The Relationship between Apoprotein A and the Osmotic Fragility of red blood cells in HCV Cirrhotic patients

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Abstract: **Aim:** To evaluate the relationship between Apolipoprotein A and osmotic fragility of red blood cells in HCV cirrhotic patients. **Methods:** We evaluated clinical, laboratory parameters, Apoprotein-A level, and osmotic fragility of red blood cells in patients with HCV cirrhosis, during the period between September 2015 and July 2016. Patients who had at admission renal failure, other causes of hemolytic anemia, hepatocellular carcinoma, sepsis, and/or active bleeding, other types of liver diseases not related to HCV e.g. HBV, autoimmune hepatitis, alcoholic, biliary, and other diseases; such as diabetes, hypertension were excluded. Fifty patients with HCV cirrhosis classified according to Child-Pugh classification, and ten controls were included. **Results:** We found a strong positive significant correlation (P value 0.000) between the Child-Pugh score and onset of hemolysis, hemolysis began in less hypotonic solution (earlier) in the RBCs of liver cirrhotic patients which shows the highly fragile nature of RBCs, a strong inverse significant correlation (P value 0.000) between the Child-Pugh score and the level of Apo-A, and a significant strong inverse correlation (P value 0.000) between the Apolipoprotein-A level and the start and the end of osmotic fragility of RBCs in HCV cirrhotic patients. **Conclusion**: The presence of increased osmotic fragility is due to lipid and Apolipoprotein A disturbances that may develop in patients with HCV cirrhosis.
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Key words: Hepatitis C; Cirrhosis; Apo A; Osmotic fragility.

Abbreviations: HCV: hepatitis C virus; HBs-Ag: hepatitis B surface antigen; ANA: anti-nuclear antibody; SCA: spur cell anemia; Apo A: Apolipoprotein A.

1. Introduction:

Cirrhosis is a general term for end-stage liver disease. It represents the final common histologic pathway for a wide variety of chronic liver diseases. Cirrhosis is defined as the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury (1). Pathological alterations in the structure or functions of RBC (Red Blood Cell) membranes are involved in the etiology of liver diseases including cirrhosis. Patients with severe liver disease may have spur cell anemia with Red Blood Cells (RBCs) that have characteristic morphological abnormalities, hemolytic anemia and altered membrane lipid composition. RBC is a complex structure composed of a lipid bilayer supported by a scaffolding of cytoskeletal proteins. Lipid composition determines the structure, function and integrity of RBC membranes. In patients with liver disease, abnormalities in the composition of the plasma lipoproteins are associated with corresponding changes in the erythrocyte membrane lipid composition and accompanying changes of their morphology. (2) These changes in membrane lipid composition can affect the structure, morphology and integrity of erythrocytes. Changes in hepatocytes are reflected by RBC changes in liver diseases. Hence the structure and functions of RBC membranes could be used to understand pathogenesis and prognosis of liver diseases. (3) The membrane function of different cells is not only dependent on the lipid composition but also on the architecture (packing and phases) of the lipid membranes.

Modifications of the lipid composition and the asymmetry of the bilayer have been shown to affect the overall shape of the erythrocyte, architecture and also the cells deformability. (4). Changes in the shape, mechanical characteristics or the integrity of the erythrocyte have severe implications on the functionality and viability of the cell, as can be seen in several dysfunctional states of the erythrocyte in diseased states. (5).

And as human Apo A-I is the most abundant protein component of HDL particles and a multifunctional apolipoprotein that plays a variety of

roles in human physiology among which are cholesterol transport and regulation of inflammation (6), we conducted our study to evaluate the relationship between Apo A and osmotic fragility of RBCs in cirrhotic patients.

2. Patients and methods:

Our study includes 60 subjects classified into 2 groups: Group 1: 50 patients with liver cirrhosis due to chronic HCV infection. Classified into 3 sub-groups according to Child-Pugh score (A, B, C), with age from 20 – 80 years. Group 2: 10 healthy subjects served as control group. Patients of other types of liver diseases not related to HCV e.g. HBV, autoimmune hepatitis, alcoholic, biliary, and other diseases; such as diabetes, hypertension and renal diseases, patients with evidence of blood loss, and patients with other causes of hemolytic anemia were excluded.

Admission etiologies of patients who were enrolled in the study were Patients with liver cirrhosis due to chronic hepatitis C, chosen on the basis of HCV-Abs by (ELISA), and positive HCV-RNA by (PCR) presented by anemia, without evidence of blood loss and other causes of hemolytic anemia. All patients were subjected to the following: Full history taking, General clinical assessment, Liver function tests (ALT, AST, ALP, GGT, total and direct serum bilirubin, prothrombin time and INR, albumin, total protein and Alfa-feto protein (AFP) levels), Lipid profile: fasting cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL), and triglycerides, Fasting and postprandial blood glucose level, Kidney functions tests (serum creatinine, blood urea nitrogen (BUN)), Complete blood count (CBC) and blood film, HBsAg, ANA, HCV Antibody for both groups and HCV PCR quantitative for patients only, Abdominal ultrasound, Osmotic fragility test, and Apolipoprotein A level by ELISA.

Statistical analysis:

All data were collected, tabulated and statistically analyzed using IBM SPSS (statistical program for social science version 20). Quantitative data were expressed as the mean ± SD & median (range), and qualitative data were expressed as absolute frequencies "number" & relative frequencies (percentage). Independent Student t-test was used to compare two groups of normally distributed data. One way ANOVA test was used to compare more than two groups of normally distributed data. Post hoc test was done by LSD for multiple comparisons. Pearson's correlation coefficient was calculated to assess relationship between Apolipoprotein A, osmotic fragility and selected study parameters. The probability of chance (P value) is used to determine the significance of data: P value ≥ 0.05 is statistically insignificant and P value < 0.05 is statistically significant. The Correlation coefficient is measured as follows: Positive correlation is considered when the correlation coefficient ranged from 0 to 1, Negative correlation is considered when the correlation coefficient ranged from -1 to 0. Strong correlation is considered when the correlation coefficient ranged from 0.66 to 1, moderate correlation is considered when the correlation coefficient ranged from 0.33 to 0.66 and weak correlation is considered when the correlation coefficient ranged from 0 to 0.33.

Ethical approval and consent:

The study protocol was approved by the Ethics Committee of the faculty of medicine Al-Azhar University. All participants signed informed consent for using their data in the study.

3. Results:

This study was performed in the department of internal medicine, Al-Hussein hospital, Al-Azhar University, Cairo, Egypt during the period between September 2015 and July 2016. In our study 50 chronic hepatitis C patients and 10 controls were included. Out of 50 patients, 32 (64%) were male and 18 (36%) were female. The mean age was 53.72 (±9.407) in both male and female. According to Child-Pugh criteria, out of the 50 patients, 19 (38%) patients belonged to Child-Pugh class A, 15 (30%) patients belonged to Child-Pugh class B and 16 (32%) patients belonged to Child-Pugh class C **(Table 1).**

The comparison between Child classes of chronic hepatitis C patients and the controls as regard lipid profile, apolipoprotein A level, and osmotic fragility of RBCs by using One way ANOVA test to compare the results between the mean of different groups revealed that there were **statistical significant** differences between the four groups in mean value of total cholesterol, triglycerides, high density lipoproteins, low density lipoproteins, very low density lipoproteins, cholesterol to high density lipoproteins ratio, apolipoprotein A level, start of osmotic fragility and the end of osmotic fragility **(Table 2) (Figs. 1, 2 & 3).**

The multiple comparisons for lipid profile, apolipoprotein A level, and osmotic fragility stratified by Child classes of chronic hepatitis C patients and controls by using Post Hoc tests for multiple comparisons using LSD test we found that **(Table 3)** there were **statistical significant** differences between the controls VS Child A in HDL level and Apolipoprotein A level. While there were **no significant** differences as regard total cholesterol, triglycerides, low density lipoproteins, very low density lipoproteins, cholesterol to high density

lipoproteins ratio, start of osmotic fragility and the end of osmotic fragility. There were **statistical significant** differences between the controls VS Child B in total cholesterol, triglycerides, high density lipoproteins, low density lipoproteins, very low density lipoproteins, apolipoprotein A level, start of osmotic fragility and the end of osmotic fragility. But, there was **no significant** difference in the cholesterol to high density lipoproteins ratio.

There were **statistical significant** differences between the controls VS Child C in total cholesterol, triglycerides, high density lipoproteins, low density lipoproteins, very low density lipoproteins, cholesterol to high density lipoproteins ratio, apolipoprotein A level, start of osmotic fragility and the end of osmotic fragility. There were **statistical significant** differences between the Child A VS Child B in total cholesterol, triglycerides, low density lipoproteins, very low density lipoproteins, apolipoprotein A level, start of osmotic fragility and the end of osmotic fragility. While there were **no significant** differences as regard high density lipoproteins, and the cholesterol to high density lipoproteins ratio. There were **statistical significant** differences between the Child B VS Child C in total cholesterol, high density lipoproteins, cholesterol to high density lipoproteins ratio, apolipoprotein A level, start of osmotic fragility and the end of osmotic fragility. While there were **no significant** differences as regard triglycerides, low density lipoproteins, and very low density lipoproteins. Also, there were **statistical significant** differences between the Child A VS Child C in total cholesterol, triglycerides, high density lipoproteins, low density lipoproteins, very low density lipoproteins, cholesterol to high density lipoproteins ratio, apolipoprotein A level, start of osmotic fragility and the end of osmotic fragility.

The correlation between the Apolipoprotein A level and the start and end of osmotic fragility showed that: (Table 4) There is a significant (P value 0.000) strong negative correlation (correlation coefficient -0.892) between the Apolipoprotein A level and the start of osmotic fragility of RBCs. There is a significant (P value 0.000) strong negative correlation (correlation coefficient -0.750) between the Apolipoprotein A level and the end of osmotic fragility of RBCs.

Table (1): Demographic data of the studied chronic hepatitis C patients (N=50) and the controls (N=10).

Demographic data	**Chronic HCV patients (N = 50)**		**Control (N = 10)**	
	NO.	**%**	**NO.**	**%**
Age (years)				
• Mean ± SD	53.72 ± 9.407		48.50 ± 12.268	
• Median (Range)	44 (30 – 74)		36 (29 – 65)	
Sex				
• Male	32	64%	5	50%
• Female	18	36%	5	50%
HBsAg:				
• Negative	50	100%	10	100%
• Positive	0	0%	0	0%
ANA:				
• Negative	50	100%	10	100%
• Positive	0	0%	0	0%
Child classification:				
• Child A	19	38%	0	0%
• Child B	15	30%	0	0%
• Child C	16	32%	0	0%

Table (2): Comparison between Child classes of chronic hepatitis C patients and the controls as regard lipid profile, apolipoprotein A level, and osmotic fragility of RBCs.

		Control	Chronic hepatitis C patients			P- value (Sig.)
			Child A	Child B	Child C	
Cholest.	Mean±SD	170.2±25.5	157.8±30.8	132.3±22.6	82.88±23.7	0.000 (S)
	Median (range)	168 (127-215)	158 (93-215)	126 (110-182)	77 (47-141)	
TGs	Mean±SD	87.5±13.8	82.6±16.02	72.0±14.49	61.88±16.3	0.000 (S)
	Median (range)	90 (65-105)	85 (55-105)	70 (50-110)	55 (35-90)	
HDL	Mean±SD	55.60±7.12	47.21±12.6	40.73±13.7	10.69±4.44	0.000 (S)
	Median (range)	52.5 (49-69)	50 (17-69)	41 (23-71)	9.5 (6-25)	
LDL	Mean±SD	96.4±25.23	93.6±22.49	77.07±21.6	59.7±19.1	0.000 (S)
	Median (range)	102 (61-131)	95 (61-131)	69 (50-124)	56 (25-98)	
VLDL	Mean±SD	17.5±2.75	16.5±3.20	14.4±2.89	12.38±3.3	0.000 (S)
	Median (range)	18 (13-21)	17 (11-21)	14 (10-22)	11 (7-18)	
CHOL / HDL	Mean±SD	3.09±0.48	3.47±0.73	3.55±1.18	7.99±2.44	0.000 (S)
	Median (range)	3.05 (2.30-3.88)	3.4 (2.30-5.47)	3.1 (2.04-6.17)	8.53 (3.91-11.8)	
APO A	Mean±SD	1.46±0.13	1.24±0.26	1.01±0.10	0.48±0.18	0.000 (S)
	Median (range)	1.4 (1.30-1.70)	1.3 (0.78-1.70)	0.98 (0.88-1.20)	0.485 (0.20-0.90)	
O.F starts	Mean±SD	0.45±0.00	0.455±0.016	0.477±0.026	0.54±0.022	0.000 (S)
	Median (range)	0.45 (0.45-0.45)	0.45 (0.45-0.50)	0.5 (0.45-0.50)	0.55 (0.50-0.55)	
O.F ends	Mean±SD	0.300±0.00	0.3105±0.02	0.327±0.026	0.350±0.00	0.000 (S)
	Median (range)	0.3 (0.30-0.30)	0.3 (0.30-0.35)	0.35 (0.30-0.35)	0.35 (0.35-0.35)	

P < 0.05 was considered statistically significant (S), and P > 0.05 was considered non statistically significant (NS).

Table (3): Post hoc test by LSD for lipid profile, apolipoprotein A level, and osmotic fragility stratified by Child classes of chronic hepatitis C patients and controls.

	Control VS Child A	Control VS Child B	Control VS Child C	Child A VS Child B	Child B VS Child C	Child A VS Child C
Cholesterol	0.233(NS)	0.001 (S)	0.000(S)	0.007 (S)	0.000 (S)	0.000(S)
TGs	0.422(NS)	0.017 (S)	0.000(S)	0.050 (S)	0.073(NS)	0.000(S)
HDL	0.047 (S)	0.001 (S)	0.000(S)	0.081(NS)	0.000 (S)	0.000(S)
LDL	0.747(NS)	0.035 (S)	0.000(S)	0.033 (S)	0.31 (NS)	0.000(S)
VLDL	0.422(NS)	0.017 (S)	0.000(S)	0.050 (S)	0.073(NS)	0.000(S)
CHOL/HDL	0.511(NS)	0.440(NS)	0.000(S)	0.866(NS)	0.000 (S)	0.000(S)
APO A	0.005 (S)	0.000 (S)	0.000(S)	0.001 (S)	0.000 (S)	0.000(S)
O.F starts	0.493(NS)	0.001 (S)	0.000(S)	0.002 (S)	0.000 (S)	0.000(S)
O.F ends	0.130(NS)	0.000 (S)	0.000(S)	0.010 (S)	0.000 (S)	0.000(S)

P < 0.05 was considered statistically significant (S), and P > 0.05 was considered non statistically significant (NS).

Table (4): Correlation between the Apolipoprotein A level, and the start and the end of osmotic fragility of RBCs in the studied chronic hepatitis C patients.

	Apolipoprotein A	
	Correlation Coefficient	Sig.
Start of osmotic fragility	-0.892	0.000 (S)
End of osmotic fragility	-0.750	0.000 (S)

P < 0.05 was considered statistically significant (S), and P > 0.05 was considered non statistically significant (NS).

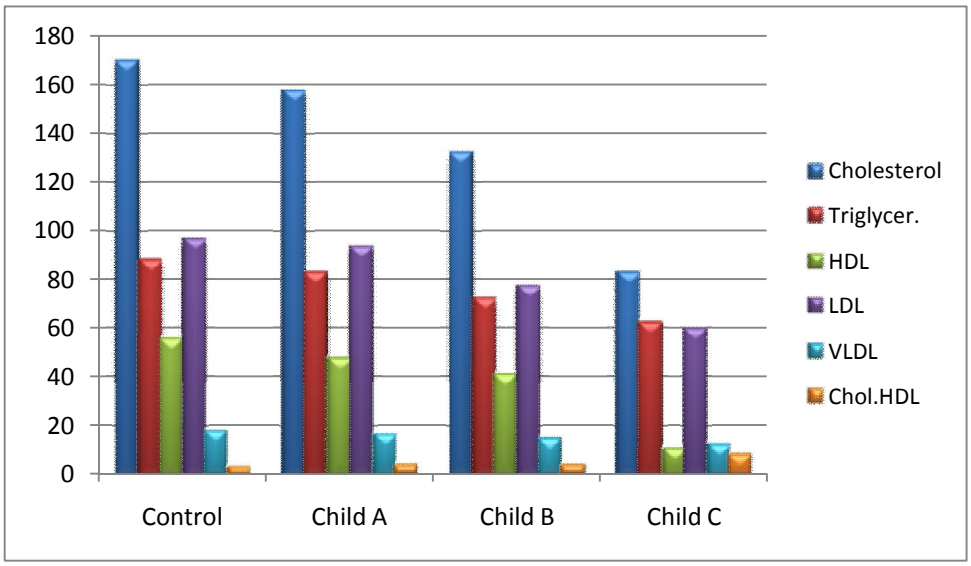

Fig. 1. Showing Comparison between Child classes of chronic hepatitis C patients and the controls as regard lipid profile.

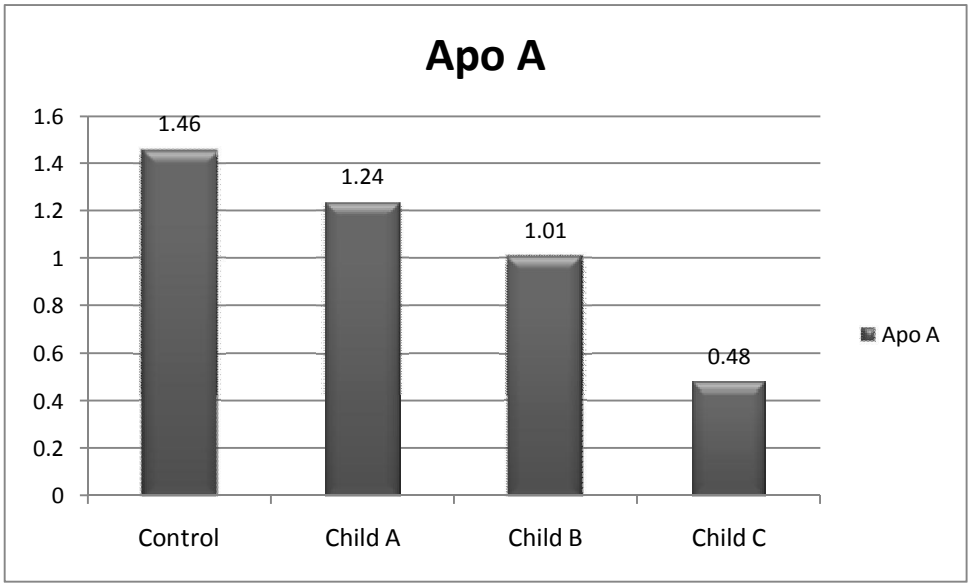

Fig. 2 Showing Comparison between Child classes of chronic hepatitis C patients and the controls as regard Apolipoprotein A.

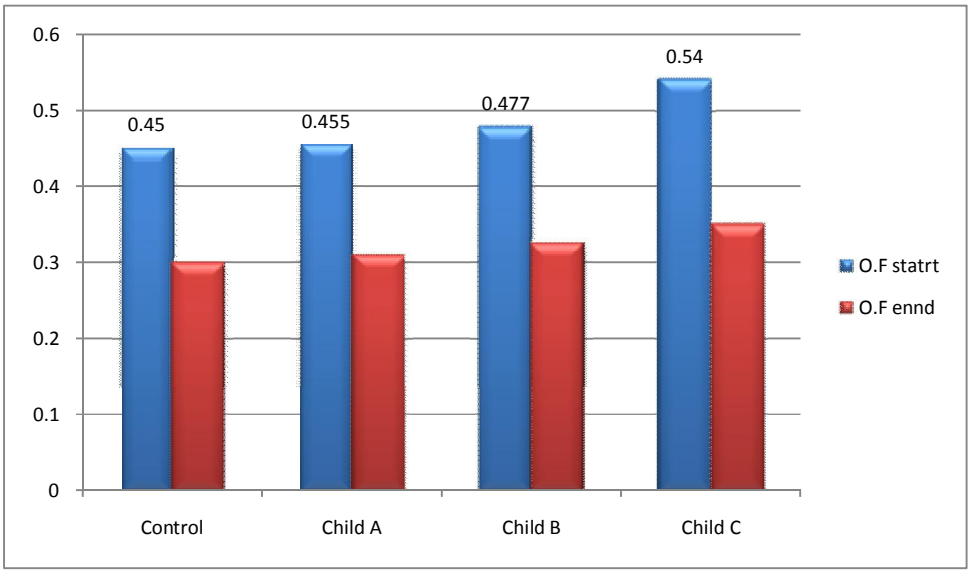

Fig. 3 Showing Comparison between Child classes of chronic hepatitis C patients and the controls as regard RBCs osmotic fragility test.

4. Discussion:

The liver plays a central role in regulating the synthesis, degradation and storage of cholesterol and lipoproteins. TC and lipoproteins have been shown to decrease with the progression of fibrosis and, further, with the onset of cirrhosis. This change in lipid levels can be used to estimate prognosis in cirrhotic patients (7). A reduction in TC serum levels is believed to be a consequence of decreased synthesis or partial blockage of the same esterification processes, likely due to a decline in the production of the enzyme ACAT (acyl CoA: cholesterol acyltransferase) (8). Decreased VLDL levels are associated with deficiencies in the microsomal triglyceride transfer protein (MTP) and a partial inhibition of cholesterol synthesis [9]. The formation of LDL is directly related to the production of VLDL and, when the metabolism of this lipoprotein is impaired, the other downstream lipid fractions also undergo changes [10]. The drop in HDL levels suggests that there is a strong correlation between prognosis and decreased synthesis of Apoprotein AI (Apo AI), the major HDL lipoprotein [11]. The prognosis of cirrhosis depends on the etiology, severity of illness, and presence of associated diseases and complications. Various laboratory and clinical evaluation systems have been developed over the years to assist in staging liver disease. The Child-Pugh classification and MELD score are among the most widely used systems [12]. Liver cirrhosis can lead to chronic liver failure with associated alterations in blood cells or hepatocellular carcinoma evolving on the basis of chronic hepatitis [13]. It has been shown that erythrocytes from patients with cirrhosis are more susceptible to lysis than normal ones and that their life span is much shorter, about 50-60% and is correlated with higher osmotic fragility [14]. Based on those facts we conducted our study to assess the relationship between Apolipoprotein A level and the osmotic fragility of RBCs in HCV cirrhotic patients.

The present study included 60 subjects; 50 chronic hepatitis C patients classified according Child-Pugh score and 10 controls. All subjects participated in the study were excluded from being patients of other types of liver diseases not related to HCV, diabetic, hypertensive, with evidence of blood loss, or other causes of hemolytic anemia.

We assessed Apolipoprotein A level in addition to osmotic fragility test in different groups.

Then we analyzed the correlation between the Apolipoprotein A level, the osmotic fragility of RBCs, and other variables in the studied group.

This study showed that low TC, VLDL, LDL, HDL and TG serum levels are associated with increased hepatic impairment. Hypocholesterolemia and hypotriglyceridemia are both significantly associated and correlated with the Child-Pugh prognostic criteria.

These findings were consistent with previous studies that showed changes in lipid metabolism in advanced stages of cirrhosis [15, 16, 17].

By analyzing the associations between the Child-Pugh classification and lipid profile, we found that the Child-Pugh score was significantly associated with a reduced lipid profile. Patients with Child-Pugh C scores had lower TC (P<0.001), VLDL (P<0.001), LDL (P<0.001), HDL (*P*<0.001) and TG (*P*<0.001) levels.

These results were consistent with the findings reported by Bassani et al. [18] and Cicognani et al. [19] who evaluated TC, VLDL, LDL and HDL levels in patients with chronic hepatitis and cirrhosis and correlated them with disease severity (Child-Pugh score). The results showed that significant reductions in TC, LDL and HDL levels were observed in patients with cirrhosis compared with other groups (chronic hepatitis and control). This reduction was related to disease progression (Child-Pugh C classification).

D'Arienzo [20] assessed the prognostic role of hypocholesterolemia in patients with advanced cirrhosis and observed a gradual decrease in plasma cholesterol in 34 patients with viral cirrhosis, Child-Pugh C classification. All patients with TC levels <100 mg/dL died within 17 months, whereas 75% of patients with TC levels >100 mg/dL survived at least 2 years.

Habib [11] analyzed 413 patients with cirrhosis of different etiologies and reported that the need for liver transplantation within 1 year was higher in patients with HDL levels <30 mg/dL.

By analyzing the associations between the Child-Pugh classification and the Apolipoprotein A level, we found a strong inverse significant correlation between the Child-Pugh score and the level of Apo-A.

These results were consistent with the findings reported by Spósitoet al. [21], Duhamel et al. [22], and Privitera & Meli [23] who showed that TC, HDL, LDL, triglycerides and Apo A-I levels were significantly reduced in cirrhotic patients compared with controls and progressively decreased with worsening severity of liver disease. These findings also concordant with results of Spadaro et al. [24].

By analyzing the associations between the Child-Pugh classification and the onset of hemolysis in cirrhotic RBCs, as we found a strong positive significant correlation between the Child-Pugh score and onset of hemolysis, hemolysis began in less hypotonic solution (earlier) in the RBCs of liver cirrhotic patients which shows the highly fragile nature of RBCs.

On the other hand we found a non-significant difference between the onset of hemolysis in controls vs Child A. So, earlier hemolysis and increasing osmotic fragility is associated with increased hepatic impairment.

These results were consistent with the findings reported by Geetha et al. [25], who reported that free radical mediated oxidative stress in severe liver cirrhosis was evidenced by the elevated levels of lipid peroxides and lipid hydroperoxides in RBCs due to imbalance between the prooxidants and/ or free radicals and antioxidizing systems. In this manner lipid peroxidation of phospholipid fatty acid causes formation of short, ionized, unsaturated fatty molecules, and hemolysis began in less hypotonic solution (earlier) in the RBCs of liver cirrhotic patients.

Vassiliadis et al. [26] reported an incidence of SCA of 16.7% in 54 cirrhotic patients studied. Patients with at least 5% of spur cells, have more advanced liver disease compared with patients with less than 5% of spur cells. Affected patients showed lower three months survival rate.

In a recent study of Alexopoulou, et al. [27] the prevalence of SCA was evaluated in 116 cirrhotic patients. The diagnosis was based on the same criteria used in the study of Sousa, et al. [28] The presence of spur cells was reported in 31% of patients and was strictly associated with mortality.

Several investigators have reported the association between alcoholism and spur cell anemia, but recent reports demonstrated that the entity might be present in liver disease regardless of etiology. It was reported that the comparison of spur cell rate in patients with alcoholic cirrhosis versus the remaining etiologies did not exhibit any difference, this was also reported by Vassiliadis et al [26].

Another crucial point is represented by the reversibility of spur cell anemia after liver transplantation. Complete resolution of SCA has been reported after LT in all the studies and case reports by Malik et al. [29]. This phenomenon might be explained to the normalization of lipid metabolism or to a decrease in portal hypertension and hypersplenism following LT.

Finally we assessed the correlation between the level of Apo A and both the onset and complete lysis of cirrhotic RBCs and found a strong significant inverse correlation.

Recommendations:

The reduced lipid profiles in patients with cirrhosis due to HCV infection were significantly associated with the Child-Pugh scores. These results suggested that the lipid profile could be used as an auxiliary tool in evaluating liver disease, given that there were statistically significant differences in these levels using instruments validated for this purpose. There is a need to perform additional larger scale

studies to verify its applicability in cirrhosis due to other etiologies. RBCs health status is crucial to the overall wellness of the liver disease patient and could represent a critical step in the assessment of cirrhotic patients with anemia. The evaluation of red blood cell health status can provide key information to create the differential diagnosis and to early identify patients with SCA to set up them into a higher priority for LT and improve peri- and post-LT survival rates.

Conclusion:

It can be concluded from the results of this study that there is a significant strong negative correlation between the Apolipoprotein-A level and the start and the end of osmotic fragility of RBCs in HCV cirrhotic patients.

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