ANSWER TO THE REVIEWERS

Dear Editor of *World Journal of Gastroenterology.*

Enclosed please find the detailed answer to the comments of the reviewers. The delay in sending you the final version of the manuscript is because we have performed genotyping experiments of HDV to answer one of the most common observations of the reviewers.

In addition we have introduced the modifications suggested by the Editor. So, we have change the tittle to avoid abreviations, and we have included the PMID and DOI numbers in the references.

Reviewer 00506552

Original comments of the Reviewer: Authors of this article (Madejón et al.) described that HBV-A genotype infection interfere viral replication than genotype D or E in HDV co-infected patients. Even though number of participated patients are not enough, it was worthwhile to investigate the genotype differences in CHB-CHD co-infected patients. If authors of this manuscript include the in vitro replication results with HBV-A only vs HBV-D only vs HBV-A and HDV vs HBV-D and HDV, it would be very interesting and certify the importance of HBV genotype in HDV co-infection. Generally, the importance of this trial is needed, but I have several suggestions. Since HBV-A DNA level was lower than HBV-D when HDV was co-infected, the authors of this manuscript should discuss the disease outcomes (HCC and/or cirrhosis) between these statues. Long term follow-up studies are needed, later. Throughout the manuscript, the authors described CHD (of course, it is no doubt), it was better to describe CHB-CHD co-infection all the time (Some readers are not from HBV research area). 16 CHD (+ CHB without HIV) patients were participated a longitudinal dynamic analysis, it would be better to include CHB (without HIV) for comparison. Results were very difficult to follow. In page 8 line 4 to 7, I cannot find where I can find in the table or Fig. In page 8, 'In all but one patient, … (Table 2 and 3)', it must be Table 3 and 4. In page 8, 'In all but one patient, HDV-RNA was higher than HBV-DNA…', I think that without copy numbers, it cannot be said that HDV-RNA was higher than HBV-DNA. Author should calculate the exact copy number of HDV-RNA and HBV-DNA. In page 9, at first paragraph, was it Table 3? In page 9, at 3rd paragraph, where can I find the result? These types of confusions are throughout the manuscript. All of Figures need to add the numeral numbers (median numbers) at of the graphs since all of Fig. were very difficult to see the numeral numbers.

Answer to the specific comments.

If authors of this manuscript include the in vitro replication results with HBV-A only vs HBV-D only vs HBV-A and HDV vs HBV-D and HDV, it would be very interesting and certify the importance of HBV genotype in HDV co-infection.

We agree with the reviewer´ comments on the potential usefulness of *in vitro* testing for analysis of the replication interference between the genotypes of hepatitis B and delta viruses. Although this experimental models would be helpful to confirm our results, in our knowledge, there are not well-established systems of HDV infection/replication

which would support the complete replication cycle of HBV and HDV in experimental conditions (needed to asses the hypothesis of our work). Thus, the use of primary hepatocytes is complex due to experimental limitations such as fast loss of infection susceptibility, experimental variation between different batches of cells and the need of addition of chemicals -such as DMSO-, which may interfere in the results. The most appropriate would be to use cell lines culture systems in which replication of both viruses can be initiated by cellular transfection with expression plasmids. However, there are not currently in vitro models of HBV infection that would ensure a similar efficiency of infection/replication of the different genotypes of HBV, a crucial aspect for comparative replication assays. We included in the Discussion section (page 14, paragraph 1, lines 6-9) a sentence indicating the future utility of these assays to elucidate the molecular mechanisms involved at different levels of HBV/HDV interactions.

Since HBV-A DNA level was lower than HBV-D when HDV was co-infected, the authors of this manuscript should discuss the disease outcomes (HCC and/or cirrhosis) between these statues. Long term follow-up studies are needed, later.

Although the rate of patients with advanced fibrosis (F3 and F4) was higher in the group of patients coinfected with HBV-D + HDV than in those with HBV-A + HDV, these differences were not statistically significant. For this reason, these data were not included in the original manuscript. A long-term follow-up of an extensive number of patients is needed, as reviewer suggests, for clarify this aspect, and this is now stated in the Discussion section (page 14, paragraph 1, lines 4-5).

Throughout the manuscript, the authors described CHD (of course, it is no doubt), it was better to describe CHB-CHD co-infection all the time (Some readers are not from HBV research area).

To clarify this aspect to those readers who are not from HBV research area, we have now included a sentence in the introduction which explains that HDV infection requires the coexistence of HBV (Page 5, paragraph 1, lines 1-3).

16 CHD (+ CHB without HIV) patients were participated a longitudinal dynamic analysis, it would be better to include CHB (without HIV) for comparison.

The inclusion of a group of CHB patients (without HDV and HIV coinfection) is optimal as control group of the HBV replicative pattern of the CHD patients. This control group (27 CHB patients) was included in the transversal study. The objective of the inclusion of a longitudinal analysis of 16 CHD patients was to perform a dynamic analysis of the simultaneous HBV and HDV replication patterns during the disease evolution, instead of focusing the analysis in the HBV replication behaviour only.

Results were very difficult to follow. In page 8 line 4 to 7, I cannot find where I can find in the table or Fig.

The overall analysis of the HBV-DNA titers in patients infected with HBV-A and D without additional stratification according the co-infection with HIV or HDV are only shown in the text, and they are not included in additional Tables or Figures to avoid data redundancy.

In page 8, 'In all but one patient, … (Table 2 and 3)', it must be Table 3 and 4.

The correct Table reference is now included (page 9, paragraph 1, line 3).

In page 8, 'In all but one patient, HDV-RNA was higher than HBV-DNA…', I think that without copy numbers, it cannot be said that HDV-RNA was higher than HBV-DNA. Author should calculate the exact copy number of HDV-RNA and HBV-DNA.

The HDV-RNA titers were expressed as copy number/ml. The HBV-DNA titters were expressed as IU/ml, the most common units, and as copy number/ml determined by an in house amplification method, in the comparative analysis of HBV and HDV replication levels. We have modified the Material and Methods section to clarify this aspect (Page 7, paragraph 4).

In page 9, at first paragraph, was it Table 3?.

The HBV genotyping results obtained by the combined use of LiPA and PCR/sequencing techniques are shown in Table 3. The reference to this table is now stated in the manuscript (page 9, paragraph 2, line 11), and the Table 3 legend has been modified to clarify the source of genotyping data (lines 4-6). The HBV genotyping results using the LiPA technique alone are only included in the text.

In page 9, at 3rd paragraph, where can I find the result?

The results of the 3rd paragraph are shown in Figure 3A. To clarify this aspect, the Table reference is now included in the first sentence of the paragraph: "Quantitative analysis of serum HBV-DNA levels also showed a different replication level according to the HBV genotype (Figure 3A):…"(page 9, paragraph 4, line 2).

<u>Reviewer 02861202</u>

Original comments of the Reviewer: The manuscript by Dr. Madejon and co-workers refers to the influence of HBV genotype on HDV replication. The subject is of interest even though it is already known that HBV genotypes may affect a different HDV replication fitness. However, in order to draw solid conclusions, it is important to analyse HDV genotypes as well, since it is well known the existence of different replication fitness either inter genotypes and intra genotypes of HDV (European genotype 1 as compared to Asian genotype 1 of HDV).

Answer to the specific comments.

However, in order to draw solid conclusions, it is important to analyse HDV genotypes as well, since it is well known the existence of different replication fitness either inter genotypes and intra genotypes of HDV

We agree with the importance of analyzing the HDV-genotype composition in our population to determine its role on the different replication behaviour of HBV in

patients with chronic hepatitis delta. In this way, the HDV genotype composition was uniform in our patients. Thus, HDV European genotype 1 was detected in the 60 Caucasian, and in 6/8 (75%) of the immigrant Subsaharian patients. In the remaining 2 patients, HDV genotypes 3 and 4 were respectively detected, one of them infected with HBV-A and the other with HBV-D. Due to the very low number of HDV genotypes other than HDV-1, no statistical analysis of the role of different HDV genotypes could be performed in this work, and the conclusions should be applied to the superinfection with HDV-1. The HDV genotype composition is now included in the final manuscript version (Table 1 legend; lines 4-6), as well as the potential importance of further studies focused in the HDV genotype to understand the HBV/HDV interference. This topic is commented in the Discussion section (page 14, paragraph 1, lines 9-11).

Reviewer 00504172

Original comments of the Reviewer: The study is really interesting and well conducted: there are only minor points to make and a few questions to ask Questions: It is very difficult to know the duration of a HBV infection and in fact only for a few patients in the "longitudinal follow-up" these data are reported. For patients in the cross-sectional study is it possible to know something more? Can there be a bias due to a different duration of infection in patients with different genotypes? Is it known if HBV-HDV infections are co-infections or super-infections? Was the presence of HBV Mediterranean variant as a possible confounding factor evaluated? (Are the subjects with anti-HBe the result of a seroconversion from HBeAg or are Mediterranean variants?) It is likely that the present HDV genotype is genotype 1, given its ubiquity, but was the HDV genotype evaluated to check if HBV replication is HDV genotype dependent as well as HBV genotype dependent? Remarks: Materials and methods Since the term of chronic liver disease is a bit too concise, more detailed clinical descriptions of case studies would be desirable. Results Since in the Discussion there is the sentence: " different HBV genotypes seem to be not related with simultaneous infection with HCV…", the data and related statistics for both the cross-sectional study and for the longitudinal follow-up should be shown. Since In the discussion there is the sentence: "different HBV genotypes seem to be not related with …..the previous treatment pressure", this has been described for the longitudinal follow-up but not for cross-sectional study. The term "tended" in the phrase " Similarly, the HDV-RNA titers tended to be higher in HIV-patients than in HIV-negatives…." gives rise to misunderstandings as p = NS. Discussion A short comment on the HBV Mediterranean variant and HDV genotypes in relation to this study may allow the reader to have a broader view of the problem.

Answer to the specific comments.

It is very difficult to know the duration of a HBV infection and in fact only for a few patients in the "longitudinal follow-up" these data are reported. For patients in the cross-sectional study is it possible to know something more? Can there be a bias due to a different duration of infection in patients with different genotypes?

As the reviewer comments, it is very difficult to obtain conclusive data about the HBV or HDV dates of infection. In the population included in the cross-sectional analysis only 25 (37%) patients had these data. No differences in the HBV duration of infection

were observed between patients infected with HBV-A or HBV-D (mean + SD: 21.5 ± 5.3 vs 23,75 ± 6.2 years, respectively). However, the low patients number with conclusive data of HBV/HDV duration of infection do not allow to perform an extensive statistical analysis. This issue is now included in the new version of the manuscript (Table 1 legend, lines 4-7).

Is it known if HBV-HDV infections are co-infections or super-infections?

Although there are no data of HDV infection date, the chronic HDV infection profile suggest that the majority (if not all) of patients were super-infected with HDV. No patients with acute HDV infection, more related with co-infection, have been included in this work.

Was the presence of HBV Mediterranean variant as a possible confounding factor evaluated? (Are the subjects with anti-HBe the result of a seroconversion from HBeAg or are Mediterranean variants?)

We agree with the potential importance of the analysis of genetic variability of HBV -beyond the determination of the viral genotype-, like the analysis of the pre-core/core variants. In this aspect, it was not possible to determine if the anti-HBe status of our patients was due to either a seroconversion or infection with the Mediterranean variant. Thus, all the patients were anti-HBe negative at the time of its inclusion, and the genetic analysis of the pre-core/core region was not available. It should be pointed out, however, that the vast majority of CHD patients are HBe-Ag negative as consequence of the suppression of HBV replication by HDV. We included in the Discussion section the potential importance of the HBV pre-core/core variants and the HDV genotype to understand the HBV/HDV interference process.

It is likely that the present HDV genotype is genotype 1, given its ubiquity, but was the HDV genotype evaluated to check if HBV replication is HDV genotype dependent as well as HBV genotype dependent?

We agree with the importance of analyzing the HDV-genotype composition to determine its role on the different replication behaviour of HBV in patients with chronic hepatitis delta. In this way, the HDV genotype composition was uniform in the population analyzed in this work. Thus, HDV European genotype 1 was detected in the 60 Caucasian, and in 6/8 (75%) of the immigrant Subsaharian patients. In the remaining 2 patients, HDV genotype 3 and 4 were respectively detected, one of them infected with HBV-A and the other with HBV-D. Due to the very low number of HDV genotypes other than HDV-1, no statistical analysis of the role of different HDV genotypes could be performed in this work, and the conclusions should be applied to the superinfection with HDV-1. The HDV genotype composition is now included in the final manuscript version (Table 1 legend; lines 4-6), as well as the potential importance of further studies focused in the HDV genotype to understand the HBV/HDV interference. This topic is commented in the Discussion section (page 14, paragraph 1, lines 9-11).

Since the term of chronic liver disease is a bit too concise, more detailed clinical descriptions of case studies would be desirable.

New data concerning HCV infection (Patients and Methods; page 6, paragraph 3, lines 8-10), duration of viral infection, time of antiviral treatment and HDV genotype (Table 1 legend, lines 4-7) are now included in the clinical description of the patients.

Since in the Discussion there is the sentence: " different HBV genotypes seem to be not related with simultaneous infection with HCV…", the data and related statistics for both the cross-sectional study and for the longitudinal follow-up should be shown.

Data about HCV infection in the cross-sectional study is now included in the Material and Methods section (page 6, paragraph 3, lines 8-10) and in the Results section (page 8, paragraph 2, lines 13-15). Data in the longitudinal study is discussed in Table 4 legend (lines 4 and 5).

Since In the discussion there is the sentence: "different HBV genotypes seem to be not related with …..the previous treatment pressure", this has been described for the longitudinal follow-up but not for cross-sectional study.

Data about previous treatment pressure in the cross-sectional study is now included in the Results section (page 8, paragraph 2, lines 15-18). Data in the longitudinal study is discussed in Table 2 legend (lines 3-6).

The term "tended" in the phrase " Similarly, the HDV-RNA titers tended to be higher in HIV-patients than in HIV-negatives…." gives rise to misunderstandings as p = NS.

This sentence was changed to avoid misunderstanding. The novel sentence is: "Similarly, the HDV-RNA were higher, but without statistical significance, in HIV-patients than in HIV-negatives….(page 8, paragraph 1, lines 7-8).

Discussion A short comment on the HBV Mediterranean variant and HDV genotypes in relation to this study may allow the reader to have a broader view of the problem.

As described above, we discussed in the new manuscript version the potential importance of the HBV pre-core/core variants and HDV genotype analysis as additional factors that should be taken into account in the HBV/HDV replication interference process.

Reviewer 00012386

Original comments of the Reviewer: It looks acceptable.

Answer to the specific comments.

Does not apply.

Reviewer 03257372

Original comments of the Reviewer: The paper by Madejon et al is interesting but could be improved Major comments: 1) That the extent of suppression of HBV

replication by HDV differs depending on the HBV genotype is something I have suspected for some time. However, I also think that the HDV genotype also plays an important role. In this respect, it is unfortunate that the HDV isolates were not genotyped. For the Caucasian patients these are almost certainly HDV 1, but the sub-Saharan patients may have "African" HDV genotypes. I think that this point of the potential role of HDV genotypes should be discussed 2) At the beginning of the Discussion the Authors make much of the fact that HDV-RNA titers are higher than HBV-DNA titers. I do not think that it is pertinent to compare the titers of two very different viruses. What is important is that HBV-DNA titers drop when there is HDV co-infection and the size of the drop seems to depend on the HBV genotype. 3) The Discussion is too long in any case and should be reduced. Minor comments: 1) In Figures 2A and 3A some of the box-plots are missing, being represented only by the thick bar that presumably represents the median value but this is not stated in the legends to the Figures. In Figure 2A, what is the meaning of the asterisk (*)? 2) There are several spelling and grammatical errors. This is especially the case for "titer" that at several places including in the Figures is spelled "titter". The paper should be reviewed by a competent English speaker.

Answer to the specific comments.

1) That the extent of suppression of HBV replication by HDV differs depending on the HBV genotype is something I have suspected for some time. However, I also think that the HDV genotype also plays an important role. In this respect, it is unfortunate that the HDV isolates were not genotyped. For the Caucasian patients these are almost certainly HDV 1, but the sub-Saharan patients may have "African" HDV genotypes. I think that this point of the potential role of HDV genotypes should be discussed.

We agree with the importance of analyzing the HDV-genotype composition to determine its role on the different replication behaviour of HBV in patients with chronic hepatitis delta. In this way, the HDV genotype composition was uniform in the population analyzed in this work. Thus, HDV European genotype 1 was detected in the 60 Caucasian, and in 6/8 (75%) of the immigrant Subsaharian patients. In the remaining 2 patients, HDV genotype 3 and 4 were respectively detected, one of them infected with HBV-A and the other with HBV-D. Due to the very low number of HDV genotypes other than HDV-1, no statistical analysis of the role of different HDV genotypes could be performed in this work, and the conclusions should be applied to the superinfection with HDV-1. The HDV genotype composition is now included in the final manuscript version (Table 1 legend; lines 4-6), as well as the potential importance of further studies focused in the HDV genotype to understand the HBV/HDV interference. This topic is commented in the Discussion section (page 14, paragraph 1, lines 9-11).

2) At the beginning of the Discussion the Authors make much of the fact that HDV-RNA titers are higher than HBV-DNA titers. I do not think that it is pertinent to compare the titers of two very different viruses. What is important is that HBV-DNA titers drop when there is HDV co-infection and the size of the drop seems to depend on the HBV genotype. 3) The Discussion is too long in any case and should be reduced.

Comparison of HBV and HDV titers have been previously analyzed (references 8-9 y 11). We have analyzed this aspect to compare the virological status of our patients with respect to those previously described in the literature. However, and in agreement with the reviewer comment, we have shortened the discussion of this issue in the Discussion section.

In Figures 2A and 3A some of the box-plots are missing, being represented only by the thick bar that presumably represents the median value but this is not stated in the legends to the Figures. In Figure 2A, what is the meaning of the asterisk (*)?

Asterisk and open circles indicates extreme values in the distribution of each group. All the figures have been reviewed.

There are several spelling and grammatical errors. This is especially the case for "titer" that at several places including in the Figures is spelled "titter". The paper should be reviewed by a competent English speaker.

The manuscript has been reviewed by an English native speaker. Their participation is pointed in the Acknowledgment section.