1 Original Research Article
 2 Validation of decision cut off values of serum albumin and prothrombin
 3 time for differentiating between compensated and decompensated liver
 4 cirrhosis.

ABSTRACT:

Aims: Serum bilirubin, albumin and prothrombin time are the most commonly measured parameters to assess the progress of hepatic cirrhosis. No widely accepted cut off values for these parameters are available for an early diagnosis of the decompensation process of hepatic cirrhosis. We aimed to establish the cut off values for these parameters with maximum sensitivity and minimum false positivity between 30 decompensated and 33 compensated cirrhosis patients through ROC curves for making a medical decision at the earliest.

Study design: It was a hospital based, cross sectional, observational study. Both of the case and control groups were selected by the method of convenience.

Place and Duration of Study: Study subjects were analyzed in the Department of Medicine (Medical Unit II), Department of Biochemistry and Department of Radiology, Mamata Medical College, Khammam, Andhra Pradesh, India between Jan 2009 and Feb 2010.

Methodology:

Sample: 63 patients (40 men, 23 women; age range 20-60 years) diagnosed as hepatic cirrhosis on the basis of clinical, biochemical and ultrasonographic features were included as the case group in the study. 35 age and sex matched normal healthy subjects were included in the control group. Patients with portal hypertension, ascitis, encephalopathy and with the medical history of gastrointestinal bleeding, were put in the decompensated group (n =30). Cases without the above criteria were selected in the compensated group (n =33).

Results:

Cirrhosis patients showed significant increase in serum bilirubin level (6.79 +/- 2.19 vs 0.86 +/- 0.02, P < 0.001) and prothormbin time (20.13 +/- 2.25 vs 15.54 +/- 0.63, P < 0.001) with opposite trend with serum albumin (3.05 +/- 0.62 vs 3.69 +/- 0.83, P < 0.001) when compared to normal control group. Furthermore, the decompensated group showed a significant rise in serum bilirubin (8.90 +/- 0.78 vs 4.80 +/- 0.79, P < 0.001) and prothrombin time (21.44 +/- 2.5 vs 18.93 +/- 0.84, P < 0.001) with a decrease of serum albumin (2.8 +/- 0.36 vs 3.2 +/- 0.76, P < 0.027) in comparision to compensated group. ROC analysis for the serum albumin level and prothrmbin time between the compensated and decompensated groups showed their cut off values to be 2.97g/dl and 19.47 seconds

respectively. No cut off value for serum bilirubin could be obtained due to non overlapping values between the two groups.

Conclusion:

The calculated cut off values of serum albumin and prothrombin time in our study indicate the transition phase between the compensatory and decompensatory groups of hepatic cirrhosis. As early detection of the decompensation process can guide the clinicians to take the proper management to reduce the complications and mortality significantly, these cut off values should provide useful diagnostic and therapeutic tools in these patients.

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Keywords: Cirrhosis, Decompensation, Medical decision values, Albumin, Prothrombin time.

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13 INTRODUCTION

In cirrhosis of liver presence of either of the jaundice, ascitis, portal hypertensive gastrointestinal bleeding or hepatic 14 15 encephalopathy together, or in isolation, is considered as a decompensated state in the disease process. These 16 manifestations appear when the disease process supersedes the compensatory mechanisms, either by progression of the 17 disease or a superimposed acute insult. Clinically and biochemically the transition from well compensated cirrhosis to 18 symptomatic decompensation is gradual and in subtle stages without any clear demarcating points. However, decompensation per se, increases the risk of mortality significantly. One year mortality in compensated cirrhosis is 1-19 20 3.4%, that in decompensated state is reported to be as high as 20-57% [1]. Moreover, decompensated cirrhosis can also 21 lead to hepatocellular carcinoma that increases the mortality rate in these patients significantly [2]. Biochemical parameters play important roles for monitoring the transition state from compensation to decompensation. Increased 22 23 serum bilirubin was observed by some authors in compensated cirrhosis with a more significant increase in its 24 decompensated state[3]. Protein energy malnutrition (PEM), in many cirrhosis patients[4] is also more pronounced, in 25 decompensated state [5]. PEM is associated with a high mortality and morbidity due to increased risk of life threatening complications resulting in poor survival and poor quality of life [6,7]. Serum albumin level have been reported to serve as 26 an important indicator of the severity of liver cirrhosis and an improvement of its level is vital for improving the prognosis of 27 28 the disease. It has been reported that the serum albumin level have decreased at a rate of 0.15 gm/dl per year in patients 29 with liver cirrhosis [8]. Some authors assume that serum albumin level above an approximate threshold of 3.5 gm/dl, might indicate transition to decompensated cirrhosis. Sato et al found that improving or maintaining serum albumin 30 31 prevented the onset of hepatic encephalopathy [9], which is the hallmark of decompensated cirrhosis. Oral supplementation with branched chain amino acid (BCAA) preparations have been reported to improve event free survival 32 33 and quality of life by raising serum albumin concentrations in patients with decompensated cirrhosis[10]. These findings 34 were strengthened by some observations that suggested a reduction in ascitis in hepatic cirrhosis after BCAA 35 supplementation [11]. On the other hand, a significantly increased prothrombin time (PT) was also found in patients with 36 chronic liver disease in comparison to controls with a significant increase in acute variceal bleeding induced mortality that ranges from 0.5% to 50% in patients with liver cirrhosis [12]. Recombinant factor vii may be useful in variceal bleeding as 37 it has been shown to normalize PT and bleeding time (BT) in cirrhosis [13]. Some authors also documented 38 hyperfibrinolytic activity and abnormal PT in cirrhosis patients [14]. Furthermore, a correlation was found between reduced 39 level of factor II,VII, IX, X in liver cirrhosis with its severity [15]. Keeping in track, the model for end stage liver disease 40 41 scoring system(MELD) has put a strong emphasis on international normalized ratio (INR) [16] for assessing the role of clotting factors in the decompensation process. Previously, Kato et al identified the characteristics of decompensated 42 cirrhosis and suggested the proper time to begin the administration of BCAA granules preparation at an albumin level 43 44 lower than 3.5g/dl and the prothrombin activity lesser than 60% [17]. Although, most of the studies reported a significantly 45 raised serum bilirubin level and prothrombin time along with a reduction in serum albumin level during the process of 46 decompensation, we could not find any study regarding the cut off value of serum bilirubin that could demarcate the decompensated hepatic cirrhosis from the compensated ones. Similarly, not many studies could report a clear cut medical 47 48 decision limits for serum albumin and PT in these patients and importantly, when any such cut off values were reported, 49 they varied significantly region wise [8,17,18]. Furthermore, some investigators evaluated definite values of different biochemical parameters obtained either as distinctive cut off values or ratios that could predict the severity of disease 50 throughout its pathological process and thus, identify the transition process between compensation and decompensation 51 states with a much more clarity [8,19]. The modified Child-Pugh is considered a good 52 classification of 53 compensate/decompensate stage of liver cirrhosis. It suggested that Identifying cirrhotic patients with high blood ammonia concentrations could be clinically useful, as high levels would lead to suspicion of being in presence of 54

55 collaterals, in clinical practice of esophageal varices, and pinpoint those patients requiring closer follow-up and 56 endoscopic screening [20]. In a similar way the presence of large shunts is clearly index of decompesation [21].

57 However, in our region also, we could find few evidences, so far, regarding the blood levels of common 58 biochemical parameters which could substantiate the disease progression from compensated to decompensated state in 59 the hepatic cirrhosis patients. So, in the present study, we assessed the serum bilirubin, albumin and PT in compensated 60 and decompensated cirrhosis patients, and made an effort to establish a cut off value of these parameters between the 61 two disease groups that could at least biochemically differentiate between the compensated and decompensated states 62 and help in initiation of proper treatment or management at the most appropriate stage reducing the mortality in cirrhosis 63 patients.

65 2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

66 2.1. Study design:

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This hospital based, cross sectional and observational study was conducted in the Department of Biochemistry, Mamata Medical College and Hospital, Khammam, Andhra Pradesh, India.

69 **2.2. Selection of study subjects:**

63 patients of liver cirrhosis (compensated-M=22, F=11.decompensated-M=18, F=12) with the age group of 20 to 60 years were selected for study from Outdoor Patient Department(OPD) and Indoor wards on convenience basis. Diagnosis of liver cirrhosis was done from detailed history, clinical and radiological examination as well as ultrasonography of liver and from relevant biochemical tests. 35 age matched, healthy subjects(not addicted to smoking,alcohol or drugs) were selected as the control group from the students, hospital staffs and persons accompanying the patients.

75 **2.2.1.Exclusion criteria**:

76 Cases: Liver cirrhosis patients associated with concomitant pathology like diabetes mellitus, hypertension, chronic 77 diarrhoea, renal failure, infective hepatitis and obstructive liver disease or taking drugs causing alteration of serum 78 albumin or prothombin time were excluded from the study. Severely ill like shock, acidosis, alkalosis, hypoglycemia, 79 unconscious, disabled and non cooperative patients were also excluded from the study.

80 *Controls*: persons with history of any type of hepatitis, alcohol addiction, habituated smoking behaviour and recent history 81 of any drug intake capable of altering liver function were not included in the control population for this study.

All study subjects were from the same geographical area with no significant difference in their food habit and drinking water quality.

84 2.2.2. Ethical consideration:

Written consents were obtained from the participants (case and control groups). The study followed the Helsinki declaration for human studies, 1975 and was undertaken after obtaining the ethical clearance from the institutional ethical committee.

88 2.2.3. Classification of subjects into study groups:

Normal control subjects were considered as Group 1(n=35). Liver cirrhosis patients with ascites, portal hypertensive gastrointestinal bleeding, and encephalopathy were considered in decompensated group or Group 3(n=30). Liver cirrhosis patients without the above mentioned features were considered in compensated group or Group 2(n=33).

92 **2.3. Measurement of study parameters:**

93 2.3.1.Sample collection:

Blood was obtained from patients and controls after an overnight fast. The blood was drawn into plastic syringes fitted with stainless steel needles, immediately transferred to metal free tubes, allowed to clot, centrifuged, and serum removed

96 to another metal free tube for storage at -20 until assayed.

97 2.3.2. Methods of measurement of analytes:

Serum bilirubin was estimated by modified Jendrassik & Grofs Method (Crest Biosystems) [22]. Albumin was estimated by
Bromocresol green (BCG) dye binding method from Crest Biosystems, India in the XL 600 autoanalyser from Erba
Transasia [23]. Prothrombin time was estimated by Quick's method using coagulometer from Erba Transasia, USA [24].
All assays were done in duplicate and average values were taken. Coefficient of variation (CV) for the control samples
(level 1 and 2 from Lypocheck, Biorad, USA) for the study parameters remained below 5% throughout the study period.

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104 **2.4. Statistical analysis:**

Statistical analysis was aimed to 1) assess the difference between the mean values of serum bilirubin, albumin and prothrombin time between the case (both group 2 and 3) and control groups by independent t test, 2) assess the difference between the mean values of serum bilirubin, albumin and prothrombin time between the two case groups

(group 2 and 3) by independent t test, and 3) obtain the cut off values for serum bilirubin, albumin and PT between the two case groups at the maximum level of sensitivity versus minimum false positivity by the receiver operator characteristic curve (ROC). All statistical analyses were done with the help of SPSS software version 17.0 for Windows. P value was considered to be significant at a value less than 0.05 for a 95% confidence limit.

3. RESULTS:

Table I: Independent t test to show the difference between the mean values of serum albumin and prothrombin time in liver cirrhosis patients (for both groups I and II, N = 63) and control subjects (N = 35)*:

	Liver cirrhosis patients in both groups (N = 63)	Control subjects (N = 35)	t value	Significance (<i>F</i> value)**
Serum Albuminin in g/dL (Mean +/-SD)	3.05 +/- (0.62)	3.69 +/- (0.83)	-4.2	<i>P</i> < 0.001
Protrombin time in secs. (Mean +/-SD)	20.13 +/- (2.25)	15.54 +/- (0.63)	11.7	<i>P</i> < 0.001
Serum bilirubin level in mg/dl	6.75 +/- (2.19)	0.86 +/- (0.02)	15.8	<i>P</i> < 0.001

It is evident from the Table I that serum albumin level is significantly decreased and the serum bilirubin and prothrombin time is significantly raised in liver cirrhosis patients in comparision to the control subjects.

Table II : Independent t test to show the difference between the mean values of serum albumin and prothrombin time in compensated (N = 33) and decompensated (N = 30) liver cirrhosis patients*:

	Compensated liver cirrhosis(n=33)	Decompensated liver cirrhosis(n=30)	t value	Significance (<i>P</i> value)**
Serum Albumin in g/dL (Mean +/-SD)	3.2 +/- (0.76)	2.8 +/- (0.36)	2.26	<i>P</i> = 0.027
Prothrombin time in secs. (Mean +/-SD)	18.93 +/- (0.84)	21.44 +/- (2.5)	-5.29	<i>P</i> < 0.001
Serum bilirubin in mg/dl	4.80 +/- (0.79)	8.90 +/- (0.68)	-21.7	<i>P</i> < 0.001

* Statistical analysis was performed by SPSS version 17.0 for Windows

**P value significant at the level of p < 0.05 at 95% confidence interval

Results in the Table II reveals a significantly reduced level of serum albumin in the decompensated liver cirrhosis patients. On the other hand, serum bilirubin and prothrombin time is found to be significantly increased in the decompensated group.

To find out the critical value of both parameters beyond which these parameters fall into decompensated group we carried out ROC analysis for these parameters against a specific sensitivity and false positivity. According to the established rules
 we obtained these cut off points from the left uppermost corners of the ROC curves (encircled points in Fig. 1 & 2) that
 suggested the cut off values with highest sensitivity with lowest false positivity (1-specificity).

Figure 1: ROC for showing the cut off value of serum albumin between compensated and uncompensated groups of liver cirrhosis.



Diagonal segments are produced by ties.

151 ROC curve in figure 1 shows the critical value of serum albumin between decompensated and compensated group of liver 152 cirrhosis to be 2.97 g/dl, with a sensitivity of 0.67 and false positivity of 0.40 with an area under curve of 0.690. It indicates 153 that decompensation process in hepatic cirrhosis is closely associated with a serum albumin level below 2.97g/dl.

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Figure 2: ROC for showing the cut off value of serum prothrombin value and serum bilirubin level between compensated and decompensated groups of liver cirrhosis.

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Diagonal segments are produced by ties.

187 188 On the other hand ROC curve in figure 2 shows the cut off value for PT to be 19.47 seconds with a sensitivity 0.8 and 189 false positivity of 0.24 with an area under curve of 0.82, that indicates the close link between decompensation process to 190 a prothrombin value greater than 19.47 seconds. On the other hand, no overlapping value and hence cut off values are 191 observed with an area under curve for serum bilirubin being 1.

4. DISCUSSION

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Decompensation per se, is a significant risk of mortality and malignancy in cirrhosis of liver [1]. Biochemical parameters are more practicable for differentiation between the decompensated and compensated phases as histopathological examinations are more invasive and complications have been reported to arise following biopsy. In our present study we made an effort to utilise three common and easily measurable parameters like serum bilirubin, albumin and prothrombin time as such biochemical parameters for an early detection of the decompensation state and hence a more favourable outcome due to an earlier intervention.

In our study, Table I have shown that the mean serum bilirubin level is increased, albumin level is reduced, and mean prothrombin time is prolonged significantly in liver cirrhosis patients than controls. All of these parameters showed similar deviations in the decompensated group as compared to the compensated hepatic cirrhosis (Table II).

Koruk M et al found the mean serum bilirubin level is 2.19 mg/dl in decompensated liver cirrhosis and 1.14 mg/dl in compensated one [3]. Those observations were strengthened by Villeneure JP et al [25] who observed the serum bilirubin level was 3.9 mg/dl in decompensated liver cirrhosis. The findings of those studies keep in tract with our observations where we found serum bilirubin level is 8.9 mg/dl in decompensated and 4.8 mg/dl in compensated liver cirrhosis.

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212 Our major aim in this study was to find out a cut off value for these three common parameters that could demarcate the 213 decompensation process at the earliest. For this we undertook the ROC analysis for these parameters in both 214 compensated and decompensated groups and determined the cut off value on the basis of maximum sensitivity and 215 minimum false positivity among the overlaps. Serum bilirubin level could not yield such a cut off point from the ROC 216 (broken lines in figure 2) as there was no overlapping value between the two groups. This observation suggests that the 217 increase in bilirubin during the process of decompensation occurs abruptly and there was no smooth and continuous 218 deterioration in its detoxification process during the decompensation process. Hence, it can be suggested that the failure 219 in detoxification process by the hepatocytes occurs much rapidly as the cirrhosis patients transit to decompensated state 220 from a compensated condition. Studies have reported strong evidences of a deterioration in alternative pathways of 221 detoxification along with the classical pathways of glucuronidation of bilirubin in cirrhotic livers that may explain the sharp 222 rise in bilirubin level during decompensation [26]. Hence, in our study we were not able to get any critical value of serum 223 billirubin between compensated and decompensated groups since there was no overlapping of values between the two 224 disease groups.

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226 However, hepatic functions related to synthesis of albumin and prothrombin did not follow this pattern as they showed a 227 relatively smoother and gradual deterioration during the process of decompensation yielding several overlapping values in 228 both group I and II patients. In the present study, ROC curves in figure 1 and figure 2 showed cut off values of serum 229 albumin and PT that can differentiate between the compensated and decompensated liver cirrhosis with the maximum 230 possible sensitivity and minimum possible false positivity. These cut off values can be considered as the critical values for these studied parameters for making appropriate medical decisions. In our study such critical values of serum albumin 231 and PT between the compensated and decompensated group of liver cirrhosis were found to be 2.97 g/dl and 19.47 232 seconds respectively (Fig. 1 & 2). Based on these results we suggest that in liver cirrhosis a reduction of serum albumin 233 234 level below 2.97 g/dl, indicates the initiation of the process of decompensation. Similarly, with a PT value more than 19.47 235 seconds features of decompensation and bleeding disorders related to complicated cirrhosis start appearing. The findings 236 of our present study can be explained through the observations found by Kiammt S et al who described a decrease in albumin binding capacity in patients with decompensated liver cirrhosis which correlated inversely with severity of disease 237 238 [27]. Some authors utilized serum albumin and PT or INR (the ratio of PT of cases to PT of controls) for prediction of the 239 severity of liver cirrhosis [28]. In agreement with these studies we observed that the serum albumin was not only 240 significantly decreased in decompensated liver cirrhosis patients in comparision to controls and compensated liver 241 cirrhosis patients, but more importantly, to a lesser level than observed in other studies (Table | &II) most probably due to 242 variations in the baseline nutritional status among different study population. These observations prompted us to search 243 for a new cut off value for this parameter in our region considering the fact that albumin has got major biochemical role as 244 a marker for several crucial hepatic functions. The results of our study indicated that appropriate time at which the BCAA 245 as well as the zinc preparations can be started might be 2.97 g/dL which is slightly lower than those found in some other studies in different regions of world. We suggest that different contributory factors like routine dietary habits. 246 247 environmental status and genetic make up may play important role in this slightly lower critical value of serum albumin in 248 our region.

Serum albumin level is not only an indicator of the synthetic function of the liver, maintenance of its optimum level is also 250 251 necessary for regulating normal trace element homeostasis in our body. Zinc is transported in plasma mostly by 252 albumin(60-70%) [29]. Hence, a lowered serum albumin level results in a reduced zinc level in body that might be 253 responsible for an increased risk of hepatic encephalopathy and hepatocellular carcinoma observed in decompensated 254 liver cirrhosis patients [29,30,31,32]. Low zinc level in liver cirrhosis patients might be the result of decreased liver 255 albumin content, poor dietary intake or protein restriction [29]. Accordingly, some reports indicated that zinc 256 supplementation may have a beneficial role in decompensated liver cirrhosis patients with hepatic encephalopathy, but further trials are necessary to have conclusive evidence [32,33]. Sato et al suggested that maintaining serum albumin 257 258 prevented the onset of hepatic encephalopathy [9].

The importance of evaluating the critical value of albumin is evident from the delay in the process of decompensation after administration of effective therapy at this juncture. Oral supplementation with BCAA preparation was reported to improve the quality of life in decompensated cirrhosis[10-11]. Hence, several authors tried to establish the proper time to begin the administration of BCAA supplementation [8,12].. So the appropriate time for BCAA administration becomes vital for therapeutic purpose in decompensated liver cirrhosis along with prevention of related complications.

266 267 Owing to their rapid degradation, the measurement of coagulation factors is one of the best indicators of liver synthetic 268 function, and is beneficial for assessing severity and following progress of liver disease [18]. Several authors have 269 described strong relationship between cirrhosis of liver and the compromised status of clotting factors along with a strong 270 correlation between decreased levels of factor II,V,VII,IX, and X and the severity of liver diseases [15]. The modified Child 271 Pugh stated PT > 11 seconds or albumin < 2.3 g/dl for identification of severely decompensated liver cirrhosis [30]. 272 Furthermore, some authors strengthened the prognostic importance of PT in cirrhosis of liver by pointing out the optimal time to initiate treatment with BCAA at a prothrombin activity of ≤60% [12].

275 Keeping all these factors in mind we tried to establish a cut off value of PT in our study population following determination 276 of the same for serum albumin. Our results indicated it to be 19.47 secs. As described earlier the cut off value of serum 277 bilirubin between compensated and decompensated group cannot be determined since the bilirubin values are not 278 overlapping each other in two disease groups. Hence we suggest that along with an albumin level lower than 2.97 g/dl, a 279 PT more than 19.47 seconds strongly indicate the progression of hepatic cirrhosis into its decompensated state in majority of the cases. Our study results are somehow different from the observations recorded by the modified Child Pugh [34] that 280 stated a PT level more than 11seconds or albumin level of less than 2.3 g/dl for progression into decompensation. 281 However, the modified Child Pugh study was carried in severely decompensated liver cirrhosis patients. Along with other 282 contributory factors the degree of decompensation might have affected the critical values of these parameters. 283 284

285 Nevertheless, the above mentioned parameters have some pitfalls for assessing the severity of the disease if considered 286 in isolation. Serum albumin being a negative acute phase reactant has been reported to be falsely decreased by IL-6 287 under inflammatory conditions. Furthermore, although in cirrhosis of liver the synthesis of albumin has been found to be 288 decreased generally, normal values or an increase has been found in some cases also. Loss of albumin in ascitic fluids 289 has been also attributed to its lowered serum value. Similarly, the proteins induced by vitamin K antagonists (PIVKA) like warfarin can elevate the PT significantly [35]. To avoid these interferences and for obtaining a more realistic state of 290 decompensation in hepatic cirrhosis, we propose to consider that the cut off values of serum albumin and PT taken 291 292 together will provide substantial help in determining the progression to decompensation at an early stage and will help the 293 clinicians to take the appropriate steps for reducing the grave consequences of liver cirrhosis. Serum bilirubin, albumin 294 and PT are the three simple and commonly performed parameters estimated in laboratories for liver cirrhosis patients. Finally, measurement of these simple parameters(bilirubin couldn't show cut off value) are easy to perform, cost effective 295 and can be carried out in almost all laboratories. 296

Limitations of the study and implications of future research: The study was done in Mamata General Hospital, Khammam which caters rural population mainly with low socio economical status. A larger sample size including the urban population as well can reflect our goals more accurately. There is a great need of broader study on liver cirrhosis and its complications which can ultimately help in achieving the goal of healthy and prosperous society.

305 4. CONCLUSION

Selecting the parameters like albumin and prothrombin time for assessing the cut off values between the compensated and decompensated groups of liver cirrhosis is a more realistic approach. Furthermore, the present study strengthens the importance of these two markers taken together for assessing the initiation of decompensation process at a greater accuracy. These cut off values of albumin and prothrombin time in liver cirrhosis patients are inherent to the local study population and thus, can help the clinicians to take the appropriate step (like to start BCAA and zinc preparations) to counteract the serious consequences of the disease. These observations, in our opinion, have significant relevance to healthcare of India; as liver cirrhosis is a common occurrence in our country.

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322 COMPETING INTERESTS

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327 AUTHORS' CONTRIBUTIONS

Dr. Kaushik Kar and Dr. Anindya Dasgupta designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Dr. Suparna Ray, Dr. Satwika Sinha and Dr. Ushasi Banerjee managed the analyses of the study. and managed the literature search. All authors read and approved the final manuscript."

333 CONSENT (WHERE EVER APPLICABLE)

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All authors declare that 'written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editorial office/Chief Editor/Editorial Board members of this journal."

341 ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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APPENDIX:NIL