Increased ascites natural killer cells in patients with chronic hepatitis B cirrhosis using lamivudine: A pilot study

Kronik hepatit B'ye bağlı sirozda lamivudin kullanımı ile artan asit natural killer hücreleri: Pilot çalışma

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Background and Aims: *Natural killer cells play a direct role in liver injury and fibrogenic response. Peripheral blood natural killer cells have been studied widely in chronic liver diseases. We aimed to evaluate the ascites natural killer cell frequency and its significance in chronic hepatitis B related cirrhosis.* ***Materials and Methods:*** *Overall, 30 patients (23 males) with decompensated chronic hepatitis B cirrhosis with ascites were included. Patients with a recent ascites infection history and other etiologic factors besides chronic hepatitis B infection were excluded. After defining the demographic characteristics of the cases, we divided patients according to their Child-Turcotte-Pugh classification scores into two groups, and their natural killer (CD3–/CD16+/CD56+) cell frequencies in peripheral blood and ascites were studied using flow cytometry. Finally, we compared the natural killer cell frequencies in peripheral blood and ascites between the Child-Turcotte-Pugh classification groups based on lamivudine use.* ***Results:*** *Median lamivudine usage duration was 60 months, interquartile range 18.7–93 months in Child-Turcotte-Pugh B and 7.5 months, interquartile range 2.2–21 months in Child-Turcotte-Pugh C (p = 0.053). Ascites natural killer cells were significantly increased in lamivudine users of Child-Turcotte-Pugh B (p = 0.049), whereas no change was observed in peripheral blood - natural killer frequency in patients using lamivudine in the Child-Turcotte-Pugh B and C groups (p = 0.574 and p = 0.174, respectively).* ***Conclusion:*** *Long-term lamivudine use might have increased the ascites natural killer frequency, whereas no change was observed in the peripheral blood - natural killer cell frequency in patients with chronic hepatitis B cirrhosis, suggesting a potential role of antivirals in ascites natural killer cell response.*

Keywords: *Chronic hepatitis B, cirrhosis, ascites, natural killer cells, lamivudine*

Giriş ve Amaç: *Natural killer hücreler karaciğer hasarı ve fibrojenik cevapta doğrudan rol alırlar. Kronik karaciğer hastalıklarında periferik kanda natural killer hücreleri bir çok çalışmada araştırılmıştır. Amacımız asit natural killer sıklığını ve bunun kronik hepatit B'ye bağlı sirozdaki önemini araştırmaktı.* ***Gereç ve Yöntem:*** *Otuz erişkin (23 erkek) ardışık asitle dekompanse kronik hepatit B sirotik hasta çalışmaya alındı. Son zamanlarda asit infeksiyon öyküsü olanlar ve kronik hepatit B etiyolojisi dışında etiyolojik faktörü bulunan hastalar çalışmaya alınmadı. Olguların demografik özellikleri tanımlandıktan sonra Child-Turcotte-Pugh puanlarına göre iki gruba ayrıldılar. Bu olguların asit ve kan örneklerinde akım sitometrisi ile natural killer hücre sıklıkları araştırıldı. Daha sonra Child-Turcotte-Pugh B ve C grupları içinde lamivudine kullanımına göre natural killer hücre sıklıklarını karşılaştırdık.* ***Bulgular:*** *Lamivudin kullanım medyan süresi Child-Turcotte-Pugh B'de 60 ay, çeyrekler arası açıklık (18.7-93), Child-Turcotte-Pugh C'de 7.5 ay, çeyrekler arası açıklık (2.2-21) idi (p=0.053). Asit natural killer hücreleri Child-Turcotte-Pugh B grubunda lamivudin kullananlarda, kullanmayanlara göre belirgin olarak artarken (p=0.049), periferik kan natural killer hücrelerinde her iki grup arasında farklılık saptamadık (sırasıyla p=0.574 ve p=0.174).* ***Sonuç:*** *Uzun dönem lamivudin kullanımı kronik hepatit B'ye bağlı sirozda periferik kan natural killer hücre sıklığında değişiklik oluşturmamakta iken, asitte natural killer hücre sıklığını arttırmış olabilir. Bu durum asit natural killer hücrelerinin antivirallere cevapta potansiyel rollerinin olduğunu düşündürmektedir.*

Anahtar kelimeler: *Kronik hepatit B, siroz, asit, natural killer hücreleri, lamivudin*

INTRODUCTION

Hepatic stellate cell activation during liver injury is the crucial factor in the development of liver fibrosis through the secretion of fibrosis-related factors (1). Natural killer (NK) cells are the third lineage lymphoid cells of the innate immune system, albeit different from the T and B cells, which recognize and kill the infected cells (2). They play a direct role in liver injury and fibrogenic response. Additionally, they exert a protective role through fibrosis induction owing to their inhibitory effect on hepatic stellate cells (3,4). Peripheral blood (PB) NK cells in chronic liver diseases have been studied widely in recent decades; however, the ascites NK cell frequency and its significance in chronic liver diseases have not been studied yet. Several studies have evaluated the effects of antivirals on PB-NK cell activation in patients with chronic hepatitis B (CHB), albeit with ambiguous findings (5-8). To our knowledge, the role of oral antivirals on the ascites NK has not been evaluated in patients with CHB. Therefore, we planned to assess the NK frequency in a limited number of patients with decompensated CHB with ascites undergoing LAM treatment.

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MATERIALS and METHODS

This cross-sectional pilot study included 30 consecutive adult patients [23 males (M), with median age 60 years [interquartile range (IQR) 50.7–66.2 years] with cirrhosis and ascites who were hospitalized for palliation of ascites. These patients were selected among patients who agreed to participate in the study. Patients with a recent peritoneal infection history and other etiologic factors besides a CHB infection were excluded. Demographic data, disease duration, and antiviral treatment history were recorded from the hospital files. For laboratory examination, 20 ml of PB and ascites samples were collected from each patient. After removing 5 ml of PB and ascites sample for flow cytometry analysis, the remaining PB sample was used for biochemistry and complete blood count, and the remaining ascites sample was used to determine total protein, albumin, and leukocyte count. We grouped the patients according to their CTP scores. We then calculated the NK (CD3– -/CD16+/CD56+) frequencies in PB and ascites through flow cytometry. Finally, we compared the NK cell frequencies in PB and ascites between the CTP groups. The demographic and biochemical characteristics of PB and ascites, and the flow cytometry results are presented in Table 1.

Our study was approved by the Institutional Review Board and Ethics Committee. All patients were informed about the study and written informed consent was obtained from them.

Table 1. Patients' demographic, peripheral blood, and ascites biochemical characteristics and flow cytometry results in both CTP groups.

Characteristics	CTP B	CTP C	p
Patients (n), (F/M)	18 (4/14)	12 (3/9)	0.858
Age*	60 (52.2-67.2)	60 (50.2-65.5)	0.767
***Peripheral Blood** *			
T Bil (mg/dL)	1.2 (1.0–12.75)	2.8 (2.1–4.7)	**0.001**
AST (U/L)	46 (33.7–88.2)	70.5 (45.7–136.5)	0.086
ALT (U/L)	36.5 (25.5–51)	35.5 (26.7–70.5)	0.626
ALP (U/L)	91.5 (67.5–121.5)	118.5 (77.2–172.5)	0.099
GGT (U/L)	49 (30.7–85)	58 (33.5–106.5)	0.597
AFP (ng/dL)	3.2 (1.9–10.8)	3.5 (2.7–4.5)	0.916
T Pro (g/dL)	6.8 (5.9–7.6)	6.4 (5.2–7)	0.227
Alb (g/dL)	3 (2.4–3.6)	2.4 (2.2–2.6)	**0.025**
Wbc (number/mm^3)	4835 (3815–5950)	3690 (3200–7300)	0.498
Platelet (number/mm^3)	93.5 (71.2–144)	50.5 (41.2–114)	**0.032**
***Ascites Values** *			
T pro (g/dL)	0.9 (0.7–1.3)	0.5 (0.3–0.8)	**0.037**
Alb (g/dL)	0.4 (0.2–0.9)	0.3 (0.2–0.5)	0.125
SAAG	2.2 (1.8–2.7)	2.1 (1.9–2.4)	0.346
Wbc (number/mm^3)	200 (130–440)	290 (105–398)	0.498
Lymphocyte cellular markers*			
CD3 plasma %	69.9 (56.6–74.1)	58.7 (48.1–70.2)	0.236
CD16 plasma %	11.1 (7.3–15.5)	13 (7.5–24.4)	0.310
CD56 plasma %	8.2 (3.8–11.5)	13.8 (9.6–16.5)	**0.014**
CD3 ascites %	17.4 (6.9–29.9)	20.7 (8.1–24.1)	0.899
CD16 ascites %	28.5 (12.2–40.3)	23 (12.7–47.8)	0.799
CD56 ascites %	7.2 (3.7–12.6)	9.2 (3.8–12.2)	0.849
Plasma NK %	6.1 (3.7–10.1)	9 (6.8–15)	0.162
Ascites NK %	6.1 (2.7–9.7)	6.6 (3.1–10.1)	0.688

*Values are median, (IQR)

CTP: Child-Turcotte-Pugh classification, CTP B: Child-Turcotte-Pugh Class B, CTP C: Child-Turcotte-Pugh Class C, F: Female, M: Male, T. Bil: Total bilirubin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma-glutamyl transpeptidase, AFP: Alpha-fetoprotein, T. Pro: Total protein. Alb: Albumin, SAAG: Serum ascites albumin gradient; Wbc: White blood cells; NK (CD3–/CD16+/CD56+): Natural killer cells. Results expressed as median and IQR. IQR: Interquartile range. Mann-Whitney U test used for comparison of two groups.

PB and ascites samples were collected in tubes containing ethylenediaminetetraacetic acid and analyzed within the first 6 hours to keep the lymphocytes viable. Two tubes each were obtained of PB and ascites sample, with the first tube used as the control, and the second tube for assay. Cytofluorimetric analyses were performed using the EPICS-XL Coulter (Coulter Diagnostics, Hialeah, Florida, USA). The NK lymphocytes in the anticoagulated PB and ascites samples were determined per the manufacturer's instructions with the following modifications: 10 µL of immunoglobulin (Ig) G1 antibody (FITC), IgG1-PE, and IgG1-PC5 were added into the control tube; 10 µL of CD3PC5, CD16FITC, and CD56PE were added into the second tube; and 100 µL of blood or ascites were added to each tube. The PB containing tubes were treated using the ImmunoPrep reagent system and TQ-prep (Beckman Coulter) to lyse the red blood cells and stabilize the white blood cells. Approximately 20 µL of antihuman monoclonal antibodies, namely antiCD3 IgG1 (FITC), antiCD16 IgG1 (PE), and antiCD56 IgG1 (PE) were added to 100 µL solution containing mononuclear cells. The samples were then analyzed using flow cytometry. Lymphocyte populations were described according to the dot plot profiles based on the forward and side scatter properties. Finally, the fluorescence densities were analyzed according to the following defined gates: 10,000 cells counted for each sample. The NK cell population was defined as the percentage of CD3–/CD16+/CD56+ cells in the lymphocyte population.

Statistical analyses were performed using the IBM SPSS Statistics for Windows, version 25.0 (Armonk, NY, USA). Descriptive statistics of variables were expressed as median and IQR values. Because the sample size in groups was small, we could not investigate the normality assumption. Mann-Whitney U test was employed to compare CTP groups regarding variables and to compare LAM users with the untreated subjects. Correlations of the numerical variables were analyzed using Spearman's rank test. A two-tailed p value of <0.05 was accepted as statistically significant.

RESULTS

The demographic data and the laboratory results of the groups are presented in Table 1. The CTP B group comprised 18 patients (14 M) and the CTP C group 12 (9 M). The median age was 60 years in both groups with IQR of 52.2–67.2 and 0.2–65.5 years, respectively. Serum albumin (p = 0.025), platelets (p = 0.032), and ascites total protein (p = 0.037) were significantly higher, whereas total bilirubin (p = 0.001) was lower in the CTP B group.

Table 2 reveals the PB and ascites NK frequency based on LAM use in groups CTP B and C.

PB-NK frequency was not different among the groups based on antiviral use. In contrast, the ascites NK frequency was significantly higher in LAM users than nonusers in the CTP B group only (p = 0.049) (Figure 1).

No significant correlation was observed among PB-NK frequency, ascites NK frequency, total serum protein, and ascites total protein levels (all with p values >0.05). AST (p = 0.019, r = –0.886) and total bilirubin (p = 0.0420, r = –0.829) reversely correlated with the ascites NK cells in patients using LAM. In addition, the ascites NK frequency negatively correlated with total bilirubin level in patients using LAM (r = –0.653, p = 0.041).

Overall, 10 patients were using LAM at the time of inclusion in the study. Patients' characteristics are presented in Table 3.

Figure 2 reveals that the LAM usage duration was almost significant in CTP B and C groups (p = 0.053).

Moreover, we did not detect a significant correlation between the PB and ascites NK frequency with LAM use (p > 0.05).

Table 2. Natural killer cell frequencies in PB and ascites according to LAM use in CTP B and C.

		CTP B				CTP C			
		n	Median	IQR	*p*	n	Median	IQR	*p*
PB-NK *	LAM (–)	12	6.1	4.5–10.3	0.574	8	7.8	4.1–15.9	0.174
	LAM (+)	6	5.3	3.1–10.5	4	11.4	9–15		
Ascites NK*	LAM (–)	12	4.5	2.3–8.2	0.049	8	7.4	4.9–14.7	0.174
	LAM (+)	6	10.5	5.7–34.2	4	4.1	2.2–8		

*Values represent the percentage of NK cells in peripheral blood and ascites.

CTP B: Child-Turcotte-Pugh Class B, CTP C: Child-Turcotte-Pugh Class C, PB: Peripheral blood, NK: Natural killer cells. LAM (–): Not using lamivudine, LAM (+): Using Lamivudine, IQR: Interquartile range. Results expressed as median values with IQRs. Mann-Whitney U test used for comparing differences among LAM users and nonusers in CTP groups.

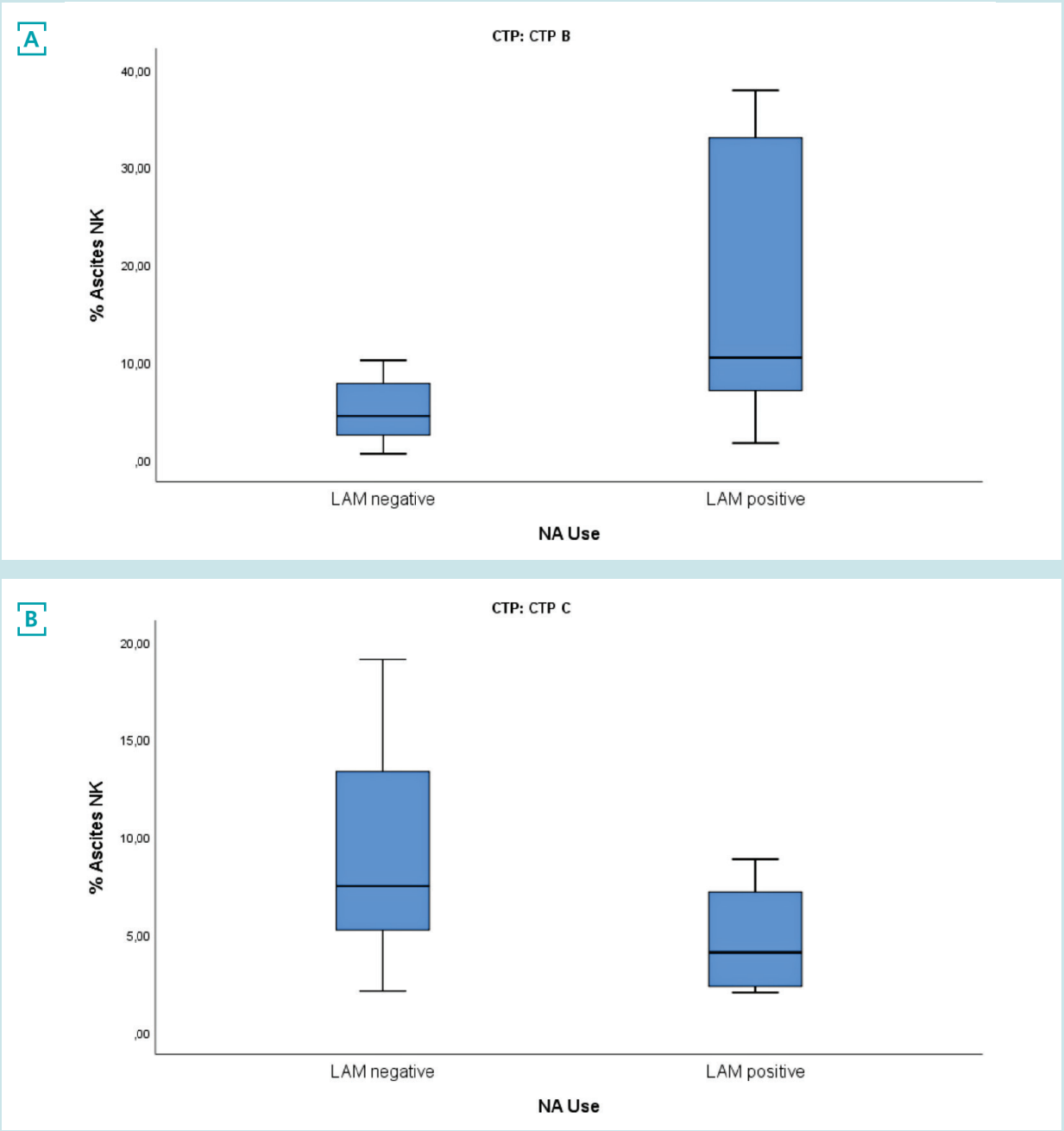

Figure 1. Ascites NK frequencies with LAM use in CTP B and C. **A.** Increased ascites NK frequency in LAM users of CTP B group. **B.** No significant difference in NK frequency with LAM use in CTP C.

NA: Nucleoside analogues, NK: Naturel killers, CTP B: Child-Turcotte-Pugh Class B, CTP C: Child-Turcotte-Pugh Class C, LAM: Lamivudine,

Table 3. Lamivudine use in CTP B and C.

Parameter	CTP B	CTP C	p
Lamivudine			
Users/Total*	6/18, (33.3)	4/12, (33.3)	0.545
Time (months)*	60, (18.7-93)	7.5, (2.2-21)	0.053*

*Values are n (%) or median, (IQR).

CTP B: Child-Turcotte-Pugh Class B, CTP C: Child-Turcotte-Pugh Class C,. IQR: Interquartile range.

*Mann-Whitney U test used for comparison of two groups.

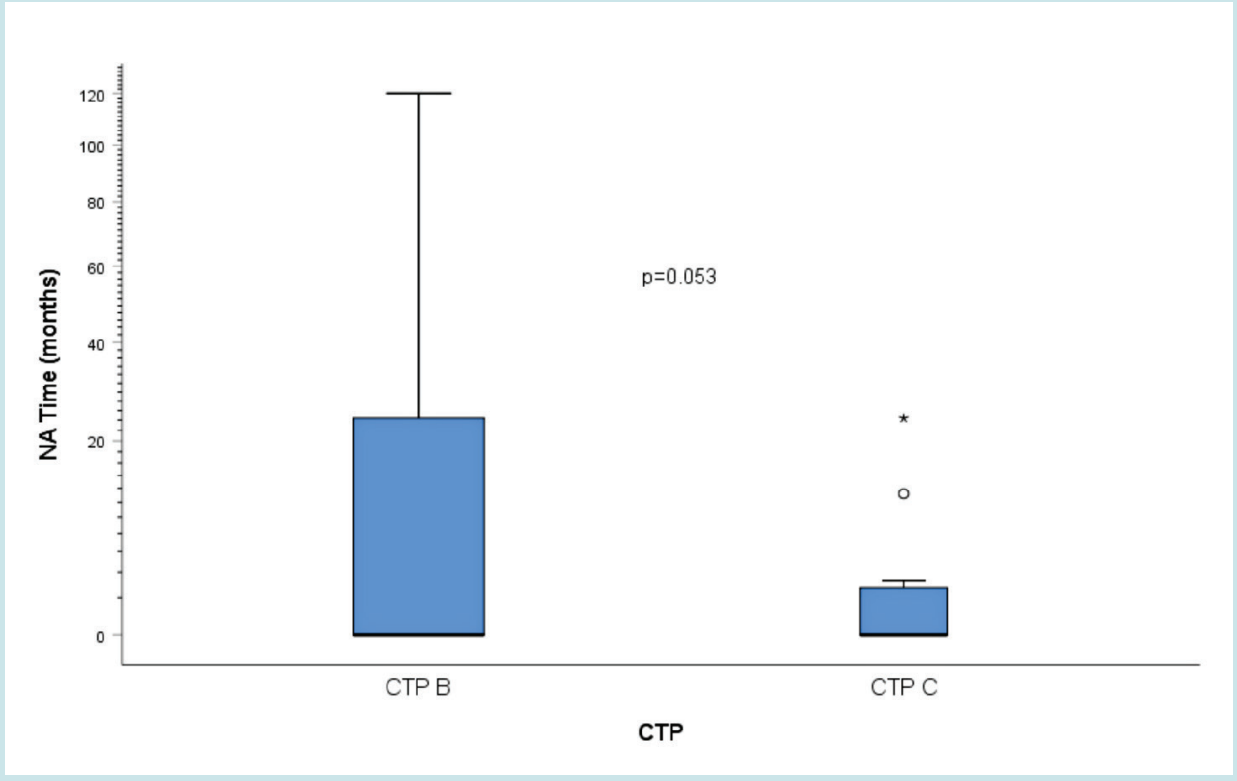

Figure 2. LAM usage duration of the groups.
NA: Nucleoside analogues, CTP B: Child-Turcotte-Pugh Class B, CTP C: Child-Turcotte-Pugh Class C, LAM: Lamivudine,

DISCUSSION

In the current study, we evaluated the role of LAM on the frequency of NK cells in the plasma and ascites of patients with decompensated CHB. Overall, 30 patients with decompensated CHB were divided into two groups based on their CTP score. Overall, 10 patients were on LAM treatment at the time of inclusion in the study. The median lamivudine usage duration was 60 months (IQR 18.7–93 months) in CTP B group, and 7.5 months (IQR 2.2–21 months) in CTP C. Lamivudine usage duration was significantly shorter in the CTP C group (p = 0.053) (Figure 2). We observed that ascites NK cell frequency was significantly higher in patients using LAM than the untreated patients in the CTP B group. However, we did not observe a significant increase in ascites NK frequency in patients using LAM in the CTP C group. This finding could probably be due to shorter LAM usage duration in the CTP C group. Furthermore, we observed beneficial effects of LAM on several laboratory parameters, such as lower AST (p = 0.015) and ALT (p = 0.007) in CTP B, lower bilirubin (p = 0.011) and ALP (p = 0.007) in CTP C, whereas the ascites albumin level (p = 0.036) was significantly higher in patients using LAM in CTP B group.

Ascites total protein concentration was reported to be related to a higher opsonic activity and survival rate in several studies (9-12). Serum and ascites total protein levels significantly correlated positively in patients using LAM (p = 0.036, r = 0.841). However, AST (p = 0.019, r = –0.886) and total bilirubin (p = 0.042, r = –0.829) exhibited negative correlation with the ascites NK cells in patients using LAM.

Based on the data presented in several studies, NK cells play a crucial role in fibrosis control and antiviral therapy, which improves PB-NK cell function (13-15). Moreover, the role of LAM and TNF on PB-NK cells was studied in patients with CHB (5,16). PB-NK cell frequency was observed to be lower in CHB and CHC patients than the healthy controls (3,16). In a study by Zheng et al. (17), NK cells were noted to migrate from the PB to the liver, thereby exacerbating hepatic injury in chronic HBV infection. This finding was also supported by another study wherein patients with CHB had a lower PB-NK frequency than healthy individuals (18). This finding was corroborated by another study that revealed LAM suppresses the hepatic

fibrogenesis (19). Oral antivirals were observed to be effective in restoring liver function and improving survival in chronic HBV decompensated liver cirrhosis (20). The regression of liver fibrosis was determined to be related to the antifibrotic activity of NK cells (21). Another study noted that the intrahepatic NKp46 high subset of NK cells was inversely associated with the stage of fibrosis (22). Several studies on HBV and HCV have attributed the effects of antiviral drugs on NK cell activation (23,24).

In the current study, PB-NK frequency was not significant in the two cirrhotic stages with or without LAM use (p = 0.574). However, we detected an increased ascites NK frequency with LAM use in CTP B (p = 0.049), whereas no significant difference was detected in CTP C with LAM use. Although not significant but close to the significance level, the CTP B LAM usage duration was longer than CTP C (p = 0.053). Therefore, this may be attributed to the relatively short-term LAM use in CTP C.

Kramer (22) reported an association between the increased AST, bilirubin, and the decreased intrahepatic NK cell frequency. Our study also detected significant inverse correlations of AST (p = 0.019, r = –0.886) and total bilirubin (p = 0.042, r = –0.829) with the ascites NK cell frequency. Nonetheless, we did not detect a significant correlation between the PB-NK and ascites NK cells with LAM treatment (p > 0.05).

Nevertheless, we acknowledge the small number of patients as the limitation of our study. To our knowledge, no previous study has assessed ascites NK cell frequency in long-term LAM users with CHB-related cirrhosis. Hence, we designed a pilot study to evaluate the frequencies of PB-NKs and ascites NKs in patients with decompensated CHB cirrhosis with or without antiviral use.

Notably, increased ascites NK cell frequency was observed in patients using long-term LAM with no change in PB-NK frequency, thereby suggesting a potential role of antivirals on ascites NK cells response.

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