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Immune changes over time and survival in patients with cirrhosis treated with non-selective beta-blockers: A prospective longitudinal study

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ABSTRACT

Background: Treatment with non-selective beta-blockers (NSBB) has been associated with anti-inflammatory and anti-cancer effects in patients with cirrhosis. This study aims to analyze the impact of chronic NSBB treatment on immune activation and disease progression in stable outpatients with cirrhosis.

Methods: In this prospective follow-up of 150 patients with cirrhosis, 39 received treatment with NSBB. Blood samples were taken every 6–9 months, and immune and adrenergic variables were measured. Mixed linear models were used to assess the effect of NSBB on these variables over time. Multivariate Cox regression was used to study associations with adverse clinical events (hepatocellular carcinoma, death, or liver transplant).

Results: Median follow-up was 1635 days. NSBB treatment was associated with significantly lower levels of IL-6 (β − 4.7; 95% confidence interval [CI] −6.9, −2.6) throughout the study. During follow-up, 11 patients developed hepatocellular carcinoma, 32 died, and 4 underwent liver transplant. Patients with higher concentrations of IL-10, IL-6 and IFN-γ developed more clinical events. Event-free survival was significantly better in patients treated with NSBB (hazard ratio 0.36, 95% CI 0.18, 0.71) in a multivariate Cox regression adjusted for Child-Pugh-Score, esophageal varices, and platelets.

Conclusion: Chronic treatment with NSBB in patients with stable cirrhosis gives rise to a different state of immune activation, characterized by lower concentrations of IL-6 over time, and it is associated with a reduced risk of adverse event (death, hepatocellular carcinoma, or transplant), after controlling for disease severity.

1. Introduction

Chronic inflammation of the liver is associated with remodeling, characterized by the accumulation of extracellular matrix protein and subsequent progression to fibrosis, cirrhosis, and occasionally hepatocellular carcinoma (HCC) [1]. Non-selective beta-blockers (NSBB) remain the main treatment for portal hypertension because of their efficacy in preventing variceal bleeding and improving survival [2]. The main mechanisms through which NSBB acts are the reduction of the heart rate and cardiac output via beta-1 blockade of cardiac receptors and the production of splanchnic vasoconstriction via peripheral beta-2 blockade, causing a reduction in portal inflow and pressure [3]. On the other hand, the ability of the NSBB carvedilol to reduce the activation of stellate cells, inflammation and the progression of fibrosis has been demonstrated in cirrhotic rat livers. [4,5]. Most hepatocellular carcinomas arise in the setting of chronic inflammation followed by compensatory liver regeneration, induction of liver fibrosis and subsequent cirrhosis, so their anti-inflammatory properties may make NSBB

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potentially active drugs for preventing hepatocellular carcinoma [6].

In this regard, observational studies have described lower rates of HCC [6–8] and infections [9] in patients with cirrhosis and esophageal varices chronically treated with NSBB. Further, numerous observational studies have shown lower rates of cancers other than HCC and better survival in patients treated with NSBB [10,11], although there is concern that these results could be biased by the retrospective nature of most studies and a possible competing risk and immortal time bias [12].

Experimental studies have found that activation of beta-adrenergic receptors expressed on tumor cells increases cell proliferation [13], whereas activation of receptors expressed on immune cells polarizes macrophages toward an M2 phenotype; impairs maturation, cytokine production, and antigen presentation by dendritic cells; suppresses NK cell activity; impairs the phagocytic efficiency of neutrophils; suppresses the function of CD8 + effector T cells; and directs the polarization of CD4 + T cells towards a Th2 phenotype [14], promoting cancer development and progression. These findings point to adrenergic signaling and its interaction with the immune system as a regulatory system potentially involved in HCC development and evolution and could explain the proposed protective effect of NSBB.

Our group has described a different immune activation profile in response to the presence of bacterial DNA in patients with cirrhosis treated with NSBB [15], along with an improved functionality of beta-adrenoceptors [16]. It is unclear whether these changes in immune system activation are associated with the progression of cirrhosis and the development of HCC. There is also no information about the temporal evolution of these NSBB-induced immune changes, as most studies have had a cross-sectional design. This study aims to analyze the impact of chronic treatment with NSBB on immune activation and disease progression in stable outpatients with cirrhosis.

2. Material and methods

We conducted an observational, prospective trial in a cohort of patients with cirrhosis included in the surveillance program for early detection of HCC at Alicante General University Hospital. Cirrhosis was diagnosed by histology or by clinical, laboratory, and/or ultrasound findings. Exclusion criteria were the presence of HCC, previous liver transplantation (LT), chronic use of immunosuppressants, HIV infection, and refusal to participate in the study. All research was conducted in accordance with both the Declarations of Helsinki and Istanbul. The Institutional Review Board of Alicante General University Hospital approved the study protocol (approval number – CEIC: PI2014/055), and all patients provided informed consent for inclusion in the study.

Follow-up and data collection: Outpatients with cirrhosis were included in the follow-up cohort upon enrollment in the surveillance program for early detection of HCC from 15 January 2015–4 December 2020. The date of HCC diagnosis, LT or death was considered the end of follow-up. The date of the last abdominal ultrasound showing no space-occupying lesions was considered the follow-up end time for censured data.

Blood samples and ultrasound examination of the liver were carried out every 6–9 months, coinciding with surveillance program visits. Serum samples were stored at − 80ºC until analysis. Drug prescription data at baseline and during follow-up were obtained prospectively from electronic hospital records and from personal testimony on each visit. Variables that change over time were also recorded at each visit.

Outcomes: The primary outcomes were the evolution over time of serum immune and adrenergic parameters according to NSBB treatment. A secondary composite outcome, event-free survival, considered HCC, all-cause death, and LT as events.

Assessment of systemic immune and adrenergic activity: Serum enzyme-linked immunosorbent assays (ELISAs) were performed to quantify TGF-β1, TNF-α, IFN-γ, IL-10 and IL-6 levels, using Human Quantikine kits (R&D Systems, Minneapolis, US), according to the manufacturer's instructions. All samples were tested in triplicate and read at 490 nm in a microplate reader. Lower detection limits of all cytokine assays were between 0.5 pg/mL and 5 pg/mL.

Plasma epinephrine (E), norepinephrine (NE) and dopamine (D) were determined using the 3-CAT RESEARCH ELISA (E-5600, Labor Diagnostica Nord GmbH & Co. KG, Nordhorn, Germany).

Nitric oxide (NO) levels in serum samples were calculated by measuring the conversion of nitrate to nitrite by the enzyme nitrate reductase, using an ELISA (KGE001, R&D Systems) based on the Griess reaction.

Peripheral blood mononuclear cells were prepared from 4 mL of ethylenediaminetetraacetic acid (EDTA) blood by Ficoll-Plaque (Pharmacia, Sweden) density gradient centrifugation (400 g, 18 °C, 30 min), washed in RPMI 1640 culture medium (Gibco, USA) twice, and then resuspended at a concentration of 2×10^6 cells/mL. A panel of markers including CD3+, CD8+, CD4+ and CD16+ was used to identify lymphocytes. Cell samples were assayed by a FACS Caliber flow cytometer (BD Bioscience), and the acquired data were further analyzed using FlowJo software. Flow cytometric results are reported as follows: CD3+ and CD3-CD16+ cells are reported as percentage of the total lymphocyte population, selected based on forward scatter and side scatter parameters. CD3+CD4+ and CD3+CD8+ are reported as percentages of the total CD3+ population.

Statistical analysis: The normality of all continuous variables was assessed using the Shapiro–Wilk test in the NSBB-treated group (N = 39) and the Kolmogorov Smirnov test in the untreated group (N = 111). Normally distributed variables are reported as mean and standard deviation (SD) while non-parametric variables are presented as median and interquartile range (IQR). Categorical variables are described with absolute and relative frequencies. Statistical differences in baseline characteristics between groups were analyzed using the chi-squared test for categorical data and the t-test or, when data were not normally distributed, the Mann-Whitney-Wilcoxon test.

The evolution of immune and adrenergic parameters over time according to NSBB treatment was evaluated using linear mixed-effects models with sampling time as a random-effects variable. Individual linear mixed-effects models were calculated for variables TGF-β1, IL-6, IL-10, IFN-γ, E, NE, D, NO, and percentages of CD3+, CD4+, CD8+, and CD3-CD16+ cell counts. For each variable, five different linear mixed-effects models were calculated, with an increasing number of independent variables associated with the prognosis of cirrhosis or differences between patients according to NSBB treatment at baseline: Baseline, Risk Factors 1, Portal Hypertension, Other Treatments, and NSBB models. This approach was implemented to avoid overfitting models with too many parameters.

In a first step, the variability of each parameter was explained by the differences in extraction times as random-effects variables (Baseline model). Then, the Risk Factors 1 model was applied, adding the variables sex, age, etiology, and creatinine to the information provided by baseline model information. The two models were compared for goodness-of-fit using the likelihood ratio test statistic, with a chi-squared distribution and degrees of freedom equal to the number of extra parameters in the more complex model. The resulting best-fit model was then compared with the Portal Hypertension model, which added the variables for esophageal varices, Child-Pugh score, and platelet count. This process was repeated successively with the Other Treatments model, which additionally considers diuretics, statins, and proton pump inhibitors (PPI), and with the NSBB model, which adds a variable for NSBB treatment.

All models were two-level growth models with random intercepts and slopes. Bonferroni's correction for multiple comparisons was used to guarantee the actual type I error probability. NSBB, diuretics, statins, PPI, Child-Pugh score, and platelet count were introduced as time-dependent variables.

Event-free survival was analyzed using five Cox proportional hazards regression models (Null, Risk Factors 1, Portal Hypertension, Other Treatments, and NSBB models) using a similar strategy to that with the

linear mixed-effects models described above. Sex, age, and etiology were included in models as time-independent variables, and NSBB, Child-Pugh score, platelet count, diuretics, statins, and PPI treatment as time-dependent covariates.

All reported p values are two-sided, and p values of less than 0.05 were considered to indicate significance. All analyses were carried out in R software (Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/).

3. Results

Patients. From 15 January 2015–29 March 2016, 170 patients with cirrhosis were initially considered for inclusion. Twenty were finally excluded: 19 for receiving selective beta-blockers, and 1 for receiving chemotherapy treatment shortly after inclusion in the study. At baseline, 39 patients (26%) were receiving NSBB treatment, all with propranolol at a dose of 20 mg/day to 120 mg/day (mean dose of 60 mg/day). Table 1 details patients' baseline characteristics according to NSBB treatment. Significantly, patients treated with NSBB at baseline were more likely to have esophageal varices; higher Child-Pugh score, creatinine, and INR levels; and reduced serum albumin and platelet counts, suggestive of increased portal hypertension. There were also significant differences between groups in terms of chronic treatments other than NSBB, such as statins, diuretics, and PPI.

Median follow-up in the untreated group of patients was 1635 days (IQR 630), similar to that observed in the NSBB group (1622 days, IQR 984.5; $p = 0.58$). In the latter group, median time on NSBB treatment before study entry was 1376 days (IQR 1345). Nine patients treated with NSBB at baseline stopped treatment at some point during follow-up, while 10 patients started treatment with NSBB during their participation in the study. During follow-up, there were 20 episodes of decompensation related to portal hypertension in the NSBB group (6 ascites, 4 encephalopathies, and 10 upper gastrointestinal bleeding) and 28 episodes in the non-NSBB group (14 ascites, 9 encephalopathies and 5 upper gastrointestinal bleeding).

Baseline immune and adrenergic status. At baseline, the NSBB group showed significantly lower serum levels of TGF-β1 (median 511.6 pg/mL, IQR 340.5) compared with the non-NSBB group (median 681.9 pg/mL, IQR 705.3; $p < 0.05$), as shown in Table 2. Serum values of the remaining cytokines and NO were similarly low in both groups. There were no differences in the CD3 + , CD3 +CD8 + , CD3 +CD4 + and CD3-CD16 + cell counts between groups at baseline (Table 2). Patients treated with NSBB showed significantly higher levels of serum catecholamines than the non-NSBB group (Table 2).

Longitudinal analysis of immune and adrenergic changes. During the first three years of follow-up, a mean 2.0 (SD 1.3) blood samples per patient were obtained. The first sample was obtained at baseline and the following samples every 6–9 months. One hundred fifty patients had at least 1 sample, 104 patients had 2 samples, 60 had 3, 25 had 4, 11 had 5, 3 had 6, and 2 had 7. Linear mixed models that included the portal hypertension variables explained the variability over follow-up in values for TGF-β1, IL-6, NE and CD3+ cells significantly better than Baseline and Risk Factors 1 models (Table 3). Specifically, TGF-β1 was directly and significantly associated with platelet count, while IL-6 and NE were associated with the Child-Pugh score (Table 3). CD3 + counts were inversely associated with esophageal varices and Child-Pugh score.

The inclusion of the NSBB variable in linear mixed models increased the ability of the models to explain the variability in IL-6 over time (Table 3). IL-6 was inversely associated with NSBB treatment (IL-6: β −4.7, 95% CI −6.9 to −2.6), with lower values throughout the entire follow-up in patients treated with these drugs (Fig. 1). No other immune or adrenergic variables showed differences between Portal Hypertension and NSBB models. Estimates of all variables analyzed using linear mixed models are shown in Table S1.

Event-free survival and immune and adrenergic changes over time in

Table 1
Baseline clinical and demographic characteristics of patients according to treatment with non-selective beta-blockers (NSBB).

	Non-NSBB (N = 111)	NSBB (N = 39)	Statistical test	df	p
Men	73 (65.8%)	26 (66.7%)	$\chi^2 = 0.01$	1	0.92
Age in years	59.0 [13.0]	64.0 [14.0]	U = 1818.5		0.14
Etiology					
Alcohol	49 (44.1%)	19 (48.7%)	$\chi^2 = 1.036$	3	0.79
Alcohol + virus	12 (10.8%)	5 (12.8%)			
Virus (HCV, HBV)	32 (28.8%)	8 (20.5%)			
Others	18 (16.2%)	7 (17.9%)			
Esophageal/gastric varices	54 (48.6%)	31 (79.5%)	$\chi^2 = 9.9566$	1	0.002
Medical history					
Upper gastrointestinal bleeding	19 (17.1%)	12 (30.8%)	$\chi^2 = 2.5009$	1	0.11
Ascites	44 (39.6%)	20 (51.3%)	$\chi^2 = 1.1586$	1	0.28
Encephalopathy	8 (7.2%)	4 (10.3%)	$\chi^2 = 0.0679$	1	0.79
Child-Pugh category					
A	93 (83.8%)	18 (46.2%)	$\chi^2 = 23.168$	2	< 0.001
B	15 (13.5%)	20 (51.3%)			
C	3 (2.7%)	1 (2.6%)			
Child-Pugh score	5.0 [1.0]	7.0 [1.0]	U = 648		< 0.001
Creatinine (mg/dL)	0.8 [0.2]	0.9 [0.2]	U = 1683.5		0.039
Urea (mg/dL)	29.0 [11.5]	31.0 [15.0]	U = 1930.5		0.59
ALT (U/L)	22.0 [16.0]	23.0 [15.0]	U = 2095		0.86
AST (U/L)	29.0 [23.0]	30.0 [13.0]	U = 1880.5		0.44
Bilirubin (mg/dL)	0.8 [0.6]	1.0 [1.2]	U = 1758		0.081
Albumin (g/dL)	3.8 [0.6]	3.5 [0.6]	U = 2838.5		0.004
INR	1.1 [0.2]	1.2 [0.3]	U = 1479.5		0.003
AFP (IU/mL)	2.7 [2.5]	2.8 [2.2]	U = 1986.5		0.98
Cholesterol (mg/dL)	166.9 ± 42.6	160.6 ± 38.7	t = 0.8217	148	0.41
Hemoglobin (g/dL)	13.8 [2.8]	13.3 [2.2]	U = 2312.5		0.25
Erythrocytes ($\times 10^6/\mu L$)	4.6 [1.0]	4.5 [1.0]	U = 2283		0.31
Leukocytes ($\times 10^3/\mu L$)	5.7 [2.4]	5.0 [1.7]	U = 2430		0.096
Platelets ($\times 10^3/\mu L$)	128.0 [85.5]	97.0 [51.0]	U = 2898.5		0.002
Previous time with NSBB (days)	———	1376.0 [1345.0]			
Follow-up time (days)	1635.0 [630.0]	1622.0 [984.5]	U = 2319.5		0.51
Other chronic treatments					
Statins	10 (9%)	10 (25.6%)	$\chi^2 = 5.5443$	1	0.019
Metformin	18 (16.2%)	5 (12.8%)	$\chi^2 = 0.0615$	1	0.80
Diuretics	33 (29.7%)	19 (48.7%)	$\chi^2 = 4.0463$	1	0.04
PPI	27 (24.3%)	17 (43.6%)	$\chi^2 = 4.2799$	1	0.039
Lactulose (on enrolment)	15 (13.5%)	1 (2.6%)	$\chi^2 = 2.5729$	1	0.11

ALT: alanine aminotransferase; AST: aspartate aminotransferase; INR: international normalized ratio; AFP: alpha-fetoprotein; PPI: proton pump inhibitor. Normally distributed continuous variables are reported as mean ± standard deviation while non-normally distributed are presented as median [interquartile range]. Categorical variables are expressed as frequency (percentage). χ2: chi-square statistic; U: Mann-Whitney U statistic; t: t test statistic; df: degrees of freedom

Table 2
Baseline immune and adrenergic variables according to treatment with non-selective beta-blockers (NSBB).

	Baseline data				
	Non-NSBB (N = 111)	NSBB (N = 39)	Statistical test	df	p
Serum catecholamines					
Dopamine (pg/mL)	50.8 [96.7]	82.3 [85.9]	U = 1472.5		0.020
Epinephrine (pg/mL)	135.6 [35.8]	154.8 [51.9]	U = 1163		< 0.001
Norepinephrine (pg/mL)	411.4 [337.7]	567.7 [472.8]	U = 1515		0.034
Serum cytokines					
TGF-β1 (pg/mL)	681.9 [705.3]	511.6 [340.5]	U = 2460.5		0.046
TNF-α (pg/mL)	0.9 [0.4]	1.0 [0.5]	U = 1927.5		0.31
IL-10 (pg/mL)	5.3 [6.0]	5.9 [5.4]	U = 1989.5		0.50
IFN-γ (pg/mL)	1.1 [3.7]	0.7 [2.8]	U = 2052		0.99
IL-6 (pg/mL)	4.1 [5.6]	4.7 [8.0]	U = 1766		0.37
Serum NO (μmol/L)	37.4 [40.2]	45.1 [73.0]	U = 966		0.59
Blood immune cells					
CD3+ (% of lymphocytes)	67.7 ± 11.2	66.1 ± 9.1	t = 0.7621	125	0.45
CD3-CD16+ (% of lymphocytes)	9.5 [10.6]	8.9 [11.9]	U = 1021		0.96
CD3+CD4+ (% of CD3+)	63.9 ± 10.7	59.4 ± 15.5	t = 1.3546	124	0.18
CD3+CD8+ (% of CD3+)	27.5 [16.0]	27.1 [22.3]	U = 884		0.33

Normally distributed continuous variables are reported as mean ± standard deviation while non-parametric variables are presented as median [interquartile range]. Categorical variables are expressed as frequency (percentage). χ2: chi-square statistic; U: Mann-Whitney U statistic; t: t test statistic; df: degrees of freedom

patients with cirrhosis and NSBB treatment. During follow-up, 11 patients developed HCC, 32 died, and 4 underwent LT. A Cox proportional-hazards model including Portal Hypertension variables (Child-Pugh-Score, esophageal varices, and platelets) and NSBB was the best fit model associated with event-free survival. Patients with esophageal varices and those who maintained high Child-Pugh scores throughout the study showed an increased risk, while treatment with NSBB reduced it (Table 4 and Fig. 2A).

High values of IL-10, IFN-γ and IL-6 throughout the study were associated with an increased risk of events after controlling for Child-Pugh-Score, esophageal varices, platelets and NSBB (Fig. 2B, C and D). None of the remaining immune and adrenergic variables were associated with event-free survival, as shown in Table S2. There were also no significant associations between the studied variables and the risk of developing HCC or death, probably due to the loss of power because of the reduced number of events when each of these variables is studied separately.

4. Discussion

Patients with cirrhosis and esophageal varices chronically treated with NSBB have shown lower rates of HCC and infections in observational studies [6,7,9,17] and meta-analyses of secondary variables collected in randomized controlled trials [8]. These effects agree with experimental data suggesting a role for NSBB in modulating systemic and liver inflammation through adrenergic block, which is sufficient to modify disease progression and reduce the number of complications and cancer [13,14]. However, the protective effect of NSBB in patients with cirrhosis has been questioned due to the observational and retrospective design of most studies and the existence of confounders [12].

In our study, patients treated with NSBB showed a reduced risk of developing an event (HCC, death, or LT) in a Cox regression controlled for confounding time-dependent covariates related to severe disease and portal hypertension, such as platelets count, Child-Pugh score, and esophageal varices. This control is essential if we consider the strong association between these variables and the development of events such as death, LT or HCC, and the significant differences in these variables observed in our sample treated with NSBB.

Similarly, variability in TGF-β1, IL6, NE and CD3+ levels over follow-up was better explained if portal hypertension-related variables were included in the model. Correlations between TGF-β1 levels and platelet count in patients with esophageal varices have been previously reported in cross-sectional studies [18]. Moreover, we observed that

Table 3
Summary table for the best-selected linear mixed models for immune and adrenergic parameters measured throughout the follow-up period.

Variable	Best model	Significant variables	β (95% confident interval)	Log likelihood	AIC	P
TGF-β1	Baseline			− 2401.5	4812.9	
	PH	Sample extraction time	− 38.0 (−69.5, −6.6)	− 2336.2	4688.3	< 0.0001
		Platelets	5.3 (4.5, 6.2)			
IL-6	Baseline			− 1038.5	2086.9	
	RF1	Creatinine	4.2 (2.7, 5.7)	− 1021.3	2064.5	
	PH	Creatinine	4.5 (3.2, 5.8)	− 991.8	2011.5	
		Child-Pugh score	3.3 (2.5, 4.1)			
	NSBB	Creatinine	4.7 (3.5, 5.8)	− 983.0	1995.9	< 0.0001
		Child-Pugh score	3.8 (3.0, 4.6)			
		NSBB	− 4.7 (−6.9, −2.6)			
NE	Baseline	Sample extraction time	− 34.04 (−63.75, −4.33)	− 2031.9	4073.8	
	PH	Sample extraction time	− 29.87 (−58.21, −1.53)	− 2009.1	4034.3	< 0.0001
		Child-Pugh score	111.30 (77.62, 144.98)			
		Platelets	0.77 (0.06 – 1.49)			
CD3+	Baseline	Sample extraction time	1.7 (0.9, 2.5)	− 843.6	1697.1	
	PH	Sample extraction time	1.5 (0.7, 2.3)	− 836.7	1689.4	0.0033
		Child-Pugh score	− 1.6 (−3.0, −0.3)			
		Esophageal varices	− 3.5 (−6.9, −0.0)			

NE: norepinephrine, CD3+: lymphocytes CD3+; Baseline model included time; Risk Factors 1 model (RF1) adds the variables sex, age, etiology, and creatinine to the baseline model; Portal Hypertension model (PH) adds the variables esophageal varices, Child-Pugh score, and platelet count; non-selective beta-blockers model (NSBB) adds the variable NSBB. Models were applied sequentially step by step to each dependent variable, comparing each model with the one selected in the previous step as the best model according to the Likelihood ratio test statistic and a chi-squared distribution with degrees of freedom equal to the number of extra parameters in the more complex model. The table shows for each variable the results of the baseline and successive significant linear mixed effects model. Coefficients and 95% confidence interval of model significant variables are shown. Log likelihood and Akaike (AIC) values are shown to indicate the goodness of fit for each model.

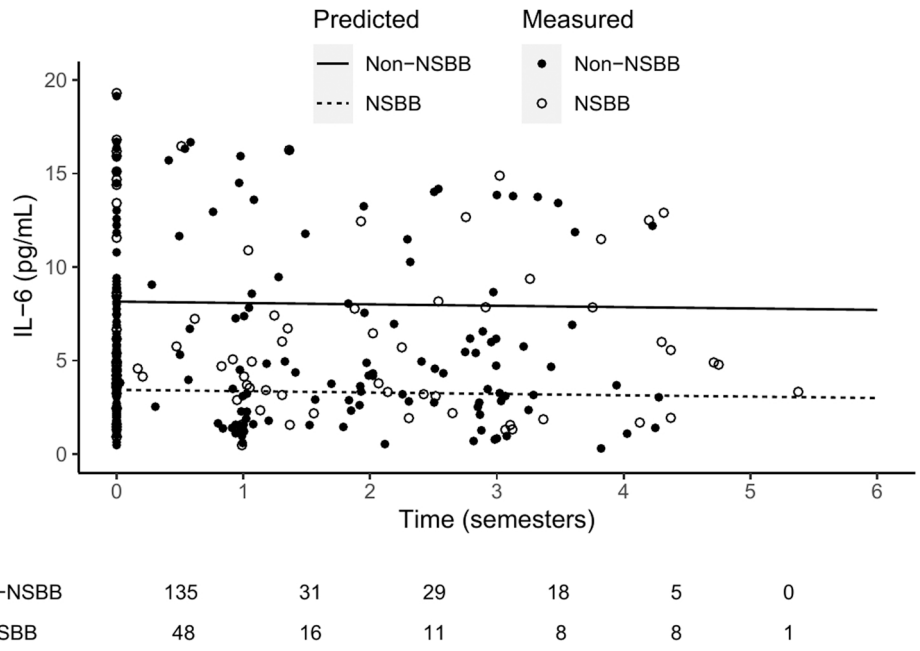

Fig. 1. Observed and predicted IL-6 levels throughout the study in patients treated (white points and dashed lines) or not treated (black points and continuous lines) with non-selective beta-blockers (NSBB). The number of samples per semester by NSBB treatment group are indicated at the middle of each semester.

Table 4
Summary table for the best fitted Cox regression model for event-free survival:.

Cox regression models	Log-likelihood	df	P value vs. previous significant model (*)
Null model	− 217.84		
RF1 model	− 210.72	6	0.027
PH model	− 198.75	3	< 0.001 * **
PH + OT model	− 198.17	3	0.76
PH + NSBB model	− 194.13	1	0.002 * *
PH + NSBB model variables	**Event-free survival HR (95% CI)**	**P value**	
Child-Pugh Score (number)	1.81 (1.45, 2.26)	< 0.001 * **	
Esophageal varices (yes)	2.92 (1.40, 6.07)	0.004 * *	
Platelets ($\times 10^5$/μL)	0.62 (0.35,1.12)	0.11	
NSBB treatment (yes)	0.36 (0.18, 0.71)	0.003 * *	

CI: confidence interval; df: degrees of freedom; HR: hazard ratio; NSBB: non-selective beta-blockers model; OT: Other Treatments; PH: Portal Hypertension. Risk Factors 1 model (RF1) includes the variables sex, age, etiology, and creatinine; PH model adds the variables esophageal varices, Child-Pugh score, and platelet count; OT mode adds the variables diuretics, statins, and PPI; NSBB model adds the variable NSBB. The five models were applied sequentially step by step, comparing each model with the one selected in the previous step as the best model according to the Likelihood ratio test statistic and a chi-squared distribution with degrees of freedom equal to the number of extra parameters in the more complex model.

changes in IL-6, NE and CD3+ levels were associated with changes in the Child-Pugh score. These longitudinal data agree with results of cross-sectional studies associating increased IL-6 levels with Child-Pugh, MELD and Clif-SOFA scores [19,20] and high NE levels with advanced disease and portal hypertension [21].

Changes in TGF-β1 and NE levels occur in parallel to disease progression, regardless of NSBB treatment. However, the change in IL-6 is much better explained if NSBB treatment is considered in addition to disease progression. This long-term immunomodulatory effect on IL-6 of NSBB is consistent with previous studies showing a reduction of IL-6 levels in cirrhotic patients treated with NSBB [22]. Some authors have suggested that NSBB could act indirectly by reducing intestinal permeability and bacterial translocation episodes and directly by reducing IL-6 production [23] through a direct effect on IL-6-producing cells [24].

Persistently high values of IL-6, IFN-γ and IL-10 were associated with an increased risk of events in time-dependent, multivariate Cox regressions controlling for the time-dependent covariates Child-Pugh score, platelet count, and NSBB treatment and the time-independent covariate esophageal varices. The hazard ratio for IL-6 was over 1, although the value did not reach statistical significance (1.25, 95% CI 0.93, 1.69), probably because treatment with NSBB and portal hypertension variables show a strong and inverse association with event-free survival on the one hand, and with serum IL-6 levels on the other. Given the complexity of these interactions, we cannot rule out that the protective effect of NSBB could, at least in part, be exerted through a chronic modulatory effect of IL-6. Previous studies have described elevated levels of IFN-γ in decompensated compared to compensated patients in response to bacterial products [25]. Similarly, serum IL-10 was higher in patients with chronic hepatitis C, showing a significant association with disease progression [26].

Taken together, these data suggest that in people with cirrhosis, NSBB reduces chronic inflammation, as characterized by high serum levels of IL-6 and other cytokines. The treatment would act through two complementary mechanisms: indirectly, as a consequence of its portal pressure-reducing hemodynamic effects, and directly, by reducing the production of cytokines by immune cells. This modulation of chronic inflammation would contribute to the reduction of morbidity and mortality observed in these patients.

Our work has several limitations, starting with its observational nature, although the prospective design reduces the inherent limitations of retrospective and cross-sectional studies. However, the existence of a selection bias cannot be excluded since the patients treated with NSBB have more advanced disease. In our study, this bias has been controlled by including the variables associated with greater portal hypertension and disease severity in the statistical analyses, although this strategy is not without limitations. The only way to safely eliminate this bias would be to perform a randomized placebo-controlled clinical trial, which is not feasible because it is unethical to use placebo in patients with cirrhosis requiring NSBB.

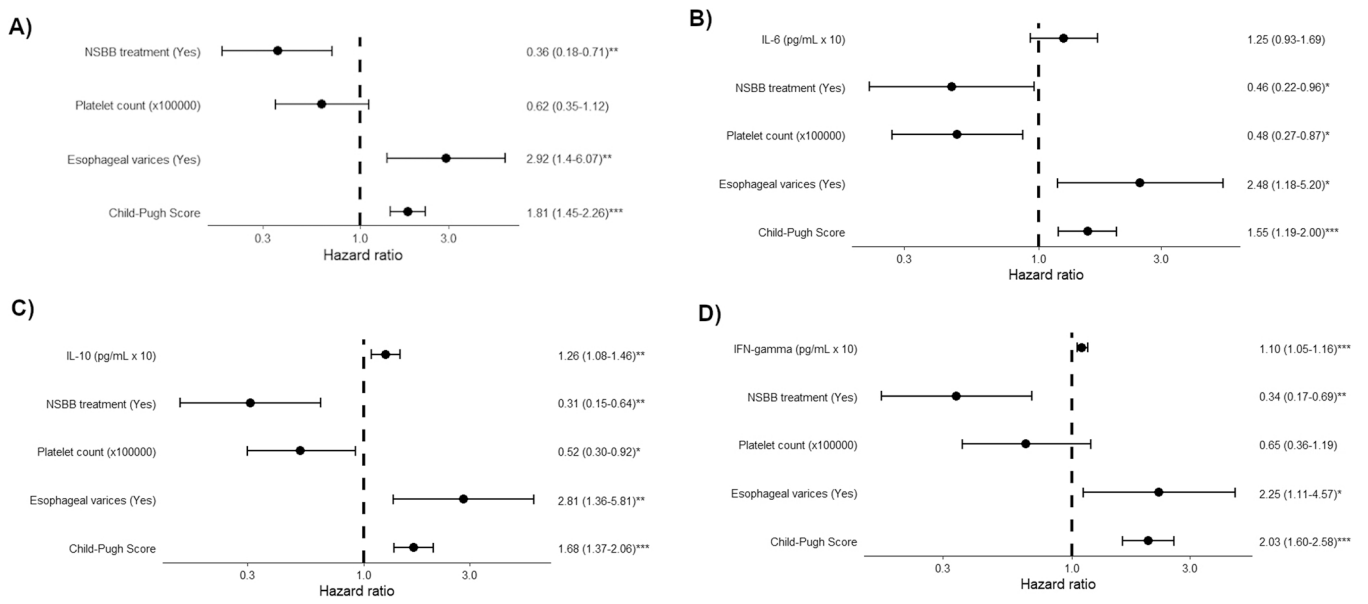

Fig. 2. Forest plot of multivariate Cox regression analysis for event-free survival non-including immune parameters (A), and including IL-6 (B), IL-10 (C), and interferon-gamma (IFN-gamma) (D) as time-dependent covariates. Events are defined as any hepatocellular carcinoma (HCC), all-cause mortality, or liver transplant (LT).

Another limitation comes from the fact that patients with cirrhosis may adhere poorly to treatment [27]. In our study, adherence to NSBB was assessed using electronic records, where prescription is automatically inactivated when patients do not pick up the medication at the pharmacy. This measure allowed us to identify the periods of non-adherence and to include drug treatment as a time-dependent variable in the statistical analysis. Finally, in the survival analysis there is a possibility of competing risk bias, as HCC would not occur if the patient dies prematurely, as may be expected in NSBB-treated patients that suffer from more advanced disease. To control for this bias, we performed an analysis incorporating the death event in the outcome, rather than considering it as censored [28]. This approach prevents us from studying HCC, death, or transplantation separately.

In conclusion, chronic treatment with NSBB in patients with stable cirrhosis gives rise to a different state of immune activation, characterized by lower concentrations of IL-6 over time, and it is associated with a reduced risk of suffering an adverse event (death, HCC, or transplant), after controlling for disease severity and concomitant treatments.

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CRediT authorship contribution statement

All authors contributed to the study conception. Material preparation and data collection were performed by Susana Almenara, Ivan Herrera, Cayetano Miralles, Pablo Bellot, Maria Rodriguez, Jose M. Palazon, Sonia Pascual y Pedro Zapater. Laboratory determinations were performed by Susana Almenara, Beatriz Lozano-Ruiz, Paula Gimenez, Favian Tarin, Hector Sarmiento, Ruben Frances, Jose M. Gonzalez-Navajas and Pedro Zapater. Statistical analysis was performed by Susana Almenara and Pedro Zapater. The first draft of the manuscript was written by Susana Almenara and Pedro Zapater and all authors commented on versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114885.

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