The Egyptian Journal of Hospital Medicine (January 2023) Vol. 90, Page 607-614

Cytokine Dependent Hematopoietic Cell Linker and Anemia in Liver Cirrhosis Patients

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ABSTRACT

Background: About 75% of people with liver cirrhosis have anemia. It significantly lowers their quality of life and raises mortality. Additionally, several etiologies may be involved. A tyrosine-phosphorylated polypeptide called cytokine dependent hematopoietic cell linker (CLNK) controls immunological receptor signalling and controls the receptor signalling of T-cells and natural killer T-cells. Tyrosine of the cytoplasmic domain band-3 is phosphorylated by oxidative stress, which is a significant factor in the development of liver cirrhosis. Tyrosine phosphorylation thus triggers the release of microparticles, local red cell membrane instability, and major changes in erythrocyte shape. It has not been previously investigated how CLNK affects liver cirrhosis and anemia caused by cirrhosis.

Objectives: The aim of the current study was to evaluate serum level of CLNK in cirrhotic patients compared to healthy controls, and its level was correlated with various hematological parameters.

Patients and methods: A case–control study was conducted on 60 liver cirrhotic patients (30 anemic and 30 not anemic) and 30 age and sex-matched healthy individuals. All patients were subjected to full history taking, complete medical examination, and thorough radiological and laboratory investigations as complete blood counts, ferritin, CRP, liver function tests, kidney function tests, and serum CLNK using enzyme-linked immunosorbent assay were done.

Results: Serum CLNK levels were significantly higher in anemic [178.86 (IQR 68.25)]) and nonanemic cirrhotic patients [138.17 (IQR 170.55)] than in controls [90.28 (IQR 10.61) with a P-value <0.001. There was no significant difference between anemic and non-anemic groups compared with each other. CLNK serum levels showed a statistically significant positive correlation with ferritin and reticulocyte count in anemic patients' groups.

Conclusion: Serum CLNK is significantly elevated in patients with liver cirrhosis and there is still some debate regarding the association between CLNK levels and the incidence of anemia in cirrhotic patients.

Keywords: CLNK, Liver cirrhosis, Anemia, Case control study, Menoufia University.

INTRODUCTION

The pathophysiological effects of cirrhosis have a major impact on the liver's immunological and synthetic activities. Hematological dysfunctions, such as anemia, are how this manifests [(1)].

Anemia may develop in 66% to 75% of liver cirrhotic patients. Notably, the cause of anemia is still unknown in the majority of cases. 53% of all cases of anemia were unknown in etiology, hemorrhage (25%) and iron deficiency (9%). Unfortunately, the high occurrence of anemia can lead to a misperception that it is a necessary component of liver disease [(2)].

Anemia brought on by cirrhosis is linked to higher mortality and morbidity rates. There may be many etiologies at play as well. Therefore, it is crucial to have a straightforward, simple to use, but informative diagnostic procedure to help with the identification and subsequent treatment of the primary cause of anemia in cirrhosis [(3)].Hepatocytes primarily generate ferritin, a sign of iron homeostasis and an acute phase reactant. A systemic review of the accuracy of ferritin analysis in cirrhotic patients showed that levels of <15 g/dL were essentially diagnostic of iron deficiency anemia (IDA) in cirrhosis whereas values of >100 g/dL practically ruled it out [(4)].

Even without particular genetic defects, iron overload has been discovered in 8% of people with an advanced liver illness comparable to hemochromatosis. An overabundance of iron, which may be identified by higher blood ferritin levels, increases the risk of HCC [(5, 6)]. The liver produces crucial mediators known as acute phase reactants during acute and chronic inflammatory diseases, which lead to a number of negative outcomes such as fever and anemia of chronic illness. Interleukin-6 is the main cytokine that stimulates the liver to make more (IL-6). Acute-phase reactants can also be induced by IL-1, tumor necrosis factor-alpha (TNF-alpha), and interferon-gamma (IFN-gamma) [(7)].

In addition to the cytokines generated in response to various stimuli and immune system dysregulation, a different class of proteins known as adaptor proteins is an essential part of signaling pathways both inside and outside the immune system [(8)].

Different hematopoietic cell types have a variety of adaptors, which refers to hematopoietic stem cells, which includes all mature cell types as well as their immature progenitors [(9)].

In cytokine-dependent lymphoid and myeloid cell lines, including neutrophils, mast cells, macrophages, platelets, T cells, and natural killer (NK) cells, CLNK, an adaptor protein, has been found to be expressed. It seems that persistent exposure to cytokines like IL-2 and IL-3 is the only factor that influences its expression. It possesses a leukocyte protein of 76 kD (SLP-76)-related molecule with a Src homology 2 domain [(10)]. The Src family of protein tyrosine kinases (SFKs) is a group

Received: 23/07/2022
Accepted: 25/09/2022

of non-receptor tyrosine kinases that are crucial for controlling membrane transport as well as hematopoietic cell activities [(11)].

Tyrosine dephosphorylation inhibition induces micro vesicle generation in vitro and may change the morphology of RBCs into echinocytes, indicating a loss of contact between the cytoskeleton and lipid bilayers and leads to local red cell membrane instability in hemolytic anemia [(12)].

Oxidative stress, which plays an important role in the course of liver cirrhosis, phosphorylates tyrosine of the cytoplasmic domain band-3 [(13)].

There are no studies that address CLNK's function in liver disease. As a result, the uniqueness of this study lies in its attempt to compare the levels of CLNK in cirrhotic patients with those in healthy controls and assess if there is a relationship between CLNK levels and anemia aggravating liver cirrhosis.

The aim of the current study was to evaluate serum level of CLNK in cirrhotic patients compared to healthy controls, and its level was correlated with various hematological parameters.

PATIENTS AND METHODS

Study design and ethical statement:

A case-control study was conducted and involved 60 cirrhotic patients due to chronic hepatitis C virus (HCV) infection (30 anemic and 30 not anemic) and 30 age and sex-matched healthy individuals. The samples were obtained from National Liver Institute, Menoufia University from December 2020 to the end of November 2021.

All patients underwent extensive medical examinations, detailed histories, and radiographic and laboratory testing. Tests on the control group's clinical and biochemical makeup revealed that they were normal.

Diagnostic criteria:

Inclusion criteria: Age requirements of at least 18 years of age, the existence of liver cirrhosis, and blood hemoglobin levels of less than 13 g/dl in men and 12 g/dl in women, as specified by the World Health Organization, . With the aid of fibroscan, ultrasound, computed tomography, or magnetic resonance imaging (MRI), liver cirrhosis was either established histo-pathologically or by obvious morphological criteria of liver cirrhosis.

Exclusion criteria: Patients who had a liver transplant, those with hepatocellular carcinoma in the early to terminal stages, those who had received direct acting antiviral medication in the past, and those who had received blood transfusions in the four weeks before to sample collection.

Methods:

Seven milliliters of venous blood were aseptically drawn from all subjects. Two milliliters were transferred to an EDTA-contained tube for hematological analysis, while the other five milliliters were transferred to a plain tube, after the blood had fully clotted, it was centrifuged at 3000 rpm for 10 min to separate the serum, which was separated and collected in two tubes. One for measurement of ferritin CRP, renal function tests (RFT) and liver function tests. The other one was kept at -80 C until used for CLNK testing.

Using a solid-phase enzyme-linked immuno-sorbent technique [Shanghai Sunred biotechnology company, Cat. No 201-12-6921, China], CLNK was identified in the patient's serum as recommended by the manufacturer: In each standard well, 50 µl of standard reagent (included in the kits) and 50 µl streptavidin-HRP were added, simultaneously 40 µl of each sample, 10 µl of anti-CLNK antibody and 50 µl streptavidin-HRP were added in each sample well followed by a 1-hour incubation time at 37°C and three automated washing cycles, next,50 µl of substrate solution A and 50 µl of substrate solution B were added to each well, and they were left to incubate in the dark for 10 minutes. The optical density (OD values) of each well was then recorded by a 450 nm ELISA reader after a stop solution had been applied to all wells. Computer-based techniques were utilized to yield the results with curve and regression analysis.

Ethical consent:

The National Liver Institute's Institutional Review Board (IRB) gave the study the go-ahead (permission number 00327/2022). Before taking part in the current investigation, all individuals provided their written informed permission. All procedures were established in accordance with relevant guidelines and regulations of (Declaration of Helsinki) for studies involving humans.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS 22.0, IBM/SPSS Inc., Chicago, IL). Descriptive statistics included estimates for summarizing the continuous data as mean and standard deviation (SD) or median and range for skewed data. Frequency with percentage (%) was used for presenting qualitative data. The hematological parameters, kidney function tests, ferritin, CRP and liver function tests as well as CLNK levels were abnormally distributed among groups; accordingly, their values were expressed as median and interquartile range. Comparisons between groups were made using Pearson Chi-square ($\chi 2$) test for categorical variables (such as gender), and Kruskal-Wallis test (for abnormal distributed parameters). Dunn-Sidak post hoc test was used for multiple pairwise comparisons after significant Kruskal-Wallis. Spearman's correlation coefficient was calculated for the variables. The results were considered significant if P value is ≤ 0.05 and highly significant if the P value ≤ 0.001.

RESULTS

Albumin when compared to control groups, patients with liver cirrhosis (both anemic and non-anemic groups) had significantly higher aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, Gamma-glutamyl transferase, urea, creatinine, and lower protein (P-value < 0.001) [**Table 1**].

Table 1: Biochemical parameters in healthy controls and in liver cirrhosis patients' groups without and with anemia.

Biochemical Parameters	Healthy controls GI (n= 30)	Non-anemic GII (n= 30)	Anemic GIII (n= 30)	Kruskal-Wallis test	Pairwise comparisons*
AST (U/L)					
Median (IQR)	16.5 (7.25)	49 (97.25)	55 (108.5)	χ^2= 43.58	
Range (min-max)	10 - 25	14 - 934	11 - 563	P-value <0.001	p1<0.001 p2<0.001 p3=0.982
ALT (U/L)					
Median (IQR)	15 (6.25)	36.5 (70.50)	28.5 (78.5)	χ^2= 28.60	
Range (min-max)	10 - 24	11 - 365	12 - 719	P-value <0.001	p1<0.001 p2<0.001 p3=0.894
ALP (U/L)					
Median (IQR)	59.5 (21.5)	139 (197.25)	157 (158.25)	χ^2= 28.23	
Range (min-max)	45 - 83	40 – 446	46 - 613	P-value <0.001	p1<0.001 p2<0.001 p3=1.000
GGT (U/L)					
Median (IQR)	17 (8.75)	77.5 (197.5)	64.5 (192)	χ^2= 37.03	
Range (min-max)	11 – 32	14 - 610	12 - 656	P-value <0.001	p1<0.001 p2<0.001 p3=0.999
Total bilirubin (mg/dL)					
Median (IQR)	0.9 (0.4)	1.9 (11.6)	1.75 (3.11)	χ^2= 19	
Range (min-max)	0.2 - 1.2	0.2 – 31	0.3 - 9	P-value <0.001	p1=0.024 p2<0.001 p3=1.000
Direct bilirubin (mg/dL)					
Median (IQR)	0.3 (0.23)	0.74 (6.47)	0.76 (1.28)	χ^2= 10.4	
Range (min-max)	0.1 - 0.6	0.09 – 15	0.07 - 5.35	P-value =0.006	p1=0.241 p2=0.003 p3=1.000
Albumin (g/dL)					
Median (IQR)	4.05 (0.83)	3.2 (1.53)	2.85 (1.03)	χ^2= 40.76	
Range (min-max)	3.5 – 5	2.2 - 4.6	2 - 3.9	P-value <0.001	p1=0.003 p2<0.001 p3=0.059
Total protein (g/dL)					
Median (IQR)	7.55 (1)	6.35 (1.47)	5.95 (1.45)	χ^2= 28.35	
Range (min-max)	6.8 - 8.3	4.6 - 10.4	4.7 - 9.2	P-value <0.001	p1<0.001 p2<0.001 p3=0.932
Urea (mg/dL)					
Median (IQR)	21 (12.5)	47.5 (93)	61 (98.05)	χ^2= 26.99	
Range (min-max)	15 - 35	15 – 260	15 - 350	P-value <0.001	p1<0.001 p2<0.001 p3=0.999
Creatinine (mg/dL)					
Median (IQR)	0.7 (0.21)	1.04 (1.17)	1.02 (0.95)	χ^2= 16.34	
Range (min-max)	0.55 - 1.18	0.6 - 8.4	0.6 - 6.87	P-value <0.001	p1<0.001 p2=0.015 p3=0.457

IQR: Interquartile range (difference between 1st and 3rd quartiles). *: Kruskal-Wallis test; if significant, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test. NS: Non significant at p-value >0.05 S: Significant at p-value ≤0.05. HS: Highly significant at p-value ≤0.01. p1:-p-value for the difference between healthy controls and non-anemic groups (GI vs. GII). p2: p-value for the difference between healthy controls and anemic groups (GI vs. GIII). p3: p-value for the difference between non-anemic and anemic groups (GII vs. GIII).

Significantly lower hemoglobin level, hematocrit, red blood cell counts and higher reticulocyte, platelet counts were present in anemic groups compared with non-anemic and control groups. There was no statistically significant changes between the analyzed groups were identified for red blood cell indices including mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration [**Table 2**].

Table 2: Hematological parameters in healthy controls and in liver cirrhosis patients' groups without and with anemia.

Hematological parameters	Healthy controls GI (n= 30)	Non-anemic GII (n= 30)	Anemic GIII (n= 30)	Kruskal-Wallis test	Pairwise comparisons*
Hemoglobin (g/dL)					p1=0.024
Median (IQR)	14 (1.33)	13.2 (1.05)	9.55 (2.63)	$\chi2$= 58.41	p2<0.001
Range (min-max)	12.5 - 16	12.5 - 15.1	4.8 – 12	P-value <0.001	p3<0.001
HCT (%)					p1<0.001
Median (IQR)	47 (5)	39.35 (5.85)	29.1 (7.7)	$\chi2$= 63.77	p2<0.001
Range (min-max)	41 - 50	36.3 - 46.3	14.3 - 36.8	P-value <0.001	p3<0.001
RBCs (10^6 cell/µL)					p1=0.024
Median (IQR)	4.95 (0.6)	4.52 (0.66)	3.17 (0.86)	$\chi2$= 57.67	p2<0.001
Range (min-max)	4.3 - 5.5	3.97 - 5.6	1.67 - 4.31	P-value <0.001	p3<0.001
MCV (fL)					p1=0.006
Median (IQR)	91.5 (9.5)	85.7 (5.47)	87.85 (6.3)	$\chi2$= 10.88	p2=0.224
Range (min-max)	83 - 101	82 - 94.6	83.3 - 100	P-value= 0.004	p3=0.121
MCH (pg)					p1=0.770
Median (IQR)	29 (4)	28.9 (2.62)	28.9 (1.75)	$\chi2$= 1.11	p2=1.000
Range (min-max)	2 - 32	23 - 32.2	27 - 33.5	P-value= 0.573	p3=0.674
MCHC (g/dL)					p1=0.467
Median (IQR)	32.5 (2.2)	33.15 (2.5)	32.55 (1.6)	$\chi2$= 1.76	p2=0.970
Range (min-max)	31 - 34.5	30.8 - 36.2	31.3 - 35.5	P-value= 0.415	p3=0.722
Reticulocytes count (%)					p1<0.001
Median (IQR)	1.1 (0.73)	1.94 (0.83)	2.84 (1.48)	$\chi2$= 36.32	p2<0.001
Range (min-max)	0.5 - 2.3	1.17 – 3	0.8 - 15.6	P-value <0.001	p3=0.006
WBCs (10^3 cell/µL)					p1=0.009
Median (IQR)	6 (2.57)	8.27 (7.02)	8.64 (8.26)	$\chi2$= 8.10	p2=0.175
Range (min-max)	4 - 9.1	4.34 - 31.57	2.61 - 25.5	P-value= 0.017	p3=0.910
Platelets (10^3 cell/µL)					p1=0.012
Median (IQR)	232.5 (136.25)	157 (118)	128 (102)	$\chi2$= 20.82	p2<0.001
Range (min-max)	118 - 457	56 - 329	30 - 394	P-value <0.001	p3=0.498

IQR: Interquartile range (difference between 1st and 3rd quartiles). *: Kruskal-Wallis test; if significant, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test. NS : Non significant at p-value >0.05 S: Significant at p-value ≤0.05. HS: Highly significant at p-value ≤0.01. p1:-p-value for the difference between Healthy controls and non-anemic groups (GI vs. GII). p2: p-value for the difference between Healthy controls and anemic groups (GI vs. GIII). p3: p-value for the difference between non-anemic and anemic groups (GII vs. GIII).

Serum CLNK, ferritin and CRP levels were significantly higher in non-anemic patients [138.17 (170.55), 272.00 (433.33) and 55.50 (152.75)] and anemic [178.86 (68.25), 345.50 (361.00) and 30.85 (64.40)] compared with the controls [90.28 (10.61), 64.50 (71.50) and 1.50 (1.60) with a *P* value <0.001 [**Table 3**].

Table 3: Investigated parameters in healthy controls and in liver cirrhosis patients' groups without and with anemia.

Investigated parameters	Healthy controls GI (n= 30)	Non-anemic GII (n= 30)	Anemic GIII (n= 30)	Kruskal-Wallis test	Pairwise comparisons*
CLNK (ng/mL)					
Median (IQR)	90.28 (10.61)	138.17 (170.55)	178.86 (68.25)	χ^2= 51.71	p1<0.001 p2<0.001
Range (min-max)	70.96 - 110.89	78.97 - 319.62	105.25 - 411.09	P-value <0.001	p3=0.451
Ferritin (µg/L)					
Median (IQR)	64.50 (71.50)	272 (433.33)	345.5 (361)	χ^2= 31.31	p1<0.001 p2<0.001
Range (min-max)	29.00 - 200.00	50 – 843	35.1 - 746	P-value <0.001	p3=0.954
CRP (mg/dL)					
Median (IQR)	1.5 (1.6)	55.5 (152.75)	30.85 (64.4)	χ^2= 46.31	p1<0.001 p2<0.001
Range (min-max)	0.2 - 4	1.2 - 322	0.5 - 311	P-value <0.001	p3=0.498

IQR: Interquartile range (difference between 1st and 3rd quartiles). *: Kruskal-Wallis test; if significant, multiple pairwise comparisons were adjusted by Dunn-Sidak post hoc test. NS: Nonsignificant at p-value >0.05 S: Significant at p-value ≤0.05. HS: Highly significant at p-value ≤0.01. p1:-p-value for the difference between Healthy controls and non-anemic groups (GI vs. GII). p2: p-value for the difference between Healthy controls and anemic groups (GI vs. GIII). p3: p-value for the difference between non-anemic and anemic groups (GII vs. GIII).

CLNK serum levels have positive correlation with ferritin and reticulocyte counts in anemic groups but no correlation with CRP. In the various study groups, there was no statistically significant association between serum CLNK and other parameters [**Table 4**].

Table 4: Correlation between CLNK (ng/mL) and various parameters in liver cirrhosis patients' groups.

Correlated Parameters	CLNK (ng/mL)			
	Non-anemic (n= 30)		Anemic (n= 30)	
	r_s	P-value	r_s	P-value
Age (years)	-0.03	0.892 ^{NS}	-0.02	0.926 ^{NS}
Ferritin (µg/L)	0.05	0.837 ^{NS}	0.44	**0.015** ^{S}
CRP (mg/dL)	0.00	0.995 ^{NS}	-0.13	0.481 ^{NS}
AST (U/L)	-0.17	0.466 ^{NS}	-0.21	0.276 ^{NS}
ALT (U/L)	-0.16	0.494 ^{NS}	-0.19	0.307 ^{NS}
ALP (U/L)	-0.33	0.160 ^{NS}	-0.06	0.739 ^{NS}
GGT (U/L)	-0.17	0.462 ^{NS}	-0.06	0.747 ^{NS}
Total bilirubin (mg/dL)	0.01	0.982 ^{NS}	-0.28	0.134 ^{NS}
Direct bilirubin (mg/dL)	-0.04	0.877 ^{NS}	-0.27	0.148 ^{NS}
Albumin (g/dL)	0.31	0.181 ^{NS}	0.29	0.120 ^{NS}
Total protein (g/dL)	0.10	0.672 ^{NS}	0.24	0.199 ^{NS}
Urea (mg/dL)	-0.02	0.929 ^{NS}	-0.22	0.238 ^{NS}
Creatinine (mg/dL)	-0.04	0.884 ^{NS}	-0.16	0.396 ^{NS}
Hemoglobin (g/dL)	-0.11	0.651 ^{NS}	-0.27	0.148 ^{NS}
HCT (%)	0.22	0.362 ^{NS}	-0.18	0.347 ^{NS}
RBCs (10^6 cell/µL)	0.01	0.985 ^{NS}	-0.14	0.460 ^{NS}
MCV (fL)	-0.07	0.768 ^{NS}	0.06	0.761 ^{NS}
MCH (pg)	-0.26	0.270 ^{NS}	-0.29	0.115 ^{NS}
MCHC (g/dL)	-0.29	0.215 ^{NS}	-0.26	0.162 ^{NS}
Reticulocytes count (%)	-0.08	0.725 ^{NS}	0.43	**0.019** ^{S}
WBCs (10^3 cell/µL)	-0.17	0.470 ^{NS}	0.15	0.415 ^{NS}
Platelets (10^3 cell/µL)	-0.29	0.210 ^{NS}	0.00	0.991 ^{NS}

r_s: Spearman correlation coefficient. ^{NS} : Non significant at p-value ≥ 0.05 ^{S}: Significant at p-value ≤0.05.

The ROC curve analysis revealed a high-predictive value of CLNK for discrimination of cirrhotic patients (either anemic or not) from controls. **Table 5** and **Figure 1** showed that in non-anemic groups CLNK had AUC (CI 95%) of 0.947 with the optimal cut-off value of ≥98.01 ng/mL (sensitivity, SS: 95.0%; and specificity, SP: 90.0%), and in anemic groups, CLNK had the AUC (CI 95%) of 0.997with the optimal cut-off value of ≥102.28 ng/mL (SS: 100%; SP: 96.7%) [**Table 6** and **Figure 2**].

Table (5): Diagnostic performance of CLNK for discrimination between non-anemic liver cirrhosis patients' groups and healthy controls

Test characteristics	Non-anemic versus Healthy controls
	CLNK (ng/mL)
Best cutoff value	≥ 98.01
AUC	0.947
***P*-value**	**<0.001** HS
Sensitivity %	95.0
Specificity %	90.0
PPV %	86.4
NPV %	96.4
Accuracy %	92.5
PPV: Positive predictive value, NPV: Negative predictive value. HS: Highly significant at p-value ≤0.01	

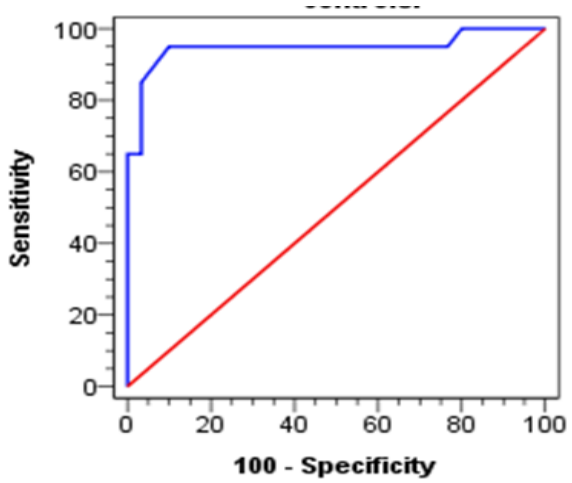

Figure 1: ROC curve for discrimination between non-anemic liver cirrhosis patients' groups and healthy controls.

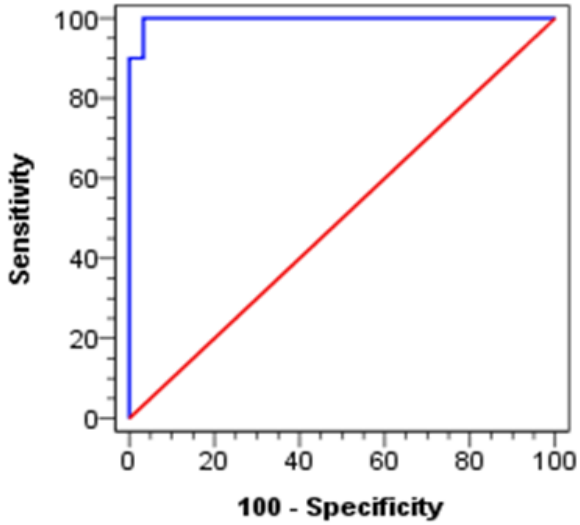

Figure 2: ROC curve for discrimination between anemic liver cirrhosis patients' groups and healthy controls.

Table 6: Diagnostic performance of CLNK for discrimination between anemic liver cirrhosis patients' groups and healthy controls.

Test characteristics	Anemic versus Healthy controls
	CLNK (ng/mL)
Best cutoff value	≥ 102.28
AUC	0.997
***P*-value**	**<0.001** HS
Sensitivity %	100
Specificity %	96.7
PPV %	96.8
NPV %	100
Accuracy %	98.4
PPV: Positive predictive value NPV: Negative predictive value. HS: Highly significant at p-value ≤0.01.	

While the predictive cut-off value for CLNK was ≥106.68 (AUC: 0.612; SS: 96.7%; SP: 35%) for discriminating between non-anemic and anemic cirrhotic patients [**Table 7** and **Figure 3**].

Table (7): Diagnostic performance of CLNK for discrimination between non-anemic and anemic liver cirrhosis patients' groups.

Test characteristics	Anemic CHC vs. Non-anemic CHC
	CLNK (ng/mL)
Best cutoff value	≥ 106.68
AUC	0.612
***P*-value**	0.181 NS
Sensitivity %	96.7
Specificity %	35
PPV %	69.1
NPV %	87.6
Accuracy %	65.9
PPV: Positive predictive value, NPV: Negative predictive value. NS: Non-significance at p-value >0.05.	

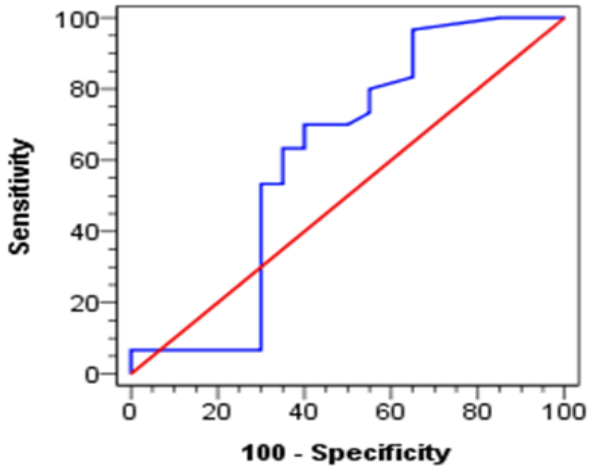

Figure 3: ROC curve for discrimination between non-anemic and anemic liver cirrhosis patients' groups.

DISCUSSION

The laboratory indicators of liver cirrhosis are primarily influenced by the degree of hepatocellular insufficiency and are independent of the etiology of the disease. Generally speaking, leukocyte, erythrocyte, and platelet sprouts of hematopoiesis all exhibit malfunction in the evident stage of liver cirrhosis [(14)].

According to the current study, the number of red blood cells, hemoglobin level, hematocrit, and platelets were extremely decrease in patients with liver cirrhosis than in non-cirrhotic individuals while the absolute number of reticulocytes and leukocytes increased. These findings were in line with **Sun *et al.*** [(15)] and **Bakhriev *et al.*** [(16)].

Interestingly, the average volume of red blood cells (MCV) did not statistically significantly increase in cirrhotic individuals with anemia compared to those without, according to the study's findings. This is consistent with the research by **Bakhriev *et al.*** [(16)] which showed that liver disease would naturally be the cause. Along with other potential causes, the hemoglobinization and maturation of erythroid components of the bone marrow is likely the main cause of the anemia in this case.

On the background of the reticulocyte formula's renewal, patients with liver cirrhosis were shown to have significantly more reticulocytes than normal (Table 2). And this is the same as **Bakhriev *et al.*** [(16)] and **Rassi *et al.*** [(17)] who recorded a rise in the overall number of reticulocytes in cirrhosis of the liver is attributable to a number of reasons. Hemolysis, which increases erythropoiesis in accordance with the feedback principle, is likely the main mechanism at play here.

The main gain of the present study is that, patients with liver cirrhosis (including those with and without anemia) have greater serum CLNK levels than the controls, as shown in (Table 3). Due to a paucity of data about the concentration of CLNK in the serum of cirrhotic patients, this result lacks a clear explanation.

Human serum CLNK has only been tested in a few number of investigations, mainly in thalassemia patients. CLNK levels increase in thalassemia patients relative to healthy controls, according to **Al-Hakeim *et al.*** [(18)], because immature erythrocytes release soluble receptors and signaling molecules like CLNK into the blood in response to immune system signals. Contrarily, patients with chronic liver illness may experience the usual side effect of portal hypertension. This disorder has the potential to cause splenomegaly, which can enhance the rate of RBC oxidation and release immature RBCs into the bloodstream, where they may release soluble receptors and signaling molecules like CLNK. This could explain what we found regarding the increase in CLNK in cirrhotic patients' blood.

Another study by **Al-Fadhel *et al.*** [(19)], focused on the rise of CLNK in type 2 diabetic patients, this phenomenon indicating the involvement of the adaptor protein level in the disease. CLNK functions as an adapter protein and is essential for the T-cell signaling pathway, which is controlled by the T-cell receptor and required for the adaptive immune response as well as vital for cell proliferation, differentiation, and cytokine production. The connection between CLNK and inflammation is the other aspect that needs to be verified [(10)]. This should be kept in mind when explaining the elevated CLNK levels in cirrhotic patients and the role of inflammation in such findings.

Unfortunately, in accordance with the data collected in the current study (Table 3), the level of CLNK did not statistically significantly differ between the cirrhotic individuals in anemic and non-anemic groups, amounted to 178.86 (IQR 68.25) and 138.17 (IQR 170.55) ng/ml, respectively. Therefore, the question about the association between CLNK levels and the incidence of anemia in cirrhotic patients remains open.

Interestingly, we found that serum CLNK and ferritin were significantly associated in anemic patients with liver cirrhosis. The overall positive correlation between CLNK and ferritin necessitates measuring the other iron status parameters to further determine whether this connection in such cases due to excess iron as reported by **Al-Hakeim *et al.*** [(18)] in thalassemic patients, or whether the association between CLNK and ferritin is within the inflammatory sequences in liver cirrhosis as the ferritin is one of the acute phase proteins.

In cirrhotic patients, **Bruno *et al.*** [(20)] observed an increase in erythropoietin when the haemoglobin level was less than 120 g/L. Additionally, patients with chronic liver illness typically have an accompanying increase in the rate of RBC breakdown and release of immature RBCs as well as reticulocyte to the systemic circulation, which is stimulated by an increase in erythropoietin (EPO) levels [(16)]. They release soluble receptors and signaling molecules, including CLNK, into the blood, which might account for the fact that

anemic patients' levels of CLNK and reticulocyte in this study showed a positive connection (**Table 4**).

CONCLUSION

Serum CLNK levels are higher in patients with liver cirrhosis compared with controls, and their higher levels are linked to higher serum ferritin levels and reticulocyte counts in anemic groups. This could be related to the production of signaling molecules such as CLNK in the bloodstream by an immunological signaling pathway and immature erythrocytes.

Our study was limited by the small number of patients we included. We envisage that future wide-reaching studies in the population with liver cirrhosis will help in evaluating the association of CLNK with liver cirrhosis and anemia complicating liver diseases.

Financial support and sponsorship: Nil.
Conflict of interest: Nil.

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