	/
HBV genotypes No	A 1; D 37; D/E 1; D/F 2; D/G 2 n.d. 8	/	/	/	/	/	/	/

p A vs B = 0.000; *p* C vs D = 0.000; *p* E vs F = 0.000;
[a]All 46 patients who had completed combination therapy were offered TDF monotherapy and 36 of them accepted.
[b]TDF monotherapy is ongoing and none of the patients dropped out; numbers refer to cases that reached the corresponding length of therapy.

during TDF treatment. Five of these patients stopped TDF treatment and changed in ETV treatment for renal impairment.
Ten patients continued LAM+ADV combination, because they did not give their consent to shift; they were not followed up at the coordinating centre and data about them are not available.

HBsAg and HBV-DNA levels
Pretreatment quantitative median HBsAg was 5660 I.U./ml. Median HBsAg quickly dropped to 2347 I.U./ml after 24 months of LAM-ADV combination treatment. HBsAg decline was progressive but slow until 48th month of LAM-ADV combination and after 24 months from switch to tenofovir monotherapy (1976 I.U./ml and 1503 I.U./ml respectively) (Figure 1).
No patient cleared HBsAg.
Median HBV-DNA was 6.12x10^5 (range 8.46x10^2-1.1x10^8) at baseline. No statistically significant correlation between HBsAg and HBV-DNA was observed.

Histology and LSM
Before starting treatment 13 (26%) patients had liver cirrhosis; histological scores (HAI, fibrosis and steatosis) of the patients are shown in Table 1. Paired liver histology (baseline and after four years of LAM+ADV treatment) was available for 10 patients; 8 of these had HBV genotype D, 1 had HBV genotype D/G and in 1 genotype was not available. A significant improvement of inflammation (p=0.000) and fibrosis (p=0.002) scores was observed while no significant changes in steatosis scores were detected (Table 2).
HBsAg was positive at baseline immunostaining in all patients and in all but one patient after 48 months of combination treatment, when HBVDNA had been negative in serum since 3 years before (Table 2).
LSM was assessed in 24 patients at baseline; the median Kpa at T0 was 8.4; 6 patients showed liver stiffness (LS) compatible with F4 Metavir, 8 with F3, 1 with F2, 9 with F0-F1. In 7 patients the test was repeated after 4 years treatment. The

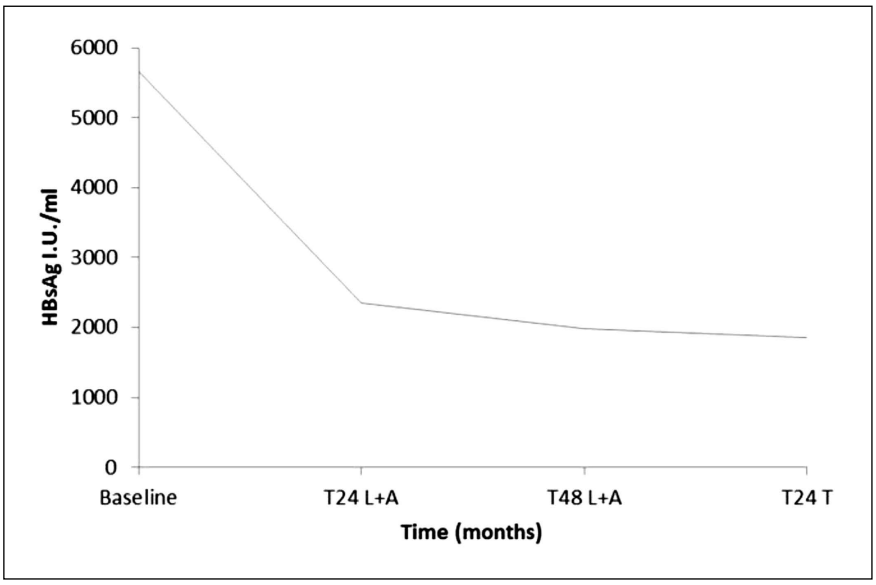

Figure 1 - HBsAg median levels in serum of 32 patients treated with LAM + ADV combination for 4 years and of 14 out of 32 who completed 24 months of subsequent TDF monotherapy (L+A: lamivudine + adefovir; T: tenofovir).

Table 2 - Histology and immunohistochemistry data of 10 patients who had paired liver biopsy taken prior to and at the end of treatment with LAM+ADV for 4 years.

No.	*Patients*	*HAI: BL*	*HAI: END*	*F:BL*	*F: END*	*HBsAg%: BL*	*HBsAg%: END*
1	CG	10	3	5	2	70 C	20 c
2	GP	9	3	4	1	20 C	0
3	FP	7	1	1	0	40 C S	50C
4	OC	2	3	2	1	30 C	90 C
5	CD	13	2	5	1	20 C	20 C
6	AM	13	IS*	3	IS*	15 C	80 C
7	TG	4	2	4	1	70 C	30 C
8	AS	5	3	4	2	5 C	15 CS
9	SD	4	2	5	3	60 C	60 C
10	MM	11	0	3	0	50 C	30 C

BL = baseline; END = end of treatment; HAI. = histological activity index; F = fibrosis; C = cytoplasmatic; N = nuclear; S = submembranosus , *Insufficient Sample.

median Kpa was 7; the Kpa was correlated with F0-F1 for 4 patients, and F2 for 3 patients. None of these patients showed values attributable to severe fibrosis or cirrhosis. These results showed an important reduction of LS after antiviral therapy (p=0.056), not significant perhaps for the small number of tested patients at the second detection.

Side Effects

Treatment with LAM+ADV was well tolerated. No significant alteration of pancreatic function emerged in all patients. Serum creatinine and creatinine clearance were significantly modified during the 4 years of LAM+ADV treatment and were accompanied by a progressive decreasing of serum phosphate and increasing of serum calcium (Table 3). In most of patients renal function improved after one year of TDF treatment. Only in four patients creatinine clearance made worse until <50 mL/min in two subsequent determinations during TDF treatment; three of them did not improve value after adjustment of TDF dosage and were shifted to ETV 0.5 mg/day, obtaining renal improvement and maintaining virological response.

Table 3 - Renal function and bone metabolism during antiviral treatment.

	LAM+ADV					*TDF on going*		
	T0	*T12*	*T24*	*T36*	*T48*	*T0 TDF*	*T12*	*T48*
Creatinine, mg/dl (mean±SD)	0,8±0,15[A]	0,85±0,16	0,92±0,19	0,94±0,24	0,95±0,27[B]	0.94±0.24	0.96±0.23	0.94±0.13
GFR, ml/min (mean±SD)	104,8±29.4[C]	98,4±25,35	97.2±31.26	96,5±31,2	92.1±32.15[D]	92.3±29.94	106.6±47.1	104±25.12
Serum Phosfate, mg/dl (mean±SD)	3,34±0,46[E]	3,21±0,58	3,14±0,69	3,29±0,72	3±0,67[F]	3.11±0.62	2.9±0.54	2.9±0.6
Serum Calcium, mg/dl (mean±SD)	9,2±0,39[G]	9.37±0.37	9.41±0.42	9.32±0.53	9.54±0.38[H]	9.57±0.36	9.57±0.32	9.27±0.46[I]

P A vs B = 0.000; *p* C vs D = 0.045; *p* E vs F = 0.016; *p* G vs H = 0.000; *p* H vs I = 0.004.

DISCUSSION

The *de novo* combination of LAM and ADV is highly efficacious in suppressing viral replication and activity of HBeAg negative CHB. Switch to TDF monotherapy maintains the effect safely.

The evaluation of pre- and post-treatment paired biopsies of ten patients showed that the LAM and ADV combination was also associated with improvement of liver histology, although the immunohistochemical demonstration of HBsAg in liver cells indicated that hepatic infection persisted.

It is difficult to compare our data on the effect of LAM and ADV on HBeAg-negative CHB with those of other studies for several reasons. Some of previous studies on the *de novo* LAM and ADV combination include patients from different geographical areas, do not give genotype, have enrolled HBeAg-positive patients or both HBeAg-positive and HBeAg-negative patients and data by the HBeAg status are not given [15-21].

In addition, duration of treatment differs from one paper to another. A paper by Wong et al. on treatment of HBeAg-negative CHB with LAM and ADV combination as compared with entecavir, for 48 weeks, showed similar efficacy and safety between the two regimens [22].

A recent meta-analysis of studies comparing *de novo* LAM and ADV and entecavir monotherapy for treatment of HBeAg-positive and/or HBeAg-negative CHB showed a superiority of the combination regimen over ETV with respect to biochemical response, HBeAg seroconversion and emergence of viral resistance [23].

The data we reported indicate that the emergence of HBV strains resistant to either drug was fully prevented by the initial use of LAM and ADV combination. In this respect results of our study cannot be compared with those obtained by adding ADV on a previous LAM course, because with this therapeutic approach, the possibility that drug resistance occurs and its storage in cccDNA cannot be excluded.

Results of the present study show that TDF monotherapy is able to maintain suppression of HBV replication already achieved by an initial therapy of LAM and ADV. This suggests that TDF-resistant mutations had not occurred under LAM and ADV *de novo* therapy. Whether a similar antiviral effect of TDF occurs when ADV is added on LAM because of LAM-resistance should be evaluated in prospective studies. Also, the data we presented cannot be compared with those showing that TDF is efficacious as rescue therapy for patients who failed previous oral treatments and have active HBV replication and active disease

In this study suppression of HBV replication and HBsAg clearance behaved differently. Indeed after the first 6 months of LAM and ADV therapy and during TDF monotherapy HBV DNA was undetectable in blood, while HBsAg, although decreased in serum, still persisted. Moreover immunohistochemistry data showed persistence of hepatic HBsAg in 9 out of the 10 patients who had been biopsied at the end of LAM and ADV therapy. The data are not surprising and may be accounted for by several factors. First, previous studies have shown that the kinetic of HBsAg decline in blood of NUC treated patients is very slow [24,25]. Second, the expression of HBsAg is a molecular event somewhat independent from HBV replication since it may derive from the translational activity of cccDNA as well as of DNA sequences integrat-

ed in the host genome both of which are not targeted by NUC therapy [26].

The aims of antiviral therapy are long-term HBV suppression, prevention of disease progression and improvement of clinical outcomes. The long-term undetectability of HBV DNA in serum indicates that prolonged suppression of viral replication is achieved by therapy. Although immunohistopathology of CHB is well categorized for HBeAg-positive and not as well for HBeAg-negative disease, the demonstration of HBsAg in livers of patients that we biopsied after four years of LAM and ADV also speaks for persistence of hepatic infection. In this respect the study of Wong et al. shows that a short-term NUC therapy achieved a great reduction of serum HBV DNA while the decrease of circulating HBsAg and that of hepatic cccDNA was far smaller [27]. The authors also showed that the titre of HBsAg in serum and the amount of cccDNA in the liver parallel one another, but the correlation between the two is not significant [28]. On the other hand the data published by Chen et al. show that lower serum levels of HBsAg and longer duration of LAM therapy are independent predictors of HBsAg loss after discontinuation of therapy [29]. All together the above data indicate that the role of serum HBsAg as surrogate marker of intrahepatic infection still remains to be elucidated and that it is conceivable that a long-term NUC treatment may result in disappearance of hepatic cccDNA.

Several studies have demonstrated that long-term antiviral treatment may achieve improvement of fibrosis regression of cirrhosis [30-32]. Our data confirm those available in the literature, because after four years of LAM + ADV *de novo* combination treatment, median fibrosis score decreased from 4 to 1 and all the three patients who initially had probable cirrhosis had no evidence of cirrhosis in the post-treatment biopsy [12]. In addition this improvement of liver fibrosis has been also observed by measuring the LS, so in absence of liver biopsy, the longitudinal assessment of LS, may be considered as a further confirmation of the laboratory tests, and an index to improvement of clinical conditions in cirrhosis.

It is most relevant that only one of 51 patients, of whom 36 switched to TDF and 10 still on LAM and ADV, developed HCC after a median length of therapy of 96 months.

Treatment was generally well tolerated. Renal insufficiency observed under LAM + ADV *de novo* combination was effectively handled by prolonging dosing interval and also improved after shift to TDF in most of the patients.

This was a pilot study and its main limitation is the small number of patients. It was designed when lamivudine and adefovir were the only available oral drugs for treatment of CHB and the emergence of HBV drug-resistant strains was shown to be associated with loss of clinical benefit. Since that time new generation antivirals became available, with greater efficacy and high genetic barrier, giving us the possibility of better manage chronic HBV infection. The present study refers to a well defined group of HBV patients successfully switched to TDF after a long-term LAM + ADV *de novo* combination treatment.

In conclusion TDF monotherapy is safe and effective in maintaining the suppression of HBV replication achieved by a long-term course of LAM + ADV *de novo* combination for the treatment of HBeAg-negative chronic hepatitis B.

Conflict of interest

- Giuseppe Ruggiero, Ilaria Rainone, Adriana Boemio, Luca Rinaldi, Lorenzo Andreana, Luigi Elio Adinolfi declare that they have no conflict of interest.
- Aldo Marrone and Rosa Zampino have received research grant from Gilead outside the submitted work.
- Barbara Guerrera, Giuseppe Pasquale and Rosa Zampino have been sponsored to research meeting from Gilead.

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