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▶ **To cite this version:**

Ruy M. Ribeiro, Georgios Germanidis, Kimberly A. Powers, Bertrand Pellegrin, Paul Nikolaidis, et al.. Hepatitis B virus kinetics under antiviral therapy sheds light on differences in hepatitis B e antigen positive and negative infections.. Journal of Infectious Diseases, 2010, 202 (9), pp.1309-18. 10.1086/656528 . inserm-00529800

HAL Id: inserm-00529800

https://inserm.hal.science/inserm-00529800

Submitted on 3 Nov 2011

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Hepatitis B Virus Kinetics Under Therapy Sheds Light on the Balance Between Replication and Immune Clearance

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Keywords: HBeAg; mathematical model; e antigen; HBV; dynamics

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Abbreviations:

HBeAg – hepatitis B e antigen; HBV – hepatitis B virus; HBsAg – hepatitis B s antigen; CTL – cytotoxic T lymphocytes;

Financial Support:

Portions of this work were done under the auspices of the United States Department of Energy under contract DE-AC52-06NA25396 and supported by NIH grants AI28433 (ASP), RR06555 (ASP), and P20-RR18754 (RMR). Portions of this work are part of the activity of the VIRGIL European Network of Excellence on Antiviral Drug Resistance supported by a grant (LSHM-CT-2004-503359) from the Priority 1 "Life Sciences, Genomics and Biotechnology for Health" programme in the 6th Framework Programme of the European Union and have been supported by the French National Agency for AIDS and Viral Hepatitis Research (ANRS).

ABSTRACT

Hepatitis B e antigen (HBeAg)-negative chronic hepatitis B has a divergent presentation and clinical course from that of HBeAg-positive infection. The former usually presents with lower viral levels, but faster progression to liver disease complications. In order to better understand the balance between replication and the immune response against hepatitis B virus (HBV), the viral kinetics in 50 HBeAg-negative patients under treatment was analyzed and compared with data in HBeAg-positive infection. The decay in viral levels under various treatment protocols with interferon-α and/or nucleos(t)ide analogues was modeled. HBV DNA level was measured frequently and the data fitted with a mathematical model of viral dynamics. A meta-analysis of all published studies of viral kinetics in HBeAg-positive and negative infection was also conducted. We found that the turnover of both HBV virions and infected cells was high in HBeAg-negative infection. Virion half-lives and infected cell half-lives were significantly faster in HBeAg-negative than -positive infection. However, the total production of virions was higher in HBeAg-positive infection due to the much higher baseline replication levels. There was also a negative correlation between baseline HBV DNA levels and infected cell half-life, suggesting that the higher the viral level, the faster the turnover of infected cells. *Conclusions:* This study indicates that the lower HBV DNA levels in HBeAg-negative infection are not due to suppression of viral replication, but rather they occur through the balance of high viral replication and fast immune clearance because the immune response against HBeAg-negative infection appears to be stronger for higher viral levels.

By some estimates, one third of the global population, i.e. approximately 2 billion people, has been infected with hepatitis B virus (HBV) in their lifetime (1, 2). Only a small percentage of infected adults develops chronic hepatitis B, but global prevalence of this disease is estimated at 350 million people (1). End-stage HBV infection can lead to cirrhosis and to hepatocellular carcinoma (HCC), and up to 1.2 million people die every year from the consequences of this infection (1).

Chronic HBV infection is usually characterized by detectable HBV DNA in serum, as well as the presence of hepatitis B surface antigen (HBsAg). Hepatitis B e antigen (HBeAg) can be present. However, a large proportion of infected individuals are HBeAg-negative (3), because they are infected with HBV variants that are unable to produce high amounts of the excreted protein that bears the HBe epitope. This results from nucleotide substitutions in the pre-core and/or core promoter region of the HBV genome that abolish or down-regulate HBe protein production (4). The most common substitution is a G to an A at nucleotide 1896 that results in a stop codon at position 28 and abolishes synthesis of the HBe protein (5, 6). Almost only HBV genotypes with a T at nucleotide position 1858 develop the G1896A mutation, corresponding mostly to genotypes B, D, and some strains of genotype C. HBV pre-core mutants are rather uncommon in North America and Northern Europe, because the predominant genotype has a C at nucleotide 1858 (3, 7-9). In contrast, the vast majority of patients with chronic hepatitis B in Southern Europe and Africa is infected with HBV variants that

express little or no HBeAg, and prevalence of HBeAg-negative chronic hepatitis B seems to be increasing worldwide (3, 8, 10). HBeAg-negative chronic hepatitis B represents a potentially severe and progressive liver disease with frequent development of cirrhosis and/or HCC (4, 6).

Hepatocytes expressing concomitantly HBcAg and HBeAg epitopes could become a preferential immune target for cytotoxic T lymphocytes (CTLs), compared with cells expressing only HBcAg epitopes (11). Cytoplasmic HBeAg is more efficiently presented to CD4-positive T cells, resulting in a stronger CD4-positive T cell immune response (12), and mutant HBeAg-negative strains may represent effective immune escape mutants (13-15). On the other hand, infection with HBeAg-negative hepatitis B variants is associated with lower serum viral levels (16-18), higher intra-hepatic necroinflammatory lesions and more severe progression of disease than infection with HBeAg-positive strains (16, 19). Thus, taken together these observations may indicate a stronger immune response against HBeAg-negative infection. Consistent with this and even though the function of the HBe protein is not clear, it has been suggested that it serves to down modulate the immune response (14, 20).

Prognosis for individuals infected with HBeAg-negative HBV is usually worse than for HBeAg-positive chronic hepatitis B. In addition, the former represent a more difficult to treat patient pool (17, 21, 22). At present, the two main strategies to treat these patients are with a protocol of one year of pegylated interferon (IFN)-α, or nucleos(t)ide analogues that inhibit HBV reverse transcriptase for an indefinite duration (17). To date, there are several approved

nucleos(t)ide analogue-based inhibitors including lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir disoproxil fumarate (17).

Modeling of different viral infections and their treatment has given insight into aspects of viral evolution, pathogenesis and the mechanisms of antiviral drug action (23-26). HBeAg-positive HBV infection has been analyzed in this way, with results that showed that this virus has a short half-life in plasma and rapid viral production. We and others have calculated that the daily production of HBV in HBeAg-positive infection is in excess of 10^{11} and that infected cell lifespan is very variable but can be as short as 2 days (27, 28). A few studies have also analyzed the effect of drug therapy on the kinetics of HBeAg-negative viral infection. Sypsa *et al.* (29) compared the viral dynamics in HBeAg-negative patients treated with lamivudine with or without pegylated IFN-α2b at two different dosages. They estimated that the median half-life of free HBV virions was 12.7 hours and that the infected cell half-life ranged from 2.7 to 75 days. In a similar study, HBV kinetics was studied in patients treated with lamivudine and pegylated IFN-α2a (30). These authors developed more complex dynamic models to analyze their viral level data. The results indicated that the half-life of free virions in patients treated with lamivudine was 9.2 hours and that of those treated with pegylated IFN-α2a and lamivudine was shorter at 5.7 hours.

Here we analyzed the dynamics of HBeAg-negative infection under a variety of drug treatments, including lamivudine, IFN-α, pegylated IFN-α2a and/or adefovir dipivoxil in different combinations and conducted an exhaustive meta-analysis of published results concerning HBeAg-positive infection. Our goal

was to understand the respective roles of viral production and the immune response in HBeAg-negative infection and compare them with HBeAg-positive infection.

PATIENTS AND METHODS

Patients

The study population included 50 patients (40 men, mean age = 40±11 years) with chronic HBeAg-negative infection related to HBV genotype D, enrolled between 2000 and 2002. Their median serum alanine aminotransferase (ALT) level was 72±64 international units (IU)/L and diagnosis of chronic hepatitis B was made according to well established criteria (31). All had well compensated active liver disease and liver biopsy showed a modified Ishak score of at least 6, with a fibrosis score of at least 1. Ten out of 50 (20%) had cirrhosis on liver biopsy and none had HCC, based on serum α1-fetoprotein determinations and liver computed-tomography scan. The patients had no coinfections with hepatitis delta virus, hepatitis C virus, or human immunodeficiency virus. They were treated with one of six therapy regimens for 48 weeks. The study was approved by the Ethical Committee of the Scientific Council of Papageorgiou General Hospital. All patients gave informed consent for the kinetic study.

Therapy

The therapy protocols used in this study were the accepted clinical practice or investigational protocols for drugs being developed at the time of patient enrollment. Twelve patients received standard IFN-α2a monotherapy, 4.5 MU tiw (IFN group), 10 received lamivudine monotherapy, 100mg qd (LAM group), 10 received a combination of both standard IFN-α2a and lamivudine at the same doses (IFN+LAM group), 6 received pegylated IFN-α2a monotherapy, 180 μg qw (PEG group), 7 received a combination of pegylated IFN-α2a and lamivudine (PEG+LAM group), and 11 received a combination of adefovir dipivoxil, 10mg qd, and lamivudine, 100mg qd (ADV+LAM group) (Table 1). Of this last group, 6 were patients whose first treatment regimen had failed and they were retreated with this second protocol: they were numbered with the suffix “b” after their number, eg. P11b. Treatment was maintained in all included patients for 48 weeks, and all patients were followed for at least 12 more months after treatment withdrawal. However, for the majority of the patients (29 patients), the biochemical response (normal ALT) and virological response (undetectable HBV DNA), as well as the complete response (HBsAg loss), were assayed at 24 months after treatment withdrawal.

HBV DNA quantification

HBV DNA was quantified by means of an in-house real-time polymerase chain reaction (PCR) assay with a lower limit of detection of 350 IU/ml using an international quantification standard (Optiqual® HBV DNA Controls, AcroMetrix,

Benicia, California). The patients were not randomized into the different treatment groups, but sequentially included according to availability of the drugs and new treatment guidelines. Therefore, the sampling protocols slightly differed between the groups. In all patients from the PEG+LAM and PEG groups and in 7 patients from the LAM group, HBV DNA was measured at treatment commencement, then at 8 hours; 1, 2, 4, 5, and 7 days; and 2, 3, 4, 5, 6, 8, 12, 18, 24, and 30 weeks after initiation of treatment. In the ADV+LAM group, the schedule was almost identical except that no samples were taken at 8 hours. Measurements were taken more frequently in the 3 remaining patients from the LAM group and in all patients from the IFN or IFN+LAM groups. HBV DNA levels in these patients were measured at the start of treatment and every 6 hours up to and including 48 hours, and every 12 hours through day 5. Measurements were then taken every second day through day 15, every third day from days 15 through day 30, and then at the end of every month through 12 months after therapy initiation.

Mathematical Model

Analysis of the dynamics of HBV under treatment was based on the standard model of viral infection, which is described by the following system of differential equations (27):

$$dT/dt = s - dT - (1 - \eta)\beta VT \qquad \text{(Equation 1)}$$

$$dI/dt = (1 - \eta)\beta VT - \delta I \qquad \text{(Equation 2)}$$

$$dV/dt = (1 - \varepsilon)pI - cV \qquad \text{(Equation 3)}$$

In the model, *T* is the number of target cells, *I* is the number of productively infected cells, and *V* is the virion concentration. The parameter *s* denotes the rate at which target cells are produced and the constant *d* represents their death rate. Target cells become infected at rate β per uninfected cell per virion, and infected cells die at rate δ. The production and release of hepatitis B virions by infected cells occurs at an average rate of *p* virions per cell per day, and clearance of these virions occurs at rate *c* per day. This model considers two possible effects of treatment: a reduction of the production of virions from infected cells by a fraction (1 - ε) and/or a reduction of the de novo rate of infection by a fraction (1 - η). Assuming that during the period of analysis, the level of target cells remains constant at its pretreatment steady state level, the solution of the equations is:

$$V(t) = \frac{1}{2}V_0[(1 - \frac{c + \delta - 2\varepsilon c}{\theta})e^{-\lambda_1(t-\tau)} + (1 + \frac{c + \delta - 2\varepsilon c}{\theta})e^{-\lambda_2(t-\tau)}], \qquad (4)$$

where

$\lambda_1 = 0.5(c + \delta + \theta)$,

$\lambda_2 = 0.5(c + \delta - \theta)$, and

$\theta = \sqrt{(c-\delta)^2 + 4(1-\varepsilon)(1-\eta)c\delta}$.

This solution includes a delay, τ, between treatment administration and its effect on viral level, and is valid for all time *t* after this delay. For $t < \tau$, $V(t) = V_0$, the initial viral level.

Data Fitting

For the estimation of patient parameters, individual non-linear least squares fits were performed, using a Levenberg-Marquardt algorithm. To maintain the validity of the assumption that the number of target cells remains constant, the fits were limited to data collected during the first four weeks of antiviral treatment. To reduce the number of parameters to fit, and because previous work indicates that η has very little influence on the data fits, we fixed it at 0.5 for all patients (27, 32). All other parameters were free, with the exception of τ for patients 12 and 32, for whom we could not find a stable value and this delay was fixed (Table 1). The virion half-life was calculated from the estimated value of c as ln(2)/c, and the half-life of infected cells was calculated as ln(2)/δ, where ln(2) represents the natural logarithm of 2.

For a few patients there was not enough data to fit the model either because the viral load went below detection too quickly, usually within the first 2 days (4 cases: P24b, P34, P36, P49), or if circumstances precluded sampling the patient at the protocol times (3 cases: P7, P33, P39).

Meta-analysis of published data

A meta-analysis was conducted to compare viral kinetics in HBeAg-positive and HBeAg-negative patients. On October 23, 2008, a Pubmed search was launched with the keywords “dynamics HBV”, “model kinetics HBV”, “mathematical model HBV”, “dynamics HBV treatment” and the same expressions with “HBV” replaced with “hepatitis B”, for papers in the English

language without restriction of dates. Of the over 140 papers retrieved, 17 papers included viral dynamics analyses similar to the one conducted in this paper. Of these, seven papers furnished individual patient's parameter estimates that could be used for our analyses. To further represent previously published results, we also produced forest plots (cite Lewis BMJ 2001) of the data in all studies that provided mean and standard deviations for the kinetic parameters of HBeAg-positive and -negative individuals (n=10 studies).

Statistical Analyses

Descriptive statistics are presented as mean±standard deviation, unless otherwise indicated. We used parametric tests, including t-test, anova and linear regression whenever the necessary assumptions were met. We checked these assumptions by analyzing normality of the data and/or the residuals of the fits as appropriate. When necessary transformations of the data (eg. taking the square root or the logarithm) were used to comply with homoscedasticity and normality of residuals. Results are presented as mean ± standard error of the mean, unless otherwise specified. Significance was assessed at the α=0.05 level and all statistics were performed using S-Plus 2000 (MathSoft Inc, California).

RESULTS

Baseline Characteristics and Outcome of Treatment

Pre-treatment HBV DNA levels, at $t = 0$, ranged from 2.8×10^3 IU/ml to 1.0×10^9 IU/ml (Table 1), with a geometric mean of 6.5±0.2 $\log_{10}$ IU/ml. There were no significant differences in average pre-treatment HBV DNA levels across all treatment groups (p=0.39).

HBV DNA levels went below the detection limit of 350 IU/ml at least once during their respective courses of treatment in 36 patients, mostly within the first 24 weeks. Relapses in viral load and biochemical parameters were observed in almost all patients within 12 months of stopping therapy. The exceptions were patient 5, who has been a sustained biochemical and virological responder for over two years, and patient 41, who remained a biochemical and virological responder 8 months post-therapy and at that time seroconverted to anti-HBs antibodies.

Baseline HBV DNA level was the most important factor predicting the patient's early response to treatment. Patients with undetectable HBV DNA at 8 or 12 weeks of treatment had significantly lower baseline HBV DNA levels, independent of treatment schedule (p=0.008), as recently reported (33).

HBV Decay Patterns

In most patients, HBV DNA levels showed a biphasic decay after treatment was initiated (Figure 1). Four patients (Figure 2) had only a single decay phase over the first 30 days (P26, P27, P38, P43), although P38 and P27 (and perhaps P26) were late responders, and thus they could have had an initial

fast phase of decay but we could not detect it due to less intensive sampling at later time points. The biphasic decay was characterized by an early, rapid decline in HBV, followed by a slower second-phase decrease. This second phase generally conformed to one of two patterns: pattern 1 was characterized by a slow second-phase decay (n=34), and pattern 2 by a flat or nearly flat second phase (n=7).

The average drop in HBV DNA level after 48 hours of treatment was 0.83 $\log_{10}$ IU/ml. This decrease over the first 48 hours differed significantly by treatment regimen: average HBV declines were 1.30 $\log_{10}$ IU/ml, 1.01 $\log_{10}$ IU/ml, 1.05 $\log_{10}$ IU/ml, 0.57 $\log_{10}$ IU/ml, 0.48 $\log_{10}$ IU/ml, and 0.45 $\log_{10}$ IU/ml for patients in the LAM, ADV+LAM, IFN+LAM, PEG+LAM, PEG alone, and IFN alone regimens, respectively (p=0.0008). These differences became less pronounced with time and, at one month, all (except the IFN) groups had similar viral declines (between -2.6 and -3.1 $\log_{10}$, p=0.31), whereas the IFN monotherapy group only had a -1.5 $\log_{10}$ decline.

Kinetic Parameters

In Figure 1, we show representative fits of the model to the data (Figure 1). We present the data and fits for all patients in supplementary information. For some patients, the viral load data could not be fitted. This happened when, during the first month, therapy induced no or minimal decay (4 cases: P3, P10, P17, P46) or when only one phase of decay was observed (4 cases: P26, P27, P38, P43) (Figure 2). The reasons why some patients present these non-

biphasic patterns are not known, but this is often the case for large viral dynamics datasets [cite papers].

Parameter estimates obtained from the non-linear least-squares fits for each patient are displayed in Table 1. The estimated parameters were not significantly different among the treatment groups (with one exception, see below). Overall, the mean±standard deviation values for the clearance rate of virions (c) and infected cells (δ) were 1.9±2.8 day^{-1} and 0.10±0.10 day^{-1}, respectively. The corresponding half-lives were 0.7±0.5 days (17±12 hours) for virions and 13.4±16.9 days for infected cells. The average efficacy (ε) of all treatment regimens was 0.90±0.14, and the average delay (τ) between treatment initiation and its effect on viral load was 0.4±0.6 day (9.6±14 hours). However, protocols including pegylated IFN-α2a had a significantly larger delay than the other treatment groups (0.86 day vs. 0.38 day, p=0.002).

Baseline HBV DNA level correlated negatively with infected cell half-life (r=-0.55, p=0.0008; Figure 3, filled circles). This result suggests that patients with higher baseline viral levels clear infected cells faster because these cells have a shorter half-life. There was also a trend for a weak negative correlation of baseline viral level with drug efficacy (p=0.08), and when three patients (two receiving IFN-α2a and one pegylated IFN-α2a) with low initial drug efficacy (ε<60%) were excluded, this latter trend became significant (r=-0.34, p=0.035). The four regimens that included lamivudine had better efficacy than the IFN alone and pegylated IFN alone regimens, with ε=97% *vs* ε=85%.

Meta-analysis comparison with other viral kinetic studies

In order to compare our results with other available HBV viral dynamics studies in both HBeAg-positive and -negative patients, a Pubmed search was conducted to identify viral kinetic studies during treatment of HBV infection (see Methods). Table 2 presents a summary of the seven published studies that include parameter estimates for each patient analyzed that were used in our meta-analysis comparing HBeAg-positive and HBeAg-negative kinetics. Those papers together with the present study have information on 79 HBeAg-positive patients and 100 HBeAg-negative patients treated with a variety of different antiviral treatment regimens. For each patient, the half-life of free virions and the half-life of infected cells were obtained. The information on baseline viral level was more difficult to use, because different HBV DNA assays were used in the seven studies (Table 2). In 4 of the studies that did not report IU/ml, we converted baseline HBV DNA levels according to the conversion factors provided by Shyamala *et al.* (34). The other two studies (27, 35) either did not report individual baseline HBV DNA levels or used in-house assays, for which the conversion factor was not reported. In any case, the estimates for the half-lives of free virions and infected cells are not impacted by the specific assay/units used in a given study.

As expected, the baseline HBV DNA levels were significantly lower on average in HBeAg-negative than in HBeAg-positive patients: means 6.3±0.1 *vs* 7.9±0.1 $\log_{10}$ IU/ml, respectively ($p<0.00001$). Moreover, the range in baseline viral levels in HBeAg-negative patients (range: 3.4 – 9.5 $\log_{10}$ IU/ml) was 100-fold

larger than that in HBeAg-positive patients (range: 5.3 – 9.7 $\log_{10}$ IU/ml). We then compared the viral kinetics parameters. Figure 4 (top panels) shows the half-lives of free HBV virions and infected cells in HBeAg-positive and HBeAg-negative infection. The half-life for free virions was significantly smaller in HBeAg-negative than -positive infection (mean=13.?±1.1 h *vs* 25.?±1.7 h, p<0.00001) and the same was true for the half-life of infected cells (mean=12.?±1.4 days *vs* 16.?±1.7 days, p=0.0001). To further assess these results, we included three other studies that reported means and standard deviations for the kinetic parameters of HBeAg-positive and HBeAg-negative individuals and represent this data as forest plots in Figure 4 (middle panels). These plots show the distribution of kinetics parameters for all studies at the same time. The diamonds representing the overall parameter estimates in HBeAg-positive and -negative subjects do not overlap, lending support to our results above.

Still, it could be argued (29) that the different sampling schedules (Table 2) and modeling approaches affect the interpretation of these estimates. Thus, we repeated these analyses with data from two studies conducted by us, which included for the most part similar sampling and, crucially, the exact same fitting procedure (present work and [cite Lau]) (Figure 4, bottom panels). The results were similar: for the free virion half-life, the means were 17.?±1.9 h *vs* 27.?±3.1 h for HBeAg-negative and -positive patients, respectively (p=0.0013); for the infected cell half-life, the means were 13.?±2.9 days *vs* 16.?±2.9 days, respectively (p=0.096). This data subset was also used to compare total viral production, calculated by multiplying the free virion clearance rate by baseline

viral load. There was a significantly larger virion production in HBeAg-positive than -negative infection (7.5±0.2 *vs* 6.4±0.2 $\log_{10}$ IU/ml/day, p= 0.001).

Taking into account all the patients infected with HBeAg-negative HBV (n=81) confirmed the result of our study, shown above, that the half-life of infected cells is negatively correlated with baseline HBV DNA level (r=-0.51, p<0.00001) (Figure 3). This was not the case in the HBeAg-positive HBV patients (n=48, p=0.73). Importantly, if we restrict the analysis of HBeAg-negative infection to those patients who have baseline viral load in a similar range to the HBeAg-positive cohort, we find that the correlation still holds (r=-0.45, p=0.0003, n=63). Thus, this relationship between infected cell half-life and baseline viral load is a characteristic of HBeAg-negative infection, and not an artifact of different viral load levels in these patients.

DISCUSSION

In this study we have analyzed the dynamics of viral turnover in HBe antigen-negative hepatitis B virus infection, based on patient response to different antiviral treatment protocols based on IFN-α and/or nucleos(t)ide analogues. By using a standard model (26) of viral infection, we were able to estimate viral clearance, infected cell loss rate and the efficacy of stopping viral production of different treatment protocols. As expected, the long-term outcome of treatment was very poor in all treatment groups after only 48 weeks of administration followed by withdrawal. Therefore, it was not possible to find any

baseline host/viral characteristic, or kinetic parameter associated with long-term responses. Overall, we did not detect significant differences in viral kinetic parameters among the different study groups. However, not surprisingly the four regimens that included lamivudine had better efficacy than the two regimens that included IFN-α2a and pegylated IFN-α2a alone. Although, this same trend has been seen in other studies [citations?], we cannot make definitive conclusions about the differences in treatment regimens, because the inclusion in each arm was not random, it rather obeyed clinical criteria.

The main objective of our study was to find how the dynamics of HBeAg-negative infection differ from those in HBeAg-positive infection. We thus conducted an extensive meta-analysis to compare our results to those of all previous viral kinetic studies that included data for individual patients (Table 2). Interestingly, we found a significantly faster viral clearance rate (and thus shorter viral half-life) for HBeAg-negative infection than for HBeAg-positive infection. The mean estimate of the viral half-life for HBeAg-positive infection was ~25 hours, whereas in HBeAg-negative infection, it was ~13 hours. This indicates that the lower viral levels found in HBeAg-negative infection are not necessarily due to lower viral production (16), but rather they occur through the balance of high viral replication and fast immune clearance, *i.e.* rapid HBV dynamics. It has been suggested that the shorter virion half-lives in HBeAg-negative infection are related to a lower viral load at baseline in these patients, as faster virion clearance has been reported to correlate with lower baseline viral loads [cite Murray]. However, we did not find such a correlation in our dataset or meta-

analyses, when we considered just HBeAg-negative or HBeAg-positive patients. The estimated half-life of infected cells is also shorter in HBeAg-negative than in HBeAg-positive HBV infection.

In our meta-analysis we included all the extant studies with viral dynamic data. This implies that we collected data generated by different groups, and with somewhat different techniques, including different treatment protocols, different assays, and different viral kinetics models for determination of virion and infected cell half-life. Since this variability could bias our results, we also repeated our comparisons of HBeAg-positive and –negative infection using two studies conducted by us, which included similar sampling and exactly the same modeling methodology. This restriction of the analyses helps control for the effect of different studies. ~~Another way would be to use random effects, unfortunately, fitting such models to this data was not statistically supported, perhaps because almost all studies (with the exception of Mihm et al [cite]) included only HBeAg-positive or only HBeAg-negative patients. Preferably, our results should be confirmed in a future study including both HBeAg-positive and –negative subjects.~~

It is known that cytoplasmic HBeAg can enter both the major histocompatibility complex (MHC) class I pathway for recognition by CD8-positive CTLs and the MHC class II pathway for recognition by CD4-positive T cells (12). On the contrary, HBcAg cannot enter the class II pathway (12). Therefore, hepatocytes producing cytoplasmic HBeAg may be targeted for destruction through both CD8-positive and/or CD4-positive pathways. It is difficult to explain

the selective advantage of an HBeAg-negative variant at the level of CTL recognition because the infected hepatocyte expresses HBcAg regardless of HBeAg expression. However, if HBcAg expression becomes limiting in terms of MHC class I loading and/or CTL recognition, then co-expression of cytoplasmic HBeAg would make the infected hepatocyte more visible to specific CTLs. In this context an HBeAg-negative virus would represent an effective CTL escape variant (16). Secreted HBeAg, in contrast, has been suggested to have a tolerogenic function, down-regulating the immune response against HBV and moderating HBeAg-specific liver injury (11, 14, 36). According to this hypothesis, the HBeAg-negative virus would escape the cytoplasmic HBeAg-targeted immune response, but the escape mutant would also lose the function of its secreted immunoregulatory protein.

In this context, our results are compatible with a stronger immune response in the setting of HBeAg-negative infection. Moreover, we found a strong negative correlation between the half-life of infected cells and the baseline viral level for HBeAg-negative infection. This could indicate that, in the absence of the immunomodulatory effects of HBeAg, the immune response is stronger for larger antigen load, in spite of its inability to fully control infection (37). The dual roles of the HBeAg explain how an immune escape variant can be more pathogenic and predispose to a more aggressive form of disease (14), and still be associated with lower viral levels.

In conclusion, analysis of HBeAg-negative early viral kinetics under various antiviral treatments in patients with HBV genotype D chronic hepatitis B

showed faster dynamics of HBV DNA and infected hepatocytes in comparison with the HBeAg-positive chronic hepatitis patients. These results reveal the dual role of the immune response in maintaining lower viral levels, but at the same time inducing faster turnover of infected cells, which may be responsible for the more aggressive nature of HBeAg-negative infection.

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FIGURE CAPTIONS

Figure 1. Representative results of the fit of the model (line) to the HBV DNA level (circles). Each row corresponds to a different treatment protocol (1st row: IFN; 2nd row: LAM; 3rd row: IFN+LAM; 4th row: PEG+LAM; 5th row: PEG; 6th row: ADV+LAM). See methods for full description of fitting procedure.

Figure 2. Data for those patients who could not be fitted in the model (note that we use the same y-axis range for all cases to allow easier comparisons).

Figure 3. Correlation between infected cell half-lives and baseline HBV DNA levels for HBeAg-negative infection. The symbols correspond to different studies as indicated in the legend (see Table 2), and the best fit regression line is also shown.

Figure 4. Comparison of the half-lives of free virions and infected cells between infections with HBeAg-negative (Neg) and HBeAg-positive (Pos) HBV strains. a) and b) present the data for all the studies in Table 2, for free virions and infected cells, respectively. c) and d) restrict the data to those studies with similar frequent early sampling (see text). The bottom and top of the box in grey represents the 25th and 75th percentile of the data, respectively; the whiskers represent 1.5 times the inter-quartile range and give an idea of the range of values in the data; the horizontal line inside the box represents the median of the data and the black box around it an approximate estimate of the 95% confidence interval for the median.

Table 1. HBV Kinetic Parameters (SD: standard deviation)

Patient	Tx	V_0 (log_{10} IU/ml)	c (day^{-1})	δ (day^{-1})	ε	τ (days)	V half-life (days)	I half-life (days)
1	IFN	7.73	2.32	0.072	0.727	1.6	0.30	9.63
2	IFN	7.81	0.42	0	0.965	0.0	1.65	
3	IFN	6.98	NA	NA	NA			
4	IFN	4.18	0.24	0.053	0.855	2.9	2.89	13.08
5	IFN	5.56	1.3	0.061	0.988	0.3	0.53	11.36
6	IFN	5.52	1.32	0.036	0.953	0.0	0.53	19.25
30	IFN	6.41	17.36	0.008	0.559	0.9	0.04	86.64
31	IFN	6.23	1.3	0.027	0.363	0.0	0.53	25.67
32	IFN	4.65	1.02	0.041	0.852	0.1	0.68	16.91
45	IFN	5.74	0.64	0.113	0.985	0.0	1.08	6.13
46	IFN	8.76	NA	NA	NA			
Mean		**6.33**	**2.88**	**0.046**	**0.805**	**0.6**	**0.91**	**23.58**
8	LAM	5.61	6.19	0.135	0.922	1.2	0.11	5.13
9	LAM	7.57	2.22	0.139	0.966	0.8	0.31	4.99
10	LAM	7.66	NA	NA	NA			
11	LAM	6.58	1.81	0.172	0.934	0.6	0.38	4.03
12	LAM	7.72	1.71	0.188	0.896	0.0	0.41	3.69
13	LAM	5.76	0.77	0.029	0.998	0.0	0.90	23.90
14	LAM	7.76	1.93	0.292	0.964	0.1	0.36	2.37
15	LAM	4.56	2.09	0	0.982	0.7	0.33	
16	LAM	4.11	0.69	0.064	0.990	0.0	1.00	10.83
17	LAM	5.02	NA	NA	NA			
Mean		**6.24**	**2.18**	**0.127**	**0.957**	**0.4**	**0.48**	**7.85**
18	IFN+LAM	7.86	0.55	0.028	1.000	0.0	1.26	24.76

19	IFN+LAM	7.48	1.58	0.133	0.779	0.4	0.44	5.21
20	IFN+LAM	6.11	0.87	0.023	0.998	0.0	0.80	30.14
21	IFN+LAM	9.00	1.49	0.184	0.801	0.3	0.47	3.77
22	IFN+LAM	7.53	1.06	0.155	0.941	0.3	0.65	4.47
35	IFN+LAM	8.26	0.81	0.189	0.996	0.0	0.86	3.67
37	IFN+LAM	6.30	0.87	0.067	0.934	0.0	0.80	10.35
Mean		**7.51**	**1.03**	**0.111**	**0.921**	**0.1**	**0.75**	**11.77**
23	PEG+LAM	5.49	0.77	0	0.999	1.8	0.90	
24	PEG+LAM	8.11	0.62	0.164	0.912	1.0	1.12	4.23
25	PEG+LAM	6.76	0.61	0	0.885	0.1	1.14	
26	PEG+LAM	9.00	NA	NA	NA			
27	PEG+LAM	7.63	NA	NA	NA			
28	PEG+LAM	3.45	0.46	0	0.983	1.1	1.51	
29	PEG+LAM	5.36	1.3	0.076	0.990	0.9	0.53	9.12
Mean		**6.54**	**0.75**	**0.048**	**0.954**	**1.0**	**1.04**	**6.67**
38	PEG	8.48	NA	NA	NA			
40	PEG	8.00	5.86	0.42	0.811	0.3	0.12	1.65
41	PEG	7.08	3.39	0.199	0.509	0.8	0.20	3.48
42	PEG	4.78	0.78	0.027	0.988	0.9	0.89	25.67
43	PEG	8.38	NA	NA	NA			
Mean		**7.34**	**3.34**	**0.215**	**0.769**	**0.6**	**0.40**	**10.27**
11b	ADV+LAM	3.78	1.54	0	0.942	0.0	0.45	
12b	ADV+LAM	7.41	1.96	0.227	0.934	0.5	0.35	3.05
14b	ADV+LAM	6.48	1.63	0.262	0.986	0.0	0.43	2.65
26b	ADV+LAM	7.61	1.27	0.094	0.980	0.0	0.55	7.37
27b	ADV+LAM	7.18	1.03	0.166	0.765	0.0	0.67	4.18
47	ADV+LAM	4.98	1.18	0.067	0.992	0.0	0.59	10.35
48	ADV+LAM	4.73	1.33	0.013	0.990	0.0	0.52	53.32

50	ADV+LAM	4.30	1.57	0	0.978	0.5	0.44	
51	ADV+LAM	6.08	0.91	0.171	0.885	0.0	0.76	4.05
Mean		**5.84**	**1.38**	**0.111**	**0.939**	**0.1**	**0.53**	**12.14**
Overall Mean		**6.52**	**1.87**	**0.100**	**0.899**	**0.4**	**0.69**	**13.39**
Overall SD		**1.49**	**2.76**	**0.096**	**0.142**	**0.6**	**0.50**	**16.94**

Table 2. Viral kinetic studies used in the meta-analysis' comparison of treatment in HBeAg-positive (+) and HBeAg-negative (-) infection (LAM: lamivudine, ETV: entecavir, FAM: famciclovir, ADV: adefovir, EMT: emtricitabine, PEG: pegylated IFN).

Study	HBeAg	n	Treatment	Sampling
Wang *et al.* (35)	+	10	100mg vs 600mg LAM	0, 6, 24, 30, 36, 72, 76, 84h, d 5, 6, 7, 10, 14, , 21, 28
Wolters *et al.* (38)	+	10	4 doses of ETV	0, 8, 24, 32h, d 3, 4, 6, 7, 10, 14, 21, 28
Lewin *et al.* (27)	+	15	LAM *vs* LAM+FAM	0, 24, 48h, d 3, 4, 5, 6, 7, 10, 14, 28, 42, 56, 70, 84
Tsiang *et al.* (39)	+	10	ADV	d 0, 7, 14, 28, 56, 70, 84
Lau *et al.* (40)	+	30	ADV *vs* ADV+EMT	d 0, 1, 3, 5, 7, 9, 11, 14, 21, 28
Mihm *et al.* (41)	+/-	8	LAM+ADV	d 0, 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, 56
Colombatto *et al.* (30)	-	72	LAM *vs* LAM+PEG *vs* PEG	0, 8, 24, 48h, d 4, 5, 7, 14, 21, 28, 35, 42, 84
Ribeiro *et al.*, present study	-	42	Multiple	See methods

Figure 1

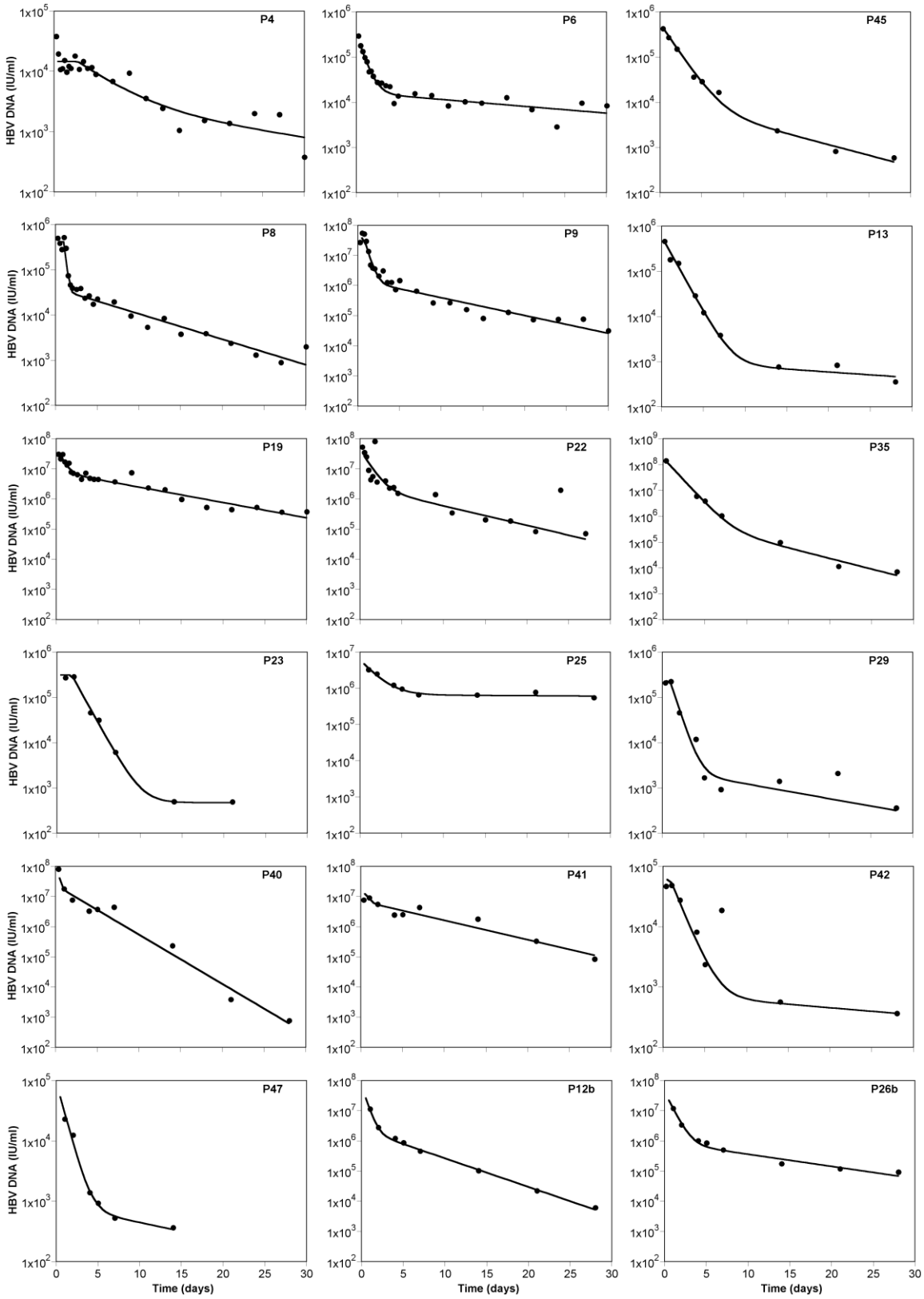

P4
P6
P45
P8
P9
P13
P19
P22
P35
P23
P25
P29
P40
P41
P42
P47
P12b
P26b
HBV DNA (IU/ml)
Time (days)

Figure 2

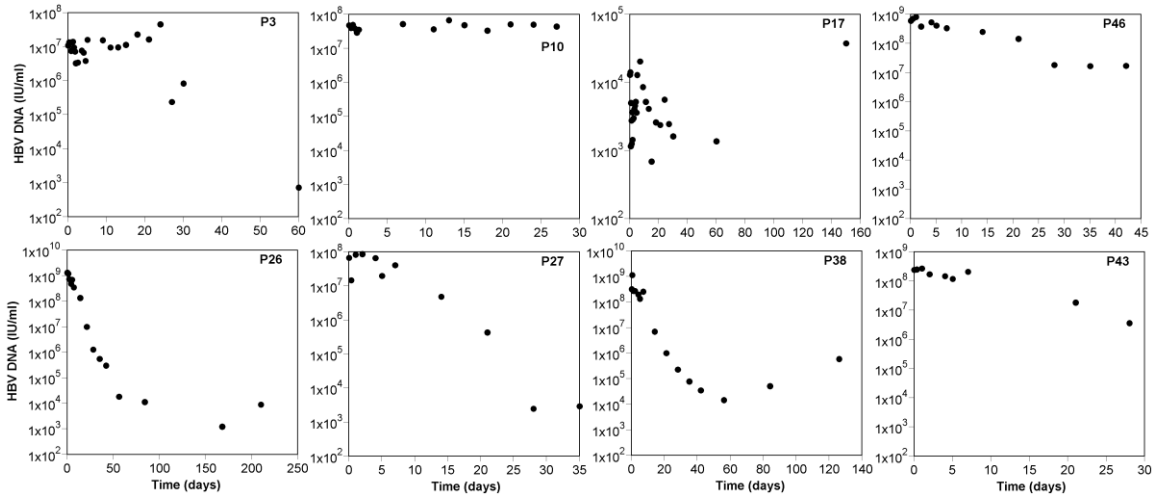


Figure 3

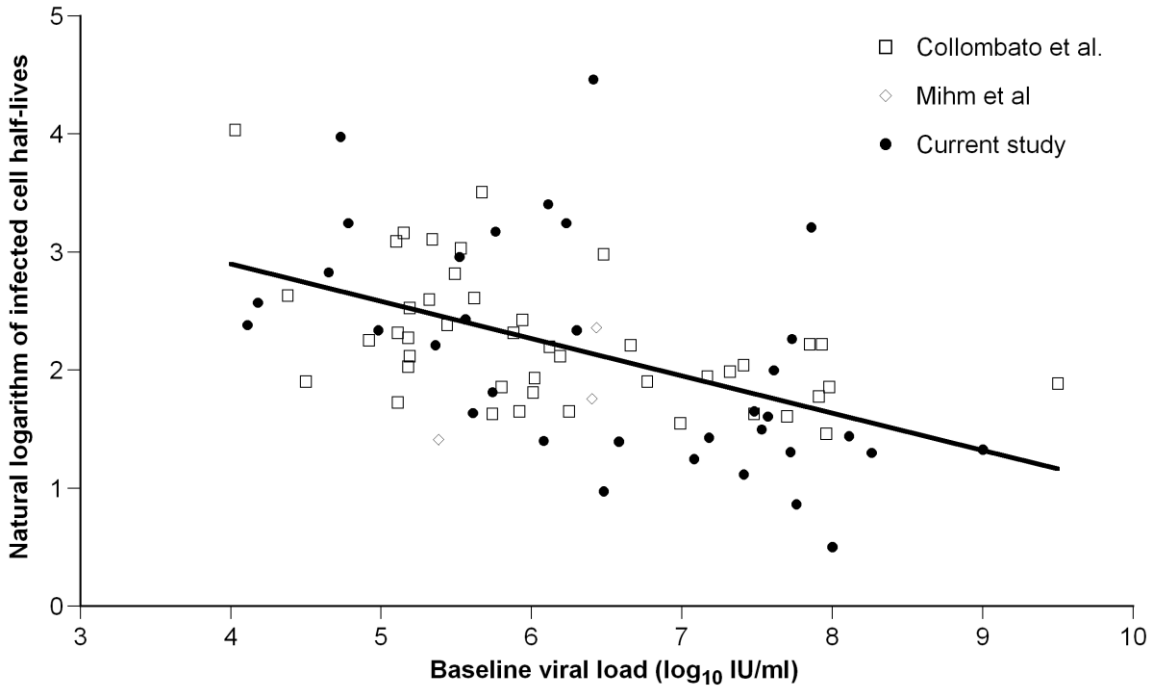
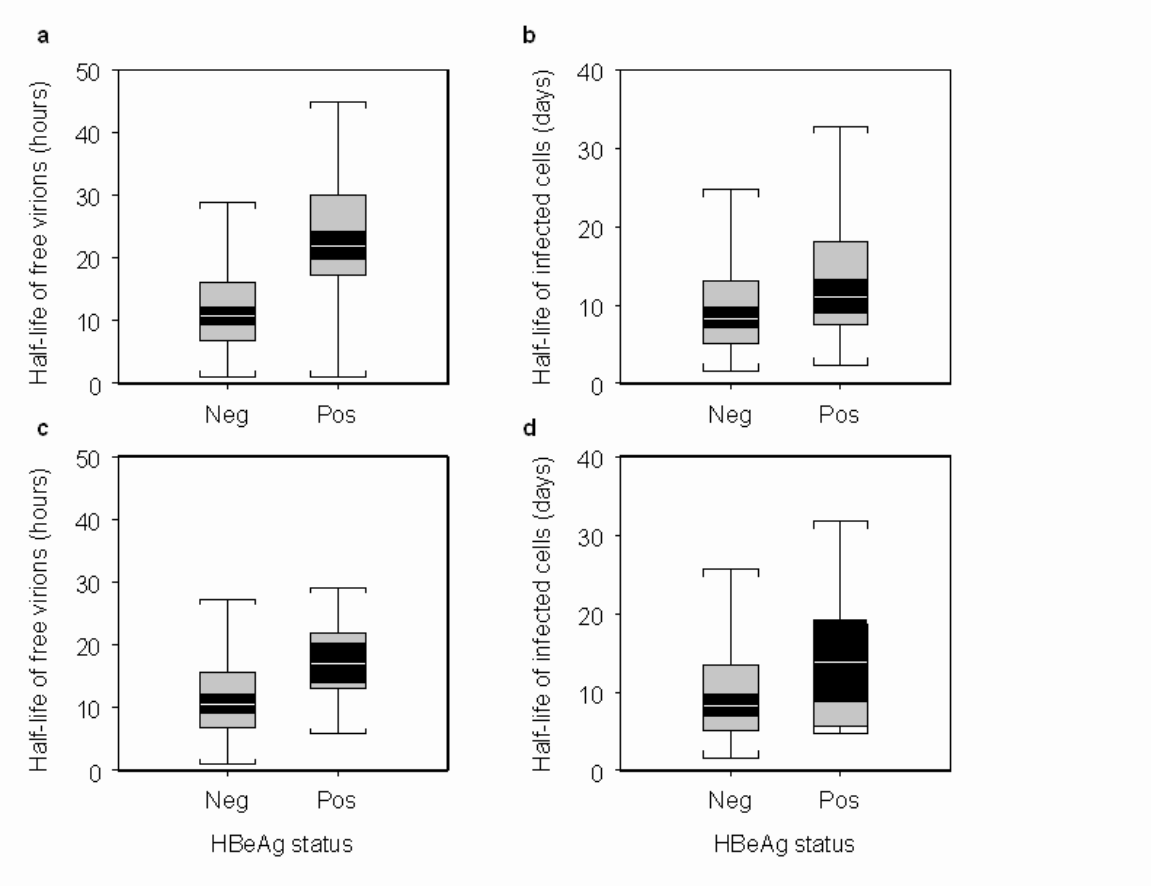
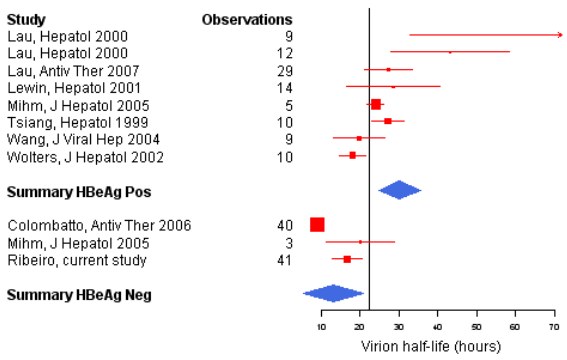
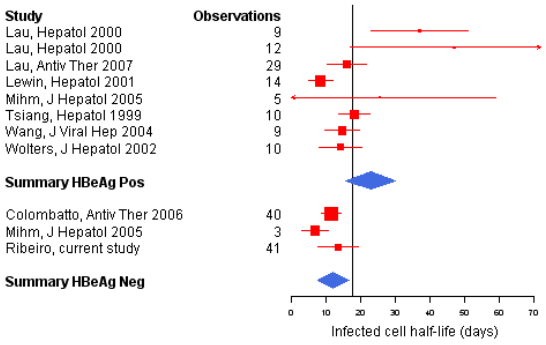

Collombato et al.
Mihm et al
Current study
Natural logarithm of infected cell half-lives
Baseline viral load (log10 IU/ml)
0
1
2
3
4
5
3
4
5
6
7
8
9
10

Figure 4


a
b
c
d
Half-life of free virions (hours)
Half-life of infected cells (days)
0
10
20
30
40
50
Neg
Pos
HBeAg status





Forest plots representing the results of different studies analyzed. The vertical black line represe the global average of HBeAg-positive (the top 8 rows) and the HBeAg-negative (the bottom 3 row Note that study [cite Lau Hepatol 2000] reported the two treatment arms separately as indicated the two first rows of the graphs.