

May 6th, 2020

Dear Profs. Ghosh and Tarnawski,

Thanks for your decision regarding our manuscript entitled “A novel noninvasive model using serum ceruloplasmin to predict liver fibrosis in hepatitis B virus-infected patients with persistently normal serum ALT” (Manuscript Number ID: 55430). We also thank the reviewers for the recognition of the scientific merits in our study and their valuable comments. We have revised the manuscript according to the reviewers’ suggestions. Revised portion are marked in red color in the revised manuscript. Also, point-to-point responses to the reviewers’ comments are listed below:

Reviewer #1:

1. Were the patients from the two mentioned previous studies included in both the training and validation groups?

Response: Thanks for your comment. Yes, in the manuscript, we reported that 15.1% of the current study population and 31.1% of the current study population were previously examined in Zeng DW et al., PLoS One 2013 and Zeng DW et al., World J Gastroenterol 2016^[1, 2], respectively. Patients from the above mentioned studies were randomly assigned to the training and validation groups.

2. Please specify what does the Abbott Architect assay detect, HBsAg or HBeAg

Response: The ARCHITECT quantitative HBsAg assay is a chemiluminescent microparticle assay, internally calibrated using WHO standard, for HBsAg. Samples were diluted 1:100 in horse serum and if the concentration was more than 250 IU/ml,

samples were retested at a dilution of 1:500. At present, quantitative HBsAg is tested by use of the Elecsys® HBsAg II quant assay or the Abbott ARCHITECT® assay. Results of Architect are comparable with Elecsys II ($r=0.96$)^[3]. Therefore, they can be substituted for each other.

On the other hand, serum HBeAg levels were measured using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories) according to the manufacturer's instructions (most widely used test worldwide). The AxSYM assay measures the ratio of the sample (S) to the cut-off (Co) (S/Co ratio) and an S/Co ratio ≥ 1.0 is defined as HBeAg-positive.

3. Was the HBV DNA test performed by PG Co, including DNA isolation and PCR? Why is the unit for DNA content Log IU/ml and not original copy number?

Response: Thanks for your comment. HBV DNA was indeed tested by PG Co, including DNA isolation and PCR. DNA value conforms to the normal distribution after logarithmic transformation.

4. Was not HAI (Knodell score) considered for determining fibrosis, if biopsies were available?

Response: Thank you for your comment. According to practice guidelines of liver biopsy^[4], complex scoring systems, such as the Knodell scoring system and the Ishak scoring system, are not highly reproducible and are only appropriate for statistical analysis of large cohorts of patients in clinical trials. In clinical practice, use of a simple scoring system is recommended, such as METAVIR scoring system. In addition, previous studies reported its efficacy in the presence of necro-inflammatory changes; therefore, it will be optimal for detecting fibrosis change in hepatitis^[5-8]. Hence, in our study, we used the METAVIR scoring system.

5. The purpose why CPHBV analysis in HBeAg+ and HBeAg- groups was

performed is not clear. Please explain it, why it is needed, since HBsAg was determined and I assume that all of the 275 patients were HBsAg positive. There is indication in the Discussion, but still a few more sentence would be good to be included, I think. Is perhaps HBeAg less sensitive than HBsAg?

Response: Thanks for pointing this out. We agree, there is no added benefit from using both values. Our results demonstrated that there was no significant difference between the CPHBV AUCs of the HBeAg-positive and HBeAg-negative patients in predicting significant fibrosis, advanced fibrosis, and cirrhosis, indicating the model is not affected by the HBeAg status. Therefore, we deleted information regarding HBeAg from the revised version.

6. Please check the numbers in Table 1-under gender, the number for female is not 71, and under HBsAg, check the numbers, because what shown is not the average.

Response: Thanks for pointing this out. We apologize for this calculation error. We revised the number of females to 81, (Table 1 of the revised manuscript).

7. In Table 2, cut-off values of CP seem to be very close between F2-F3-F4

Response: We agree. Our results demonstrated the valuable role of CP as a strong indicator for the assessment of liver fibrosis in HBV-infected patients with PNALT. Till now, there is no single serum parameter that can accurately or reliably predict liver fibrosis^[9], including serum CP. Therefore, in the current study, we constructed a novel noninvasive model containing CP to predict different stages of fibrosis. Therefore, although the cut off values seem close, the improved AUC, sensitivity and specificity of the prediction model will overcome this point. Future studies should follow up participants to validate our results.

8. Regarding CPHBV, for weighing the parameters, how did the calculation come from on top of page 10?

Response: Thanks for your comment. We explained how to weigh the parameters of the non-invasive CPHBV model in page 9-10, lines 259-268 in the revised manuscript. Briefly, we first analyzed the correlation between various biochemical parameters and significant fibrosis (Table 3). Univariate analysis revealed that the CP, albumin, gamma glutamyl transpeptidase (GGT), total cholesterol (TCHO), cholinesterase (CHE), HBsAg, and PLT levels were significantly different between individuals with non-significant and significant fibrosis ($P<0.05$). Then, these variables were subjected to a multivariate stepwise logistic regression (**Supplementary Materials, Table 1**). After analysis, CP, PLT and HBsAg were identified as independent predictors and we constructed the new diagnostic indexed named CPHBV model for the prediction of significant fibrosis in HBV-infected patients with PNALT as follows: $37.122 - 10.072 \times \text{Log CP (mg/L)} - 4.291 \times \text{Log PLT (10}^9\text{/L)} - 0.958 \times \text{Log HBsAg (IU/mL)}$. Statistical analysis was applied by use of SPSS v23.0

9. Regarding Table 3, the SD values are relative high for CP, HBsAg and PLT. Could this be a reason behind the false positivity in F3-F4 cases?

Response: Thank you for your careful review. We agree the relatively high SD value of CP, HBsAg and PLT could be a potential reason for the false positive in F3-F4 cases.

10. Regarding Table 4, it would be good to provide the original table for each column indicating the number of positive, false positive, total positive, false negative, negative, total negative, total F2/F3/F4 positive and total F2/F3/F4 negative cases.

Response: Thanks for your suggestion. We added the original table to the Supplementary Materials, Table 2 in the revised manuscript.

11. Correlation between CP and fibrosis is not shown, only p value is provided.

Response: Thanks for your comments. Our study showed that serum CP levels are negatively correlated with with hepatic fibrosis ($r=-0.6$) (page 9, line 253 of the revised manuscript).

12. The false positivity and negativity cases phenomenon is not discussed.

Response: Thanks for your comment. We added these points to the discussion section of the revised manuscript [Page12, lines 332-340].

Simple cut-off values of the CPHBV were chosen for clinical practice (**Supplementary Materials, Table 2**). In the training group, patients with CPHBV of 0.0304 or less, [46 out of 60 (76.7%)] would not have significant fibrosis. Among the 64 patients who did not significant fibrosis, 18 patients (28.1%) will be presented with a CPHBV value higher than 0.0304. Applying the score 0.496 or less, 74 (94.9%) out of the 78 patients with CPHBV showed non advanced fibrosis in the liver biopsy, and 20 patients out of 94 (21.3%) without advanced fibrosis with a score higher 0.496 would be classified incorrectly. For patients with a score below 0.553, 80 out of 81 patients (98.8%) will not develop cirrhosis. Applying the higher cut off 0.553, 32 of 112 (28.6%) without cirrhosis would be classified incorrectly.

In the validation group, 49 (70.0%) out of the 70 patients without significant fibrosis (detected by liver biopsy) were identified correctly by applying below the score 0.174. Only 12 (19.7%) out of 61 patients without significant fibrosis will have a CPHBV higher than 0.174 and those patients were misclassified as they had fibrosis stage 2 detected by liver biopsy. Applying a score of 0.176 or less, 66 (93.0%) out of the 71 patients with CPHBV showed not advanced fibrosis in the liver biopsy. In addition, 25 (27.5%) out of the 91 patients without advanced fibrosis with a score higher 0.176 would be classified incorrectly. For patients with a score below 0.206, 72 out of 73 (98.6%) would not have cirrhosis. Applying the higher cut off 0.206, 30 out of 102

(29.4%) without cirrhosis would be classified incorrectly.

13. Check reference number 6

Response: Thanks for your comment. We revised reference number 6 as follows:

6 **Kelleni MT**, Ibrahim SA, Abdelrahman AM. Effect of captopril and telmisartan on methotrexate-induced hepatotoxicity in rats: impact of oxidative stress, inflammation and apoptosis[J]. *Toxicology Methods*, 2016; 26(5):371-377[PMID: 27269004 DOI: 10.1080/15376516.2016.1191576]

Reviewer #2:

1. MATERIALS AND METHODS Study population =All individuals were randomly stratified into a training and a validation group....what do you mean???

Response: Thanks for your comment. For the purpose of internal verification, all individuals were randomly stratified into a training and a validation group using SPSS v23.0. The specific details of random process is as follows:

1) Set seed

Transform→Random Number Generators

✓ Set Starting Point

⊙ Fixed Value

Value: 12345

2) Generate random numbers:

Transform→Compute Variable

Target Variable: random

Function Group: Random Number

Functions and Special Variables: Rv.Uniform

Numeric Expression: RV.UNIFORM (1,100)

3) Rank random numbers

Transform→Rank case

Random→Variable(s)

At this point, the data window generates another column of R Random

4) Random number rank order: Sort in ascending order according to the random number rank, stipulate that ranks 1 to 138 fall into the first group, and 139 to 275 fall into the second group.

Transform→Recode into Different Variables

R Random→Numeric Variable ->Output

Output Variable, Name: group→Change

Old and New Values→Recode into Different Variables: Old and New Values

5) Randomly arrange processing factors: randomly determine group 1 as the training group and group 2 as the validation group.

2. REFERENCES Very reluctantly written, almost all of them need to be re-written and reformatted as in the Instructions to Authors e.g.: = Reference 2 needs correction (missing the Journal name) = Reference 4 needs correction (missing the Journal name) = Reference 12 needs correction (missing the Journal name) and why is there % mark??? = Reference 18 needs correction (missing the Journal name) =Reference 24 needs correction (Book or Journal???) =Reference 33 needs correction (missing the Journal name)

Response: Thanks for your comment. We revised our references according to the journal guidelines (references number 2, 4, 12, 18, 24 and 33 were modified) (page 16-19, lines 470-473, 479-483, 513-515, 539-543, 563-567, 603-606 of the revised manuscript)

Reviewer #3:

1. In Methods, in Quantification of liver fibrosis, which classification did the

authors adopted to evaluate inflammatory activity of liver tissues? Metavir scoring system classify activity grade A0, A1, A2 and A3. Metavir score does not assume A4.

Response: Thanks for your comment. Scheuer scoring system was adopted to evaluate inflammatory activity of liver tissues. Our manuscript is mainly concerned with the evaluation of liver fibrosis, and therefore, the description of inflammation grade can be deleted.

2. In Methods, the authors evaluated statistical significance of data using parametric or non-parametric tests depending the data distribution. How did the authors test whether the data distributed normally or not?

Response: Thanks for your careful review. Non-parametric data are attributed to non-normal distribution. We performed Mann–Whitney test to investigate the differences in non-normal distribution variables. Further, we adopted Kolmogorov-Smirnov to test whether the parameters conform to the normal distribution. If the parameters conform to the normal distribution and homogeneity of variance, we used the Student's t-test.

3. In Methods, the authors compared AUCs using Z-test. As you know, Z- test should be applied on data with normal distribution. The authors should present evidences that AUCs in the current study distributed normally.

Response: We adopted Kolmogorov-Smirnov to test whether CPHBV, APRI, FIB-4, GPR, Forn's index, and S-index to confirm the normal distribution, $Z=0.048, 0.036, 0.039, 0.044, 0.047, 0.034$, respectively. Moreover, a $P>0.5$, showed that the AUCs in our study was distributed normally.

4. In Results, the authors established an equation; $37.122-10.072 * \text{Log CP (mg/L)} - 4.291 * \text{Log PLT (10}^9\text{/L)} - 0.958 * \text{Log HBsAg (IU/mL)}$. The authors should

describe how to generate the equation.

Response: Thanks for your comment. We explained the detailed development of the non-invasive CP-HBV model in page 9-10, lines 259-268 in the revised manuscript. Briefly, we first analyzed the correlation between various biochemical parameters and significant fibrosis (Table 3). Univariate analysis revealed that the CP, albumin, gamma glutamyl transpeptidase (GGT), total cholesterol (TCHO), cholinesterase (CHE), HBsAg, and PLT levels were significantly different between individuals with non-significant and significant fibrosis ($P<0.05$). Then, these variables were subjected to a multivariate stepwise logistic regression (**Supplementary Materials, Table 1**). After analysis, CP, PLT and HBsAg were identified as independent predictors and we constructed the new diagnostic indexed named CPHBV model for the prediction of significant fibrosis in HBV-infected patients with PNALT as follows: $37.122 - 10.072 \times \text{Log CP (mg/L)} - 4.291 \times \text{Log PLT (10}^9\text{/L)} - 0.958 \times \text{Log HBsAg (IU/mL)}$. Statistical analysis was applied by use of SPSS v23.0

5. In Results, the authors should present the median value of CPHBV with interquartile range.

Response: Thanks for your comment. The interquartile range is adopted to describe skewness distribution. However, CPHBV conforms to normal distribution, Therefore, our manuscript does not present the median value of CPHBV with interquartile range.

6. In Results, the authors compare the diagnostic ability of CPHBV with ceruloplasmin alone, platelet count alone and HBsAg alone.

Response: Thank you for your suggestion. Indeed, we compared of CPHBV with ceruloplasmin, platelet count and HBsAg for the evaluation of significant fibrosis. The CPHBV model had a significantly higher AUC value for significant fibrosis in HBV-infected individuals with PNALT compared with the ceruloplasmin, platelet count and

HBsAg. Table and Fig are as follows:

Table. Comparisons of CPHBV with ceruloplasmin, platelet count and HBsAg for assessing F \geq 2 (n=275)

	AUC (95% CI)	Youden index	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	P- value
CPHBV	0.835 (0.788-0.882)	0.501	77.6	72.5	70.3	79.4	
CP	0.770 (0.713-0.827)	0.446	76.3	67.3	66.2	77.7	0.034
PLT	0.707 (0.581-0.715)	0.321	61.6	70.5	63.6	68.6	<0.001
HBsAg	0.648 (0.644-0.770)	0.262	72.0	54.5	56.3	70.5	<0.001

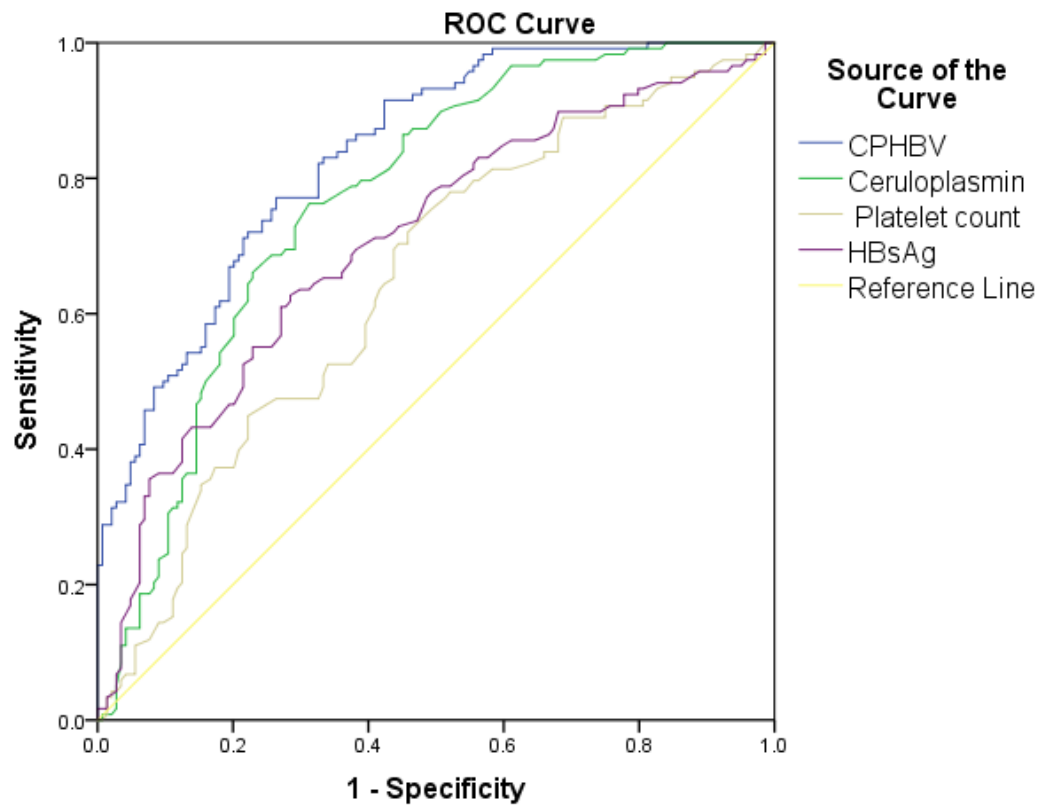


Figure. ROC curves for the CPHBV, Ceruloplasmin, Platelet count, HBsAg in all the study subjects.

7. In Discussion, the authors should add other limitation of the study. The study lacks data of liver elastography, ELF score or WFA-M2BP.

Response: Thanks for your suggestion. We added the suggested limitation in page 13-14, lines 375-383 of the revised manuscript.

Transient elastography (Fibroscan), enhanced liver fibrosis (ELF) test and Wisteria floribunda agglutinin-positive Mac-2 binding protein(WFA+-M2BP) are accurate diagnostic tests for monitoring regression of fibrosis in patients with chronic HBV infection^[10-14]. However, we did not compare the accuracy of our model against the above mentioned tests due to the unavailability of equipment at the time of data collection. Further, serum indicators such as hyaluronic acid (HA), procollagen III amino-terminal peptide (PIIINP), metalloproteinase 1 (TIMP-1), and WFA+-M2BP are not routinely tested in our hospital.

8. In Tables 4-6, the authors should revise the data of Youden Index. Youden index means a representative value of ROC curve, which maximize the value= 1 - (sensitivity + specificity). Usually, a cut-off value based on Youden index is similar to a cut-off value determined by selecting a point nearest to sensitivity =1 and specificity = 1. However, in tables 4-6, Cut-off were quite different from Youden index. I guess the authors should revise how to use Youden index in ROC curve analysis.

Response: Thanks for your comment. Youden index = sensitivity - (1 - specificity), we calculated the maximum value of Youden index corresponding to the optimal cut-off. As shown in below, in the advanced fibrosis stage of the training group, the maximum Youden index of the CPHBV model was 0.696, and the corresponding optimal cut-off was 0.496.

Figure: CPHBV predict the cut-off and Yoden index of advanced fibrosis

A	B	C	D	E
Cut-off	sensitivity	1 - specificity	Youden index	
.4960	.909	.213	0.696	
.4732	.909	.215	0.694	
.8772	.818	.129	0.689	
.4538	.909	.226	0.683	
.5977	.864	.183	0.681	
.8267	.818	.140	0.678	
.9450	.795	.118	0.677	
.0808	.977	.301	0.676	
.4064	.909	.237	0.673	
.5530	.864	.194	0.670	
.6742	.841	.172	0.669	
.7750	.818	.151	0.668	
.9110	.795	.129	0.666	
.0613	.977	.312	0.665	
.9719	.773	.108	0.665	
.1444	.955	.290	0.664	
.3711	.909	.247	0.662	
1.3263	.705	.043	0.662	
.5200	.864	.204	0.656	

Editorial Office's comments:

1. I found the title is more than 12 words. The title should be no more than 12 words

Response: Thank you for your suggestion. We revised our title as follows “Serum ceruloplasmin can predict liver fibrosis in hepatitis B virus-infected patients”.

2. I found the authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s)

Response: Along with the revised version, we will upload the approval documents provided by our funding agencies with the following file names: Science and Technology Department of Fujian Province (2018J01164) and Quanzhou Science and Technology Bureau Planning Project (2019N019S), respectively.

3. I found the authors did not provide the original figures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Response: Thanks for your comment, We will revise and upload figure 1 as a PowerPoint file.

4. I found the authors did not add all the PMID and DOI in the reference list. Please provide all the PubMed numbers and DOI citation numbers to the reference list

Response: Thanks for your comment, we will revise and update our reference list accordingly.

5. I found the authors did not write the “article highlight” section. Please write the “article highlights” section at the end of the main text.

Response: We will add the following article highlight in page 15, lines 406-418.

- 1) Serum ceruloplasmin (CP) had an inverse correlation with liver fibrosis and can be used as a promising predictive marker for liver fibrosis among hepatitis B virus (HBV)-infected individuals with persistently normal serum alanine transaminase levels (PNALT).
- 2) In this study, we developed a noninvasive model using CP, platelet, and HBsAg levels to predict various stages of fibrosis among HBV-infected individuals with PNALT.
- 3) The established noninvasive model has shown good performance in the prediction of significant fibrosis in HBV-infected individuals with PNALT and it was superior to previous models like APRI, FIB-4, GPR, Forn’s index, and the S index.

6. Re-Review: Required

Response: We carefully checked and revised the language, tables, figures and references of this manuscript. Further, the manuscript had been edited by *Medjaden*

Bioscience limited. We hope that the revised manuscript meets the requirements of *World Journal of Gastroenterology*.

We would like to thank you and the reviewers for the constructive comments. We hope that the revised manuscript is now acceptable for publication in the *World Journal of Gastroenterology*. We look forward to hearing from you.

Best Regards,

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