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Hepatology International

ISSN 1936-0533
Volume 14
Number 5

Hepatol Int (2020) 14:858-868
DOI 10.1007/s12072-020-10083-5

Springer

Hepatology International (2020) 14:858–868
https://doi.org/10.1007/s12072-020-10083-5

ORIGINAL ARTICLE

Functionality of beta-adrenergic receptors in patients with cirrhosis treated chronically with non-selective beta-blockers

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Received: 13 June 2020 / Accepted: 17 August 2020 / Published online: 3 September 2020

Abstract

Background In patients with cirrhosis, beta-adrenoceptors expressed on peripheral blood mononuclear cells have a reduced response to catecholamine stimulation. This study aimed to determine if chronic treatment with beta-blockers influences these changes.

Methods Blood samples were collected from patients with cirrhosis treated in outpatient clinics. Differences in cyclic AMP production before and after stimulation of mononuclear cells with epinephrine and/or *N*-Formylmethionine-leucyl-phenylalanine (fMLP) was used as a marker of beta-adrenoceptors activity in patients treated ($N=19$) versus not treated ($N=55$) with beta-blockers. In addition, we studied the gene expression of different types of adrenoceptors and possible associations with the activity of beta-adrenoceptors, the serum concentrations of catecholamines and cytokines, and the presence of bacterial antigens such as DNA or gram-negative bacterial endotoxin in patients' blood.

Results The increase in intracellular cAMP concentrations after stimulation of adrenergic receptors with epinephrine was significantly higher in samples from patients treated with beta-blockers. Older patients showed lower responses to epinephrine stimulus, while the response increased linearly with the duration of the beta-blocker treatment. mRNA expression levels of adrenoceptors β1, β2, β3 and α1-A, B and D showed no significant differences according to treatment with beta-blockers. Neither serum cytokines nor catecholamines levels were significantly associated with the intracellular production of cAMP after adrenergic stimulation.

Conclusion Chronic treatment with beta-blockers in patients with cirrhosis enables beta-adrenoceptors to respond to catecholamine stimulation irrespective of the degree of systemic adrenergic or immune activations of the patient at the time of sampling.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12072-020-10083-5) contains supplementary material, which is available to authorized users.

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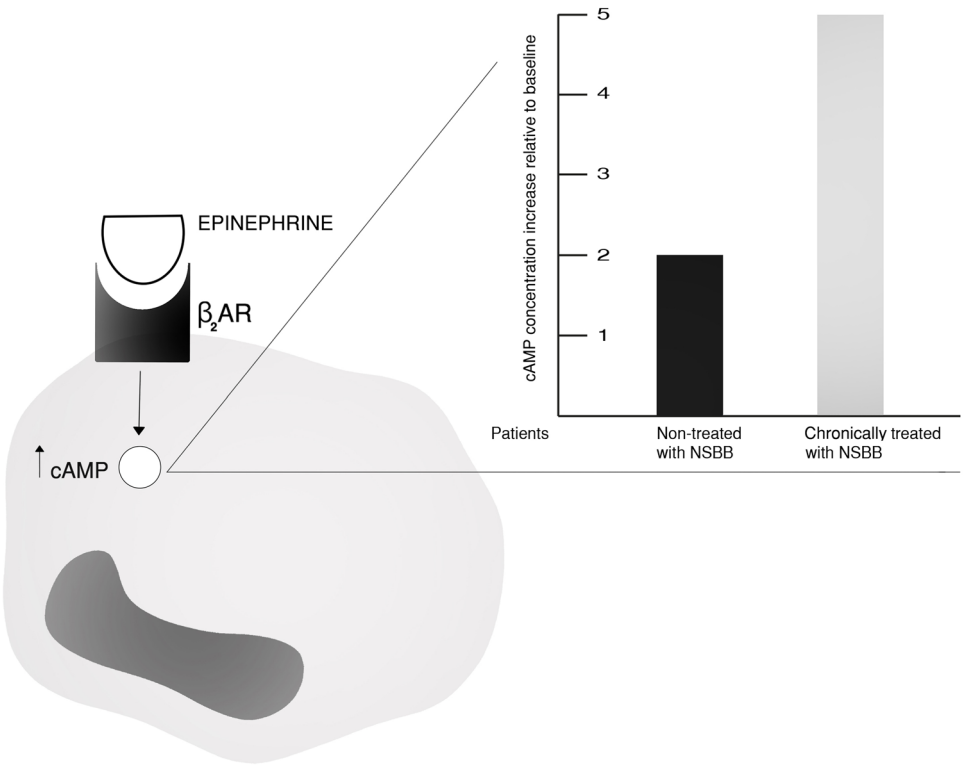
Graphic abstract



Keywords Portal hypertension · Beta-blockers · Beta-adrenoceptors · Peripheral blood mononuclear cells · cAMP · Catecholamines · Bacterial DNA · Endotoxin · Cytokines · Sympathetic nervous system

Introduction

The hyperdynamic circulatory state characteristic of patients with cirrhosis and portal hypertension is the result of splanchnic and peripheral vasodilation and increased plasma volume due to an excessive production of vasodilators [1]. Patients with cirrhosis present an increased activity of the sympathetic nervous system (SNS) during decompensation episodes in the form of ascites [2, 3]. However, this higher SNS activity fails to reverse the vasodilation, attributed to the large and sustained increase of vasodilators such as a nitric oxide (NO) [1] and the existence of dysfunctional alpha and beta-adrenergic receptors [4–6]. The presence of bacterial DNA fragments (bactDNA) circulating in blood and ascites in patients with cirrhosis and non-infected ascites is a surrogate marker for episodes of bacterial translocation [7–9]. In these patients, the presence of bactDNA is associated with a soluble immune response not related to endotoxin [10] but equivalent to that observed in patients with spontaneous bacterial peritonitis [11]. Moreover, circulating bactDNA fragments during an episode of ascitic decompensation are associated with decreased survival the following year [12]. Our group has shown that systemic inflammatory and phagocytic activity during an ascites episode was higher in patients with cirrhosis and presence of bactDNA and chronically treated with non-selective beta-blockers (NSBB) [13], suggesting the participation of adrenergic activation in the immune response to bacterial translocation. Indeed, treatment with NSBB has been associated with lower rates of infection in these patients [14].

Stimulation of beta-adrenergic receptors (β-AR) on immune cells by systemic catecholamines is involved in cytokines production and regulation of immune homeostasis [15]. Mitogen-induced in vitro production of interferon-gamma (IFN-γ) and other cytokines by peripheral blood mononuclear cells (PBMC) is partly suppressed by catecholamines, and this effect is reverted in the presence of a beta-blocker [16]. Beta-adrenergic receptors couple to G_S protein and activate adenylyl cyclase, leading to the accumulation of cyclic AMP (cAMP), a potent negative regulator of T cell immune function [17]. In monocytes and most other immune cells, intracellular cAMP is degraded mostly by phosphodiesterase 4. Evidence has been obtained in immune cells showing that cAMP level is increased by activation of β-AR

and also by activation of histamine, prostaglandin E_2 and adenosine receptors. Activation of these receptors results in the inhibition of TNF-α and IL-12 while increasing IL-10 production [18].

A previous study from our group showed that β-AR expressed on erythrocyte membranes from patients with cirrhosis and esophageal varices have a reduced ability to produce cAMP in response to catecholamine stimulation compared with healthy volunteers or from patients without esophageal varices. Included patients were either untreated or discontinued beta-blockers treatment at least 7 days before the β-AR functionality was analyzed [5]. In a second study, the functionality of β-AR of PBMC was also reduced in both patients with cirrhosis and patients with osteoarthritis compared with healthy volunteers [6]. Neither osteoarthritis patients nor healthy volunteers were treated with NSBB and it was not possible to analyze whether treatment with these drugs influences changes in β-AR functionality.

It is still unknown whether these changes in the function of the adrenoceptors and in the production of cAMP by the immune cells of cirrhotic patients are related in vivo to factors such as treatment with NSBB, PAMPs-induced immune response or the severity of cirrhosis and portal hypertension. It is also unclear whether the immunomodulatory effects of NSBB reported in these patients are associated with changes in β-AR activity on immune cells.

The aim of this study was to characterize β-AR activity on PBMC in response to catecholamine stimulation in cirrhotic patients with or without NSBB treatment.

Material and methods

We included patients treated for cirrhosis in the outpatient clinics at Hospital General Universitario de Alicante. Cirrhosis was diagnosed by histology or by the clinical, laboratory, and/or ultrasonographic findings. Exclusion criteria were the presence at baseline of hepatorenal syndrome or organic renal insufficiency, multinodular hepatocellular carcinoma and/or portal thrombosis, previous liver transplantation, transjugular intrahepatic portosystemic shunt (TIPS), alcoholic hepatitis, and refusal to participate in the study.

After signing informed consent, blood samples were collected from the patients after spending at least 30 min in a supine position in a quiet atmosphere. Blood samples were inoculated, under aseptic conditions, in rubber-sealed sterile Vacutainer SST II tubes (BD Diagnostics, Belgium), which were never exposed to free air. One aliquot of plasma was treated with ethylenediaminetetraacetic acid (EDTA; final concentration 1 mM) and sodium metabisulfite (final concentration 4 mM) to prevent catecholamine degradation. Serum and plasma samples were stored at − 80 ºC until analysis.

Adenylate-cyclase activity in PBMC

Adenylate-cyclase activity in PBMC was evaluated by determining cAMP production before and after stimulation with epinephrine and/or with the polymorphonuclear leukocyte chemotactic factor and macrophage activator N-formylmethionine-leucyl-phenylalanine (fMLP). Briefly, human PBMC were isolated using a Ficoll gradient. Cells (0.5×10^5 cells/mL) were then suspended in 1 mL 48-well plates filled with phosphate-buffered saline (PBS) containing the competitive non-selective phosphodiesterase inhibitor isobutyl-methyl-xanthine 10^{-3} M (IBMX). The non-selective beta-adrenergic antagonist propranolol (1 μM) was added according to the experimental protocol, and plates were incubated for 30 min at 37 ºC. The adrenergic agonist epinephrine (10 μM), the immune activator fMLP (1 μM) or both were added and incubated for 15 min at 37 ºC. Samples were sonicated three times for 10 s and centrifuged for 3 min at 13,000 rpm at 4 °C. The supernatant was frozen and stored at − 80 °C. Levels of cAMP were measured in 50 μL of supernatant using an ELISA assay (581,001, Cayman Chemical Company, Ann Arbor, Michigan, USA) and recorded as pmol/mL. Results of each experimental condition (stimulation with epinephrine, fMLP or epinephrine + fMLP in the presence or absence of propranolol) are expressed as the log of the fold increase in cAMP concentration over baseline conditions.

Serum epinephrine, norepinephrine, and dopamine were measured as biomarkers of in vivo sympathoadrenal activation by commercially available immunoassays in EDTA plasma (catecholamines) according to the manufacturer's recommendations: plasma epinephrine, norepinephrine and dopamine were determined using the 3-CAT RESEARCH ELISA (E-5600, Labor Diagnostica Nord GmbH & Co. KG, Nordhorn, Germany; lower limits of detection: 50 pg/mL, 20 pg/mL and 50 pg/mL for A, NA and D, respectively).

To detect the presence of bactDNA fragments in blood, a broad-range polymerase chain reaction (PCR) was performed according to the methodology described previously [7]. PCR amplicons were loaded onto DNA Laboratory-on-chips (Agilent Technologies, Palo Alto, CA, USA) and quantified in an Agilent 2100 BioAnalyzer (Agilent Technologies). Samples and reagents were handled in an airflow chamber and processed with pyrogen-free material tested by manufacturers.

The endpoint chromogenic Limulus Amebocyte Lysate test was used to quantify gram-negative bacterial endotoxin (LPS) in serum using a commercially available kit, according to the manufacturer's protocols (QCL-1000, Lonza Group Ltd., Basel, Switzerland).

Enzyme-linked immunosorbent assays (ELISAs) for the quantitative measurement of TNF-α, IFN-γ, IL-10, IL-6 and TGF-beta1 levels were carried out in serum specimens

using Human Quantikine kits (R&D Systems, Minneapolis, US), according to manufacturer's instructions. All samples were tested in triplicate and read at 490 nm in a microplate reader. Lower limits of detection of all cytokine assays were between 0.5–5 pg/mL. NOx levels in serum samples were calculated by measuring the conversion of NO^{3-} to NO^{2-} by the enzyme nitrate reductase using an ELISA assay (KGE001, R&D Systems, Minneapolis, USA) based on the Griess reaction that absorbs visible light at 540 nm. Standard curves were generated for each plate, and the average zero standard optical densities were subtracted from the rest of the standards, controls and samples to obtain a corrected concentration for all cytokines.

Isolation of RNA and quantitative real-time PCR

PBMC were stabilized in RNAlater (Qiagen, Hilden, Germany) for subsequent RNA isolation using a RNeasy Mini Kit according to the manufacturer's instructions. For gene expression analysis, 10 ng of RNA sample was then used for one-step qPCR using QuantiTect SYBR Green RT-PCR kit. GAPDH expression was used as internal reference in all experiments, and all samples were tested in duplicate. RT-PCR primers for each specific target gene were designed based on their reported sequence (Supporting Information Table S1). Calculation of mRNA fold induction was performed using the $2^{-\Delta\Delta Ct}$ (Cycle threshold) method. The average ΔCt of the control group in each experiment was used as the reference value to calculate the ΔΔCt for each individual sample in each group. Results are expressed as the log of the ΔΔCt.

Statistical analysis

Continuous variables are reported as mean ± standard deviation and categorical variables as frequency or percentages. Statistical differences between groups were analyzed using the chi-square test for categorical data and the Student's *t* test for quantitative data. Statistical differences between more than two groups were analyzed using the ANOVA test and Bonferroni correction. Linear regression was used to study the association between different variables and the times increase in cAMP concentration.

We used log-transformed data for our analysis since the fold increase in cAMP concentration and the level of catecholamines and cytokines were positively skewed. Visual inspection showed that transformed data fitted well to normal distribution. Log-transformed data are expressed as geometric mean and 95% confidence intervals (CI).

A propensity score matching using the nearest neighbors method and a one-to-one matching approach was performed to compare patients treated and not treated with NSBB with similar severity of the disease. All clinical and demographic variables that showed statistically significant differences between patients treated and not treated with NSBB were included as covariates in the propensity score matching.

All reported *p* values are two sided, and *p* values lower than 0.05 are 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 https://www.R-project.org/).

Results

Seventy-four patients with cirrhosis were included in the study, 19 (26%) of whom were being treated with NSBB (18 with propranolol at a dose ranging from 20 mg/day to 80 mg/day and 1 with carvedilol at a dose of 12.5 mg/day), with a median treatment duration of 15 months (interquartile range [IQR] 19.5 months). The characteristics of the included patients are detailed in Table 1. All patients treated with NSBB had gastroesophageal varices, compared with 64% (35/55) of participants not receiving NSBB. Patients treated with NSBB had significantly higher liver stiffness, as assessed by FibroScan, and lower counts of platelets and Quick index values than patients not on NSBB. Moreover, the NSBB group received proton pump inhibitors (PPIs) more frequently than patients not treated with beta-blockers. The bacterial DNA detection rate was nearly 20% and similar in both groups.

Adenylate-cyclase activity in PBMC of patients with cirrhosis

The mean cAMP production of PBMC obtained from patients was 4.0 (CI 95% 3.2–4.9) pmol/mL. This figure did not change according to NSBB treatment or the presence of bactDNA in blood. The increase in intracellular cAMP concentrations after stimulation of adrenergic receptors on PBMC membranes with epinephrine was significantly higher in samples from patients chronically treated with beta-blockers (5.0 vs. 2.5 times; Fig. 1a). This effect was suppressed by the presence in the medium of propranolol, showing that most of the epinephrine-induced c-AMP increase is due to beta-adrenergic stimulation. On the other hand, the stimulation with the polymorphonuclear leukocyte chemotactic factor and macrophage activator fMLP produced minimal changes on basal cAMP production and did not change the stimulant effect of epinephrine. Increases in cAMP concentrations after epinephrine or fMLP stimulus were similar in PBMC from patients with and without the presence of bactDNA fragments in plasma (Fig. 1b).

Very similar increases (4.9 vs. 2.9 times; supplementary Fig. 1) in intracellular cAMP concentrations after

Table 1 Patient characteristics according to treatment with non-selective beta-blockers

	Treatment with NSBB		*p* value
	No (*N* = 55)	Yes (*N* = 19)	
Age (years), mean ± SD	58.6 ± 7.7	58.0 ± 7.6	0.789
Female, *N* (%)	15 (27.3)	3 (15.8)	0.487
Etiology of cirrhosis, *N* (%)			0.478
Alcohol	25 (45.5)	8 (42.1)	
Alcohol + virus	6 (10.9)	4 (21.1)	
Virus	21 (38.2)	5 (26.3)	
Others	3 (5.5)	2 (10.5)	
Previous episodes of			
Ascites, *N* (%)	21 (38.2)	9 (47.4)	0.666
Encephalopathy, *N* (%)	1 (1.8)	2 (10.5)	0.325
Upper digestive bleeding, *N* (%)	11 (20.0)	6 (31.6)	0.473
Child–Pugh score, mean ± SD	5.7 ± 1.2	6.2 ± 1.4	0.111
Ascites, *N* (%)	1 (1.8)	2 (10.5)	0.325
Gastroesophageal varices, *N* (%)	35 (64)	19 (100)	0.006
Stiffness Fibroscan (kPa) mean ± SD	38.6 ± 7.5	48.1 ± 11.8	0.007
Diuretics at inclusion, *N* (%)	13 (23.6)	9 (47.4)	0.097
PPIs at inclusion, *N* (%)	10 (18.2)	9 (47.4)	0.027
SID at inclusion, *N* (%)	2 (3.6)	0 (0.0)	0.982
Hemoglobin, g/dL, mean ± SD	13.8 ± 2.1	13.6 ± 2.2	0.723
Hematocrit, %, mean ± SD	42.2 ± 5.5	40.6 ± 6.2	0.305
Platelets, $\times 10^9$/mm^3, mean ± SD	145 ± 63	99 ± 34	0.004
Leukocytes per mm^3, mean ± SD	6.3 ± 2.2	5.7 ± 3.0	0.361
Quick index, mean ± SD	80.4 ± 16.8	71.3 ± 15.2	0.046
Albumin, g/dL, mean ± SD	3.78 ± 0.59	3.47 ± 0.69	0.073
ALT, IU/L, mean ± SD	25.8 ± 16.7	30.6 ± 26.9	0.376
Creatinine, mg/dL, mean ± SD	0.85 ± 0.44	0.83 ± 0.23	0.873
Glucose, mg/dL, mean ± SD	112 ± 35	101 ± 20	0.214
Serum sodium, mEq/L, mean ± SD	140.4 ± 2.9	139.7 ± 1.9	0.329
Serum potassium, mEq/L, mean ± SD	4.44 ± 0.44	4.34 ± 0.45	0.417
Total bilirubin, mg/dL, mean ± SD	1.09 ± 0.94	1.67 ± 1.85	0.086
Presence of bactDNA, N (%)	10 (18.2)	4 (21.1)	1.000

PPIs proton pump inhibitors, *SID* selective intestinal decontamination, *ALT* alanine aminotransferase, *bactDNA* bacterial DNA, *NSBB* non-selective beta-blockers

stimulation of adrenergic receptors with epinephrine were observed when comparing the 19 patients treated with NSBB with 19 untreated patients matched by clinical and demographic variables at baseline (supplementary Table 1).

In univariate linear regression analysis, the magnitude of increase in intracellular cAMP levels after epinephrine stimulation was significantly associated with the treatment with NSBB ($\beta = 3.6$, $p = 0.0008$), the age of patients ($\beta = -0.19$, $p = 0.0013$) and the days of treatment with NSBB ($\beta = 0.0068$, $p < 0.0001$). In multivariate analysis, the significance of patient age ($\beta = -0.10$, $p = 0.0454$) and the duration of NSBB treatment ($\beta = 0.0066$, $p < 0.0001$) were maintained. Older patients showed lower responses to epinephrine stimulus (Fig. 2a). Conversely, the production of cAMP after epinephrine stimulation of PBMC from patients treated with NSBB increased linearly with the duration of the treatment (Fig. 2b).

Adrenoceptor expression on PBMC of patients with cirrhosis

Levels of mRNA expression for adrenoceptors β1, β2, β3 and α1-A, B and D in PBMC showed no significant differences according to NSBB treatment (Table 2). A significant decrease in the mRNA expression of β3-adrenoceptors was observed only in PBMC from samples of patients without bacterial DNA presence in serum, although the magnitude of this difference was small (Table 2).

Relationship between systemic adrenergic activation and adenylate-cyclase activity in PBMC of patients with cirrhosis

No significant differences in serum concentrations of catecholamines were observed according to NSBB treatment or the presence of serum bacterial DNA (Table 3). None of the descriptive variables of the patient characteristics (Table 1) were significantly related to the concentrations of catecholamines. There were no significant relationships between the concentrations of the different catecholamines and the production of cAMP in PBMC after epinephrine- or fmlp-epinephrine stimulation.In addition, no association was observed between the concentrations of catecholamines and cytokines studied.

Systemic immune activation and adenylate-cyclase activity in PBMC of patients with cirrhosis

Serum levels of TGF-β1 were lower in patients treated with NSBB ($p = 0.058$; Table 3). In univariate regression analysis, TGF-β1 levels were significantly associated with variables related to the severity of cirrhosis in addition to the treatment with NSBB (meld score, platelets, albumin, gastroesophageal varices, creatinine). In the multivariate analysis, only platelet counts maintained a significant association ($\beta = 5.48$, $p < 0.0001$), suggesting that the difference observed according to NSBB treatment was really a marker of clinical severity and not a direct consequence of treatment. None of the studied cytokines and immune parameters were significantly associated with the production of cAMP in PBMC after adrenergic stimulation.

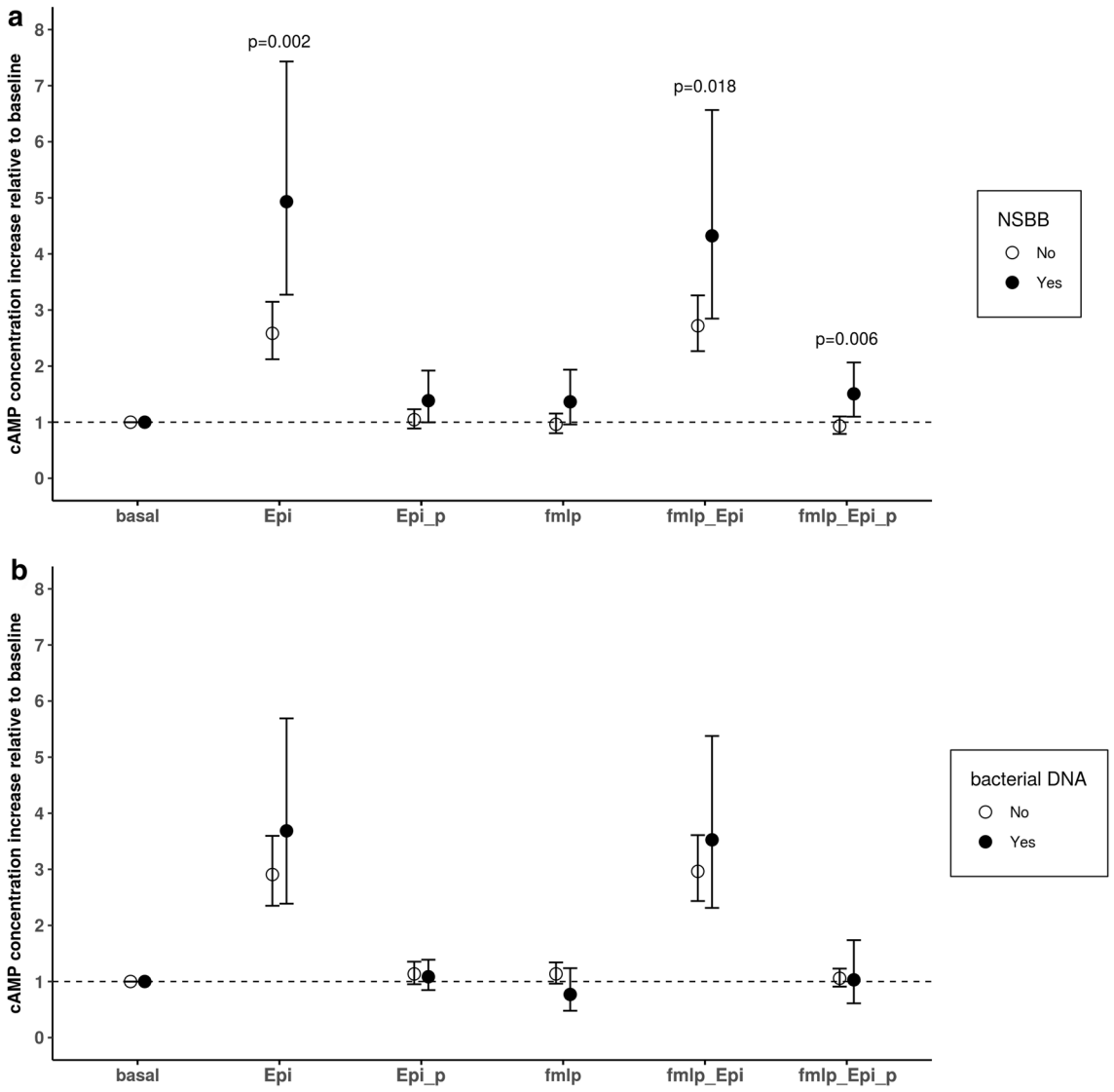


Fig. 1 Adenylate-cyclase activity in PBMC of patients with cirrhosis treated ($N=19$) vs. not treated with NSBB ($N=55$), expressed as the number of times increase in cAMP concentration over baseline conditions after adrenergic or fMLP stimulus. Each condition was evaluated in 0.5×10^5 cells/mL suspended in 1 mL 48-well plates filled with PBS containing IBMX 10^{-3} M. **a** Comparison between treated and untreated patients with NSBB. **b** Comparison between patients with and without bacterial DNA in blood. Data are geometric mean ± 95% confidence interval. *PBMC* peripheral blood mononuclear cells, *cAMP* cyclic AMP, *NSBB* non-selective beta-blockers, *Epi* epinephrine (10 µM), *fmlp N*-formylmethionine-leucyl-phenylalanine (1 µM), *Epi_p* epinephrine (10 µM) + propranolol (1 µM), *fmlp_Epi* fMLP (1 µM) + epinephrine (10 µM), *fmlp_Epi_p* fMLP (1 µM) + epinephrine (10 µM) + propranolol (1 µM). Significant p values between NSBB-treated and non-treated groups are represented.

Discussion

According to previous studies by our group, β-AR of patients with cirrhosis have a reduced ability to respond to adrenergic stimulation [5, 6]. In these studies, patients were not treated with NSBB or the treatment had been discontinued before starting the study, so it was unknown whether NSBB treatment interfered with the adrenergic signal in PBMC.

Experimental studies have shown that β-AR stimulation by catecholamines, and the consequent intracellular elevation of cAMP, suppresses LPS-stimulated production of TNF-α, IL-1β and IL-12 and the expression of surface markers involved in regulating T helper-cell differentiation and balance (I-CAM1, CD40, and CD14) in PBMC [15, 19]. From these data, it is reasonable to hypothesize a possible immunomodulating role of catecholamine-stimulated β-AR

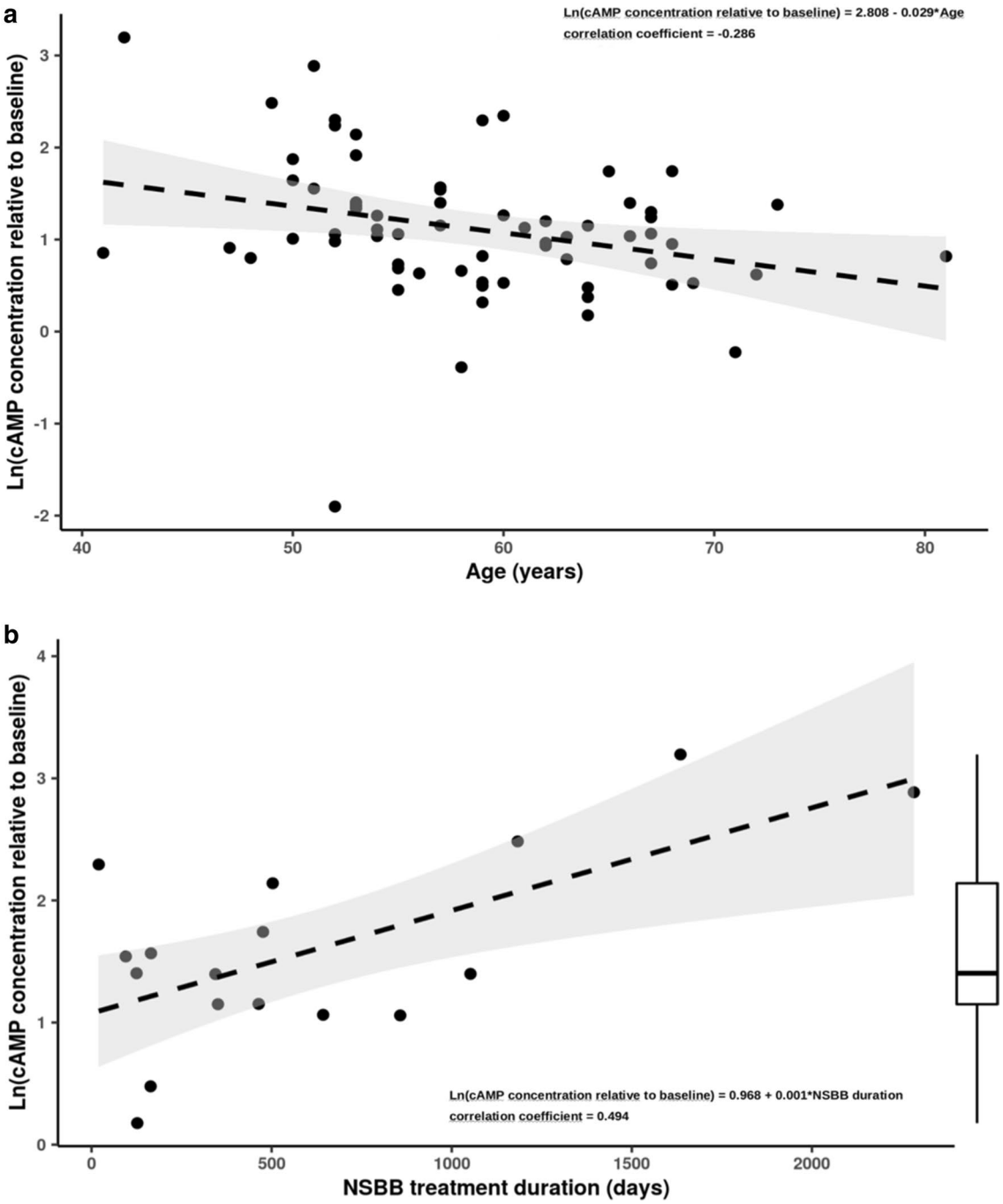


Fig. 2 Relationship between age and: **a** length of treatment with NSBB (in days); and **b** the increase in intracellular cAMP levels (data are logarithmic values) after epinephrine stimulus (10 μM) in PBMC from patients with cirrhosis. NSBB: non-selective beta-blockers; PBMC: peripheral blood mononuclear cells. In **b**, values of increases in intracellular cAMP levels corresponding to patients treated with NSBB are represented as dots while values corresponding to patients non-treated with NSBB are resumed as boxplot on the right side of the plot. Regression equation and Pearson correlation coefficient are shown in the figures

in immune cells of patients with cirrhosis. However, it is unknown whether this immunomodulatory action is involved in the differential rate of infections and tumors reported in patients treated with NSBB [14, 20, 21].

We observed that PBMC obtained from patients with cirrhosis and chronically treated with NSBB (mostly propranolol) enabled them to better respond to beta-adrenergic stimulation compared with patients who were not on NSBB.

Table 2 mRNA levels for β1, β2, β3, α1A, α1B y α1D-adrenoceptors, in peripheral blood mononuclear cells from patients with cirrhosis

	Treatment with NSBB		*p* value	Presence of bacterial DNA		
	No ($N=55$)	Yes ($N=19$)		No ($N=60$)	Yes ($N=14$)	*p* value
β1-adrenoceptors	3.3 (1.7–6.7)	2.0 (0.6–7.2)	0.478	2.8 (1.5–5.3)	3.3 (0.4–26.4)	0.866
β2-adrenoceptors	2.1 (1.8–2.3)	2.1 (1.7–2.6)	0.996	2.1 (1.9–2.4)	1.8 (1.3–2.3)	0.226
β3-adrenoceptors	2.6 (2.2–3.1)	2.0 (1.4–2.7)	0.129	2.6 (2.2–3.0)	1.7 (1.1–2.5)	0.047
α1A-adrenoceptors	11.5 (7.2–18.1)	7.1 (3.8–13.3)	0.209	9.7 (6.4–14.8)	11.0 (4.7–26.0)	0.782
α1B-adrenoceptors	8.0 (4.8–13.3)	5.0 (2.2–11.0)	0.302	6.2 (3.9–9.9)	12.1 (3.4–42.3)	0.291
α1D-adrenoceptors	2.3 (2.0–2.8)	1.6 (1.1–2.3)	0.059	2.1 (1.8–2.4)	2.1 (0.8–5.4)	0.977

Data are geometric mean (95% confidence interval). Data were calculated as $2^{-\Delta\Delta Ct}$ vs. GAPDH as reference gene

These changes were not associated with variations in the expression of adrenergic receptors (beta and alpha).

This increased response was significantly related to patients' age and the duration of treatment with NSBB. Neither clinical variables such as Child score nor analytical parameters are suggestive of a more developed disease correlated with this increased response. Further, this increased response observed in patients chronically treated with NSBB compared with those not treated was maintained after controlling for confounding or background variables using a propensity score matching. According to previous studies by our group, β-AR of patients with cirrhosis have a reduced ability to respond to adrenergic stimulation [5, 6]. Data from the present study show the ability of chronic treatment with NSBB to reverse this condition at a cellular level. Specific studies carried out in non-cirrhotic patients treated with NSBB are needed to know if this effect is exclusive to patients with cirrhosis or can occur in other pathologies.

We did not observe any significant association between serum catecholamine levels and cAMP production in PBMC in response to adrenergic stimulus. In patients with cirrhosis, a positive relationship between circulating catecholamines and the progression of the disease has been described. Patients with a high Child score, marked portal hypertension, fluid retention, and hepatorenal syndrome have the highest plasma levels of catecholamines [22]. In peripheral venous samples of patients with decompensated cirrhosis, norepinephrine levels were around 3 nmol/L while in compensated patients these figures were lower than 2 nmol/L [2]. Norepinephrine levels higher than 2.5 nmol/L have been related to reduced survival [23], a higher degree of portal hypertension and worse renal function [24]. Patients in our study showed serum levels of norepinephrine around 3 nmol/L, which is consistent with previous reports in decompensated patients, and these levels did not change with chronic NSBB treatment. This situation is different than that described 1 to 2 h after a single oral dose of 80 mg of propranolol, where an increase of norepinephrine and epinephrine concentrations were observed, probably due to compensatory hemodynamic mechanisms [25]. Accordingly, our study demonstrates that chronic administration of NSBB in patients with cirrhosis doesn't change the high levels of catecholamines observed in these patients and that NSBB-induced modifications in the activity of adrenergic receptors on immune cells are independent of the degree of systemic adrenergic activation.

Patients treated with NSBB showed lower levels of serum TGF-β1. These reduced levels were also associated with other variables related to the severity of cirrhosis. In fact, in the multivariate analysis the only variable associated with these low levels was the platelet count. TGF-β1 is recognized as a profibrogenic cytokine and also as a suppressor in the early stages of liver cancer development, although in later phases it may contribute to tumor progression [26–28]. Therefore, it is not surprising that patients with more advanced disease and higher portal hypertension showed higher variations in serum TGF-β1 levels. Consequently, a possible effect of NSBB either directly on TGF-β1 production or indirectly through its hemodynamic effects might not be identified. Only a randomized clinical trial in patients with the same degree of disease progression could elucidate the effect of NSBB on TGF-β1.

Patients with cirrhosis treated with non-selective beta-blockers (NSBB) show an increase in systemic inflammatory and phagocytic activities during an ascites episode with the presence of bactDNA in blood [13]. In our study, outpatients were included and their disease was stable at the time of blood sampling. Although bactDNA was detected in 18% of these patients, the systemic inflammatory activation was lower than that observed in patients with cirrhosis at the time of an ascites episode [11–13]. In our work, changes in PBMC beta-adrenoceptors activity were not related to the presence of bacterial DNA, LPS or cytokine concentrations in patients' blood. In a situation of low systemic inflammation, as in our patients, we cannot rule out an added difficulty in identifying a relationship between changes in the immune system and the activity of beta-adrenergic receptors.

In our study, chronic NSBB treatment was not associated with changes in gene expression of beta-adrenergic

Table 3 Systemic adrenergic and immune activation in patients with cirrhosis included in the study according to treatment with non-selective beta-blockers and presence of bacterial DNA fragments in blood

	Treatment with NSBB		*p* value	Presence of bacterial DNA		*p* value
	No	Yes		No	Yes	
	(*N* = 55)	(*N* = 19)		(*N* = 60)	(*N* = 14)	
Adrenergic activation						
Norepinephrine	548 (465–644)	573 (406–808)	0.804	555 (470–655)	552 (390–781)	0.979
Epinephrine	62 (45–84)	73 (47–115)	0.515	70 (52–95)	45 (30–67)	0.067
Dopamine	110 (95–127)	96 (84–111)	0.188	105 (93–120)	109 (81–146)	0.820
Immune activation						
TNF-alpha	0.84 (0.72–0.97)	0.82 (0.69–0.98)	0.891	0.8 (0.7–0.9)	1.0 (0.8–1.2)	0.059
TGF-β1	703 (586–844)	492 (354–684)	0.058	645 (536–777)	626 (450–871)	0.866
IL10	5.2 (4.3–6.2)	5.7 (4.5–7.2)	0.534	5.7 (4.9–6.5)	3.9 (2.3–6.6)	0.162
IFN	0.0 (0.0–0.2)	0.0 (0.0–0.2)	0.963	0.0 (0.0–0.1)	0.0 (0.0–0.2)	0.955
IL6	3.2 (2.4–4.3)	3.0 (1.9–4.9)	0.844	2.9 (2.2–3.9)	4.4 (3.1–6.5)	0.071
NO	59 (52–67)	52 (41–67)	0.344	57 (50–65)	59 (48–72)	0.790
LPS	0.34 (0.28–0.42)	0.26 (0.20–0.33)	0.089	0.33 (0.27–0.40)	0.28 (0.18–0.44)	0.473

Units of data are pg/mL. Data are geometric mean (95% confidence interval). *TNF* tumor necrosis factor, *TGF* transforming growth factor, *IFN* interferon, *IL* interleukin, *NO* nitric oxide, *LPS* gram-negative bacterial endotoxin

receptors, suggesting that changes in response to the adrenergic stimulus occur beyond the receptor, probably at the level of G proteins or Adenylate Cyclase.

Heart failure and ageing are both conditions associated to elevated circulating catecholamines levels similar to those seen in cirrhosis. Studies performed in heart failure patients have shown that chronic stimulation of cardiac beta-adrenoceptors reduces its responsiveness such as we have seen in PBMC from patients with cirrhosis [29]. Cardiomyocytes where beta-adrenoceptor system was desensitized showed upregulation of G-protein subunit Gαi and G-protein coupled receptor kinase-2 (GRK2) activities. In these studies, exercise and beta-blockers were able to resensitize cardiac beta-adrenoceptors [29] in a similar way to that observed in our study in patients with cirrhosis treated chronically with NSBB. Therefore, it is possible that NSBB treatment restores the responsiveness of beta-adrenoreceptors through the modulation of Gαi and/or GRK2 in PBMCs.

The results of this study can be best understood by considering its limitations. The first is a consequence of the baseline differences between treated and untreated patients that we have tried to control by means of multivariate regression analysis and propensity score matching. Second, the lack of a control group of non-cirrhotic patients treated with NSBB prevents us from knowing whether the observed effect of NSBB is generalized or limited to patients with cirrhosis. Third, the cross-sectional nature of the study prevents us from knowing whether the speed or intensity of changes over time in the activity of the immune or adrenergic systems influences the magnitude of the beta-adrenergic response observed in PBMCs. It is also not possible to study how this response changes over time after the start of treatment with NSBB.

Chronically elevated serum catecholamine levels seen in patients with cirrhosis have been associated with detrimental hemodynamic and immunologic changes [13]. In these patients, the treatment with NSBB counteracts the damaging effects of catecholamines and improves the hemodynamic function preventing variceal bleeding. Also, a reduction in the risk of infections and hepatocellular carcinoma has been described [13]. Theoretically, an increased response of beta-adrenoceptors with the NSBB chronic treatment could worsen the clinical condition of patients subjected to stressful situations with higher catecholamines concentrations that could overpass the beta-blocker effect. In fact, an increased risk for hepatorenal syndrome and death has been described in patients with cirrhosis and spontaneous bacterial peritonitis treated with NSBB [30]. Also, a worsening of the hemodynamic situation of patients treated chronically with NSBB after their withdrawal is possible.

In conclusion, chronic treatment with NSBB enables PBMC beta-adrenoceptors to respond to catecholamine stimulation irrespective of the degree of systemic adrenergic or immune activations of the patient at the time of sampling.

Funding This work has been supported by Grants PI14/01090 from Instituto de Salud Carlos III, Madrid, Spain. This work was awarded the "Miguel Pérez-Mateo Regadera" Prize granted by the Royal Academy of Medicine of the Valencian Community.

Compliance with ethical standards

Conflict of interest The authors Susana Almenara, Beatriz Lozano, Paula Gimenez, Ivan Herrera, Cayetano Miralles, Pablo Bellot, María Rodríguez, Rubén Francés, Jose M. Gonzalez-Navajas, Sonia Pascual and Pedro Zapater have no conflict of interest.

Ethical approval This study was approved by Institutional Review Board of Hospital General Universitario de Alicante.

Informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study. All authors reviewed and approved the final version of the manuscript.

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