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Quantitation of neutrophil extra cellular traps (NETs) in liver cirrhosis patients and their relation to the incidence of different complications

Enaam Ali ElSayed Al Mowafy[1] and Salma AbdelGhany Shawkat[2*]

Abstract

Backgrounds and aim: Neutrophil extracellular traps (NETs) have been shown to play an important role in inflammatory and thrombotic processes. Investigating the presence of NETs in liver cirrhosis to detect any contribution to occurrence of complications may help predict or prevent those complications.

Methods: Among 78 cirrhotic patients, the serum NETs level was measured using ELISA and compared to different etiologies of liver cirrhosis (Viral, HCC, Bilharzial, NASH, cardiac cirrhosis and undetermined etiology) as well as markers of inflammation and complications in those patients.

Results: We found that NETs are substantially found in LCF and have a significant relation to malignant portal vein thrombosis but not other studied complications or etiology of liver cirrhosis.

Conclusion: NETs however found in liver cirrhosis patients may not play as a significant role in occurrence of complications as thought. So, NETs cannot be reliably used as a biomarker or predictor for occurrence of thrombosis in liver cirrhosis patients.

Lay summary: Liver cirrhosis patients have many factors at play that lead to development of thrombosis. NETS may play a role with the development of malignant thrombosis in those patients. Further evaluation for their level and action should be studied before considering NETs as a key player in development of complications.

Keywords: Liver cell failure, Malignant portal vein thrombosis, Neutrophil extracellular traps, Neutrophils, Inflammatory process

Introduction

Liver cirrhosis and liver cell failure (LCF) continues to be a major health problem that plagues Egypt. It remains to be among the top 10 causes of death worldwide [1]. Liver cell failure are accompanied by several complications aggravating the clinical condition including jaundice, esophageal varices, hepatorenal syndrome, and subacute bacterial peritonitis [2]. Cirrhosis of any etiology is bound to cause inflammation, driving the inflammatory system (both humoral and adaptive arms) with the release of inflammatory mediators and active/stimulated cells [3].

Neutrophils are the most abundant leukocyte in the human body, acting in getting rid of pathogens via phagocytosis and degranulation. In the process of degranulation, neutrophils also release of some DNA contents together with histones and other granule proteins (neutrophil elastase and myeloperoxidase among others); these structures together results in web-like

*Correspondence: salmaabdelghany@med.asu.edu.eg

[2] Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo 11591, Egypt
Full list of author information is available at the end of the article

creations referred to as neutrophil extracellular traps (NETs). Increased NET formation and release have been associated with multiple pathological conditions, most famously thrombotic conditions, with NETs being the scaffold driving coagulation cascades, trapping blood cells in between [4].

NETs were known to be increased in inflammatory conditions, and liver cirrhosis of any etiology is bound to cause inflammation, driving the inflammatory system in both humoral and adaptive immune arms with the release of inflammatory mediators and active cells [3]. NETs released in the inflammatory process are associated with liver cirrhosis as many other conditions are postulated to further promote inflammation and lead to thrombosis [4].

Studying NETs and its relation to the occurrence of those complications can give insight to knowing if NETs can promote the occurrence of one complication over the others.

The elevation of NETs levels in liver cirrhosis patients when compared to healthy controls is well agreed upon [5]. Their relationship to the different complications occurring in LCF especially thrombotic or malignant complications can be of value to predict the occurrence of these complications. Exploring NET serum levels as a biomarker in liver cirrhosis patients is an idea that can help in management of those patients.

Methods

Study deign and setting

This cross-sectional study was conducted among 78 cirrhotic patients (randomly selected) with whom we encountered in our outpatient clinic and tropical department at the Demerdash Hospital Ain Shams University from the time period between March 2021 and November 2021. All patients were given an informed consent, and all the procedures were well explained to all patients. The Ain Shams University, Faculty of Medicine, ethical committee approved our work prior to commencing any data collection.

Inclusion and exclusion criteria

We included 78 patients diagnosed with liver cirrhosis by laboratory, sonographic, and clinical data on admission.

We excluded patients presenting with any malignancy other than hepatocellular carcinoma as well as Budd-Chiari syndrome.

Study procedures

Patients were diagnosed with liver cirrhosis based on clinical, sonographic, and laboratory tests, with serum NETs measured by ELISA Kit (Glory Science Co. LTD, Shanghai, China). Abdominal ultrasound and upper gastrointestinal endoscopy was performed for all patients. Child classification was also calculated for each patient.

NETs quantitation

At inclusion, venous blood was drawn to chemistry tubes. The tubes were centrifuged at 2000 g for 10 min within 1 h of sampling and serum separated. Serum was frozen at − 80° until analysis. NETs were measured according to ELISA based protocol reported in pamphlet of kit "ELISA NET ELISA Kit (Glory Science Co. LTD, Shanghai, China). A Sandwich ELISA kit for quantitative determination of NET in serum using log/log curve determining absorbance value was used. Results were reported in milligrams per deciliter. ELISA data was interpreted in comparison to the standard curve (a serial dilution of a known concentration of purified antigen), and concentrations of NETs was calculated in various samples. The results above the linearity of the kit (4500 mg/dL) were diluted to achieve linearity, and results were multiplied by dilution factor. Kit sensitivity was 10 mg/dL.

Statistical analysis

The data were analyzed employing Microsoft Excel 2010 (Microsoft Corporation, Seattle, WA, USA) and the Statistical Package for Social Sciences version 22 (SPSS Inc. Chicago, IL, USA). The continuous variables are shown as the mean values ± SD, and the categorical variables are expressed in frequencies and percentages. In addition to the descriptive analysis, the unpaired *t*-test was done for normally distributed continuous variables, the Mann–Whitney test was applied for the non-normally distributed continuous variables, and chi-square test was utilized for the categorical variable.

Data availability

The data that support the findings of this study are readily available from the corresponding author, upon reasonable request.

Consent to participate

An informed consent was administered and signed by the patients before agreeing to be included in this study. The study and the consent were revised and approved by the ethics committee of the Ain Shams University (EMASU R 55/2021).

Results

The demographic and mean values of the variables of the study are shown in Table 1, with HCV being the most common etiology (78.2%).

Table 1 Etiology of chronic disease for the study group

Etiology	Number of cases	Percentage
HCV only	61	78.2%
Viral hepatitis and HCC	23	29.5%
Bilharzial	6	7.7%
HBV	4	5.1%
NASH	1	1.3%
Cardiac cirrhosis	1	1.3%
Undetermined etiology	9	11.5%

Child scoring system was calculated in all patients, with most patients having Child score B (52.6% of our patients).

Different complications occurring in patients of this study are shown in Table 2 with edema (ascites/lower limb edema) being the most reporting complication in our sample (93.6%).

Mean values of NETs in our sample was 630.4 mg/dL. It was found that there is no significant difference between NET levels among different etiologies or among different groups of child scoring system (Child A, B, or C). The results of Mann–Whitney test are shown in Tables 3 and 4.

However, when comparing to the occurrence of complications, it was found to be significantly correlated to the occurrence of malignant portal vein thrombosis with *p* value 0.035 with results shown in Table 5. The relation between NETs and different complications occurring in our patients are shown in Table 5.

Discussion

In this study, NETs were measured in plasma of 78 patients with liver cirrhosis with different etiologies and with or without complications. NETs levels were observed and compared to these different parameters.

Table 2 Percentages of liver cirrhosis complications reported in our study group

Complication	Number	Percentage
Spontaneous bacterial peritonitis	14	17.9%
Infections other than SBP	21	26.9%
Hepatic encephalopathy	27	35.1%
Edema (ascites/lower limb edema)	73	93.6%
Gastric congestion	56	73.7%
Esophageal varices	49 (12 requiring band ligation)	64.5% (40.8%)
Benign PVT	8	10.3%
Malignant portal vein hypertension	12	15.4%
Renal impairment	25	32.1%

Table 3 Relation between NET levels and etiology of chronic liver disease

	Number	NETs (mg/dL)	Mann–Whitney test		
		Median (IQR)	z	*p*-value	Sig
HCV	61 (78.2%)	320 (300–640)	−0.408	0.683	NS
Viral and HCC	23 (29.5%)	450 (300–1500)	−1.025	0.305	NS
Bilharzial	6 (7.7%)	900 (450–1200)	−1.229	0.219	NS
HBV	4 (5.1%)	240 (165–825)	−1.119	0.263	NS
Nash	1 (1.3%)	300 (300–300)	−0.714	0.475	NS
Cardiac cirrhosis	1 (1.3%)	320 (320–320)	−0.048	0.962	NS
UN-diagnosed	9 (11.5%)	300 (270–390)	−0.904	0.366	NS

More than 60% of our study group have liver cirrhosis due to chronic hepatitis C infection. This is most probably due to the high prevalence of hepatitis C-induced cirrhosis in Egypt [6], but there was no statistically significant difference between NETs levels and chronic viral hepatitis C. The role of NETs in viral hepatitis has not been clearly explained yet [7].

The comparison of NETs levels in various etiologies of liver cirrhosis has not yielded any significant results in our study population.

There was also no statistically significant difference in NETs levels in different Child score groups in our study. However, NETs were more elevated in cases in Child A than those with Child B and C. These results are similar to that found by Agraz-Cibrian et al., who found that NET release capability decreased with advanced liver disease [3] and postulated that it may explain high incidence of bacterial infections in cases of advanced liver cell failure [8].

In our study, there was no significant difference in NET levels in patients with concomitant spontaneous bacterial infection or elevated WBC and patients with no signs of infection. This can be explained by cirrhosis-associated immune deficiency, where continuous stimulation of the immune system may lead to immune cells exhaustion [9, 10]. Elevated CRP was not also found to be significantly related to NETs in our patients, but this may be due to the fact that CRP is a poor inflammatory indicator in LCF patients since its production may be hindered due to poor liver function [9]. An important factor to be taken in consideration is that the ability of neutrophils in a liver cirrhosis patient to perform NET release is not optimum; this was shown in in vitro studies on neutrophils of those patients [3].

Although NETs may propagate portal hypertension by promoting sinusoidal microvascular thrombosis raising portal hypertension volume and pressure [11], we did not found significant difference in NET levels among patients with and without portal hypertension.

Table 4 Relation between NET levels and Child score

		Number of subjects	NETs (mg/dL)	Kruskal–Wallis Test		
			Median (IQR)	H	*p*-value	Sig
Child score	A	6	910 (300–1650)	1.589	0.452	NS
	B	41	320 (300–470)			
	C	31	450 (300–770)			

Table 5 Relation between NETs and complication of the disease

	Number	NETs (mg/dL)	Test of significance		
		Median (IQR)	Value	*p*-value	Sig
SBP	14 (17.9%)	310 (300–1060)	$z = -0.014$	0.989	NS
Other infections	21 (26.9%)	320 (180–1060)	$z = -0.025$	0.98	NS
Hepatic encephalopathy	27 (35.1%)	450 (300–600)	$z = -0.398$	0.691	NS
Edema (ascites, LL edema)	73 (93.6%)	320 (300–630)	$z = -0.394$	0.693	NS
Esophageal varices	49 (64.5%)	450 (300–900)	$z = -1.158$	0.247	NS
Benign PVT	8 (10.3%)	1200 (300–1250)	$z = -1.494$	0.135	NS
Malignant PVT	12 (15.4%)	1050 (450–1500)	$z = -2.112$	0.035	S
Renal impairment	25 (32.1%)	385 (300–1060)	$H = 0.732$	0.694	NS

There was also a lack of significant difference in NET levels among patients with and without portal vein thrombosis in our study population. Although there is an established relation between NETs with thrombus formation. It may be argued that the event of thrombosis is LCF patients has other factors as key player in thrombosis. This is also seen in the study performed by Zenlander et al. and in Blasi et al., where NETs were not a factor in the activation of coagulation [5, 12].

The only significant difference in NET levels was found in Whaong patients with malignant portal vein thrombosis (HCC complicated by portal vein thrombosis). This is keeping with the thrombogenic effect NETs have in cancer-associated thrombosis [13–15].

Recently, there is growing evidence that patients with HCC has elevated levels of NETs, and it is thought that NETs have a role in cancer invasion and progression by eliciting an angiogenic process [7]. However, NET levels are not directly related to HCC (as a consequence to viral hepatitis) as an etiology of LCF; they have shown to have a correlation to malignant portal vein thrombosis as a complication that is specifically related to malignancy in our population.

Conclusion

In conclusion, we have demonstrated that NET levels in cirrhotic may not be a driving factor in most complications occurring in those patients. However, they may have a role when linking thrombosis and malignancy demonstrated by the significant difference in their levels found in malignant portal venous thrombosis in cirrhotic HCC patients. Serum NET levels should not be used as a biomarker when predicting complications occurrence in cirrhotic patients. Having a more careful look at the role of NETs in liver cirrhosis patients due to hepatic malignancy will result in understanding in the role of malignancy-associated thrombosis.

Abbreviations

CRP: C reactive protein; ELISA: Enzyme-linked immunosorbent assay; NETs: Neutrophil extracellular traps; LCF: Liver cell failure; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus.

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Authors' contributions

Both authors were actively involved in collection of data, sampling, procedures, statistical analysis, and manuscript writing. All authors read and approved the final manuscript sent for publishing.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Approval was obtained from the Ethics Committee of the Faculty of Medicine, Ain Shams University (FWA 000017585), number (EMASU R55/2021). Informed written consent was obtained from each participant before enrollment in the study. This study was performed in accordance with the 1975 principles of the Declaration of Helsinki and its appendices.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Tropical Medicine, Faculty of Medicine, Ain Shams University, Cairo 11591, Egypt. [2]Clinical Pathology Department, Faculty of Medicine, Ain Shams University, Cairo 11591, Egypt.

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