

Bacterial load and cytokine profile in patients with cirrhosis following therapy with proton pump inhibitors: a prospective cohort study

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Abstract

Background The aim of this study was to explore the presence of bacterial products and the cytokine profile in outpatients with cirrhosis before and after short-term (4-8 weeks) administration of proton pump inhibitors (PPIs).

Methods Seventeen patients with cirrhosis—male/female: 12/5; age: median 59.2 years (49-65); etiology: HBV±HDV 23.5%, HCV 17.7%, alcohol 41.2%, other 17.6%; Child-Pugh score: median 7.5 (5-12); Model for End-stage Liver Disease: 10.5 (7-21); ascites (%): 3 (17.7)—attending the outpatient clinics were included. None had hepatocellular carcinoma. Indications for PPIs were: esophagitis (n=6, 35.3%), peptic ulcer (n=10, 58.6%) and other (n=1, 5.9%). Bacterial DNA in serum and the levels of endotoxin, lipopolysaccharide binding protein, transforming growth factor- β , interleukin -1 β , -6, -8, -12, -10, tumor necrosis factor- α and nitric oxide were assessed at baseline (time 1) and at the end of treatment (time 2). The Wilcoxon signed rank test was used to evaluate significant differences in the parameters assayed before and after PPI administration.

Results No patients developed infection during the study period. Bacterial DNA was not detected before or after treatment. No significant differences were observed between the concentrations of any indices between times 1 and 2 ($P>0.05$). Subgroup analysis according to Child-Pugh stage yielded similar results.

Conclusion Short-term administration of PPIs had no effect on bacterial DNA, bacterial products or cytokine concentrations in patients with liver cirrhosis.

Keywords Bacterial translocation, cytokine profile, infection, liver cirrhosis, proton pump inhibitors

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Introduction

Liver cirrhosis is associated with a type of immune dysfunction referred to as cirrhosis-associated immune dysfunction syndrome [1]. Altered small bowel motility, small intestinal bacterial overgrowth and increased intestinal permeability lead to increased bacterial translocation and gut-related infections [2]. Bacterial infections influence hemodynamics [3] and coagulation status [4] and represent one of the most important reasons for the progression of liver failure, development of liver-related complications and mortality in patients with cirrhosis [5].

Proton pump inhibitors (PPIs) are widely used in patients with cirrhosis [6] because of the frequently observed mucosal abnormalities following screening endoscopy [7] and their use as prophylaxis for bleeding from post-banding esophageal

ulcers [8]. Although there are data to suggest that PPIs increase the risk of infection [9-21], this positive association is not universal [22-24]. In a study of 607 consecutive patients with cirrhosis undergoing their first paracentesis at a tertiary center, the authors observed no association between PPIs and spontaneous bacterial peritonitis (SBP) or other infections, or mortality, suggesting that the severity of liver disease and other factors, rather than PPI treatment *per se*, may predispose to infectious complications [25]. Lastly, in a recent large, multicenter, prospective study, which included 519 patients with decompensated cirrhosis, PPI therapy was not found to be associated with a higher risk of SBP [26].

The interpretation of the published data is difficult because most of the studies were retrospective [9,10,13-16,18,21,22,25], the use of PPIs was inappropriate in up to half of the patients [27], and the details about the duration of their administration before the diagnosis of the infection were unknown in many cases. We conducted this prospective study, in which we included patients with cirrhosis, with or without ascites, who underwent an upper gastrointestinal endoscopy and received "appropriate" treatment with PPIs, and we investigated the effect of PPIs on bacterial load and inflammatory cytokine production.

Patients and methods

This prospective cohort study was performed at the University Hospital of Patras (Patras, Greece) between February 2010 and October 2011. Patients with a previous history of SBP, liver transplantation, human immunodeficiency virus infection or any kind of immunosuppression, gastrointestinal bleeding, antibiotic prophylaxis during the last 2 weeks, hepatocellular carcinoma that exceeded the Milan criteria, portal vein thrombosis, and previous portosystemic shunt were excluded. We evaluated 45 consecutive patients with cirrhosis attending the hepatology outpatient clinic, who had no clinical, bacteriological or radiological signs of urinary tract, respiratory or systemic infection, and who underwent an upper gastrointestinal endoscopy. Finally, 17 patients were enrolled (15 patients received antibiotics during the last 2 weeks or after endoscopy, 2 had hepatocellular carcinoma that exceeded the Milan criteria and the others refused to participate in the study). Informed consent was obtained from all individual participants included in the study.

The characteristics of PPI use in terms of indication for administration, duration of therapy and the scheduled dose were recorded. "Appropriate" PPI use was defined as the use for the following indications: gastro-esophageal reflux disease, *Helicobacter pylori* (*H. pylori*) eradication, Barrett's esophagus, Zollinger-Ellison syndrome, prevention of non-steroidal anti-inflammatory drug-induced ulcers, and peptic ulcer disease [28-32]. Prophylaxis following variceal band ligation was also considered "appropriate" for the purposes of this study [8]. None of the included patients had previously received PPI treatment. Time 1 was defined as baseline and time 2 concerned the length of PPI treatment, which ranged

from 4-8 weeks. The study population was followed up after the end of PPI treatment for a median period of 14 months (12-17).

Cirrhosis was diagnosed based upon histological findings, clinical evaluation, laboratory data or imaging findings compatible with a diagnosis of cirrhosis [33]. The severity of liver disease was assessed by the Child-Pugh classification and the Model for End-stage Liver Disease (MELD) at baseline. In addition, routine clinical, biochemical and radiological analyses (abdominal ultrasound and/or computed tomography) were performed. The patients with alcoholic cirrhosis were abstinent for at least three months before their inclusion in this study. After baseline examination, patients were followed in the hepatology clinic at regular intervals according to current guidelines [33]. The presence of bacterial DNA in serum and the levels of endotoxin, transforming growth factor (TGF)- β , tumor necrosis factor (TNF)- α , interleukin (IL) -1 β , -6, -8, -10, and -12p70, lipopolysaccharide binding protein (LBP) and nitric oxide (NOx) were assessed at baseline (time 1) and at the end (time 2) of treatment with PPIs.

Determination of cytokine, LBP, endotoxin and NOx serum levels

Peripheral blood samples (3-4 mL) from 17 cirrhotic patients were drawn before and after treatment with PPIs. Blood samples were centrifuged and the sera were collected and stored aliquoted at -80°C until further use. The serum levels of cytokines IL-1 β , -6, -8, -10, -12p70 and TNF- α were determined by a cytometric bead array method, using the Human Inflammatory Cytokine CBA kit (BD Bioscience, San Diego, USA). The measurements were performed on a BD FACS Array Bioanalyzer. The raw data were analyzed using the Flow Jo V7.5 software (Tree Star Inc., Ashland, OR, USA). Further analysis of the data was performed using the Curve Expert 1.4 software. Serum levels of TGF- β and LBP were determined by ELISA, using a multi-species TGF- β ELISA kit (Invitrogen Corporation, CA, USA) and a human LBP ELISA kit (SunRed Biological Technology, Shanghai). NOx levels were measured by a nitric oxide quantitation kit (Active Motif, Belgium). Endotoxin levels were measured using a LAL Chromogenic Endpoint assay (Hycult biotech, The Netherlands). Data analysis was performed using the Curve Expert 1.4 software. The detection limits of the methods employed were: IL-8=3.6 pg/mL, IL-1 β =7.2 pg/mL, IL-6=2.5 pg/mL, IL-10=3.3 pg/mL, TNF- α =3.7 pg/mL, IL-12p70=1.9 pg/mL, LBP=0.135 μ g/mL, TGF- β =15.6 pg/mL, NOx <1 μ M, endotoxin=0.04 EU/mL.

Bacterial DNA

Bacterial DNA was detected as described previously [34]. Briefly, 400 μ L of human serum samples were centrifuged at 15,000 g for 15 min. The supernatant was decanted and the remaining pellet was reconstituted in 1 mL of molecular biology grade water and centrifuged at 15,000 g for 10 min. The supernatant was discarded and the pellet was again reconstituted in 40 μ L of molecular biology grade water

followed by boiling in a water bath at 100°C for 10 min. The mixture was then cooled on ice and centrifuged at 15,000 g for 10 sec; 2.5 µL of the supernatant containing the bacterial DNA was used for subsequent PCR reactions. Amplification of a 540bp segment of the 16S rRNA gene was performed with a pair of universal primers (F: 5'-AGAGTTTGATCATGGCTCAG-3', R: 5'-ACCGCGACTGCTGCTGGCAC-3') on a My Cycler thermal cycler (Biorad, USA) with 2.5 µL of 10x PCR buffer, 0.75 µL of 50 mM MgCl₂, 2.0 µL of 2.5 mM each of dNTPS, 0.5 µL of 25 µM each primer and 0.625 U of Recombinant DNA Taq Polymerase (Invitrogen, Carlsbad, CA, USA) in a total volume of 25 µL. The thermal conditions for the above reaction were as follows: 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The amplicons were visualized on a 2% agarose gel stained with ethidium bromide under ultraviolet illumination.

Statistical analysis

Numerical data were expressed as mean ± standard deviation if parametric or as median and range if non-parametric. The distribution of variables was tested for normality using the Kolmogorov-Smirnov test. Categorical data were expressed as counts and percentages and compared using the chi-square and Fisher's exact test. Continuous variables with and without normal distribution were compared using Student's *t*-test or the Mann-Whitney *U* test, respectively. Spearman's correlation coefficient (*r*) analysis was used for correlation of MELD score and cytokine/bacterial indices. The Wilcoxon signed rank test was used to evaluate significant differences in laboratory measurements before and after PPI administration. Significance testing was two-sided and set to <0.05. Analysis was performed using the SPSS statistical package (version 22.0, IBM, New York, NY, USA).

Institutional review board statement

The study was reviewed and approved by the Ethics committee of the University Hospital of Patras.

Results

Baseline characteristics of study population

Seventeen patients were included in the study. The majority were male (71%) and the median age was 59 (49-65) years. The baseline demographic and clinical characteristics of the study participants are shown in Table 1. None of the patients had hepatocellular carcinoma at baseline. The indications for PPI administration were esophagitis (n=6, 35.3%), peptic ulcer (n=10, 58.6%) and other (n=1, 5.9%). Bacterial DNA was not detected before the initiation of PPIs. The baseline median serum levels of bacterial products and cytokines are shown in Table 2.

Correlation of cytokine and bacterial indices with the severity of liver disease

Spearman's correlation analysis showed a positive, moderately strong correlation between MELD score and

Table 1 Baseline characteristics of the study population

| | N or median | Percentage or range |
|--|-------------|-------------------------|
| Age (years) | 59.2 | 49-65 |
| Sex (M/F) | 12/5 | 70.6/29.4 |
| Current smoking | 5 | 29.4 |
| Etiology of liver disease (alcohol/HBV/HCV/autoimmune/other) | 7/4/3/2/1 | 41.2/23.5/17.7/5.9/11.8 |
| β-Blockers | 9 | 52.9 |
| PT (sec) | 14.65 | 11.9-21 |
| INR | 1.2 | 1.06-1.87 |
| Creatinine (mg/dL) | 0.85 | 0.7-1.5 |
| Urea (mg/dL) | 29.5 | 3.7-103 |
| Albumin (g/dL) | 3.8 | 2.4-4.9 |
| Total bilirubin (mg/dL) | 1.4 | 0.67-11.15 |
| Sodium (mmol/L) | 139.9 | 131-143 |
| Ascites | 3 | 17.7 |
| Encephalopathy | 1 | 5.9 |
| CP score | 7.5 | 5-12 |
| CP class (A/B/C) | 7/6/4 | 41.2/35.3/23.5 |
| MELD score | 10.5 | 7-21 |
| Months of follow up | 14 | 12-17 |

PT, prothrombin time; INR, international normalized ratio; CP, Child-Pugh; MELD, model for end-stage liver disease

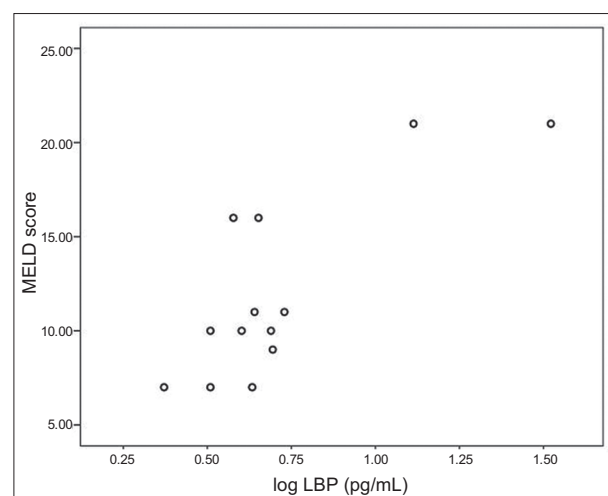


Figure 1 Scatter/dot plot presenting the correlation of baseline lipopolysaccharide binding protein (LBP) levels with Model for End-stage Liver Disease (MELD) score ($r=0.63$, $P=0.02$)

Table 2 Serum levels of cytokines and bacterial products at baseline (Time 1) and following short-term PPI administration (Time 2)

| Parameters measured | Median (range) | | Z value | P value |
|---------------------|-------------------|----------------------|---------|---------|
| | Time 1 | Time 2 | | |
| Endotoxin (EU/mL) | 3.19 (1.03-4.33) | 2.76 (1.49-86.55) | -0.12 | 0.91 |
| LBP (µg/mL) | 4.08 (3.23-12.99) | 4.65 (0-17.53) | -0.414 | 0.68 |
| NOx (µM) | 9.04 (2.44-15.72) | 7.36 (0.56-23.08) | -0.98 | 0.326 |
| TNFα (pg/mL) | 3.05 (0-8.26) | 5.4 (0-60.98) | -0.454 | 0.65 |
| TGFβ (ng/mL) | 4.85 (2.43-16.64) | 3.49 (1.54-15.94) | -0.497 | 0.619 |
| IL-1 (pg/mL) | 4.7 (0-11.95) | 8.57 (0-184.8) | -0.345 | 0.73 |
| IL-6 (pg/mL) | 4.37 (0-16.09) | 10.29 (0-31.5) | -0.227 | 0.82 |
| IL-12 (pg/mL) | 7.69 (0-25.88) | 15.96 (0-159) | -0.785 | 0.433 |
| IL-10 (pg/mL) | 6.56 (0-27.87) | 5.16 (0-34.8) | -1.07 | 0.285 |
| IL-8 (pg/mL) | 103.4 (0-281.4) | 64.45 (18.99-2892.4) | -0.308 | 0.758 |

LBP, lipopolysaccharide binding protein; NOx, nitric oxide; TNF, tumor necrosis factor; TGF, tumor growth factor; IL, interleukin

baseline LBP ($r=0.63$, $P=0.02$) levels (Fig. 1), but no other correlations were observed. Child-Pugh score was not correlated with any of the evaluated indices. No differences were observed between patients with and without ascites (data not shown).

Changes in the bacterial profile and cytokine concentrations following short-term PPI administration

No patient developed infection during the study period. Bacterial DNA was not detected during the following 4-8 weeks of PPI treatment and the serum levels of cytokines and bacterial products were not significantly different from baseline (Table 2).

Discussion

We conducted this prospective study taking into consideration the need to use PPIs in patients with cirrhosis [35], the conflicting data in the literature [22-26], and the fact that only a few studies have examined the potential association between PPIs and a broad range of bacterial infections, with controversial results [10,24]. We found that the "appropriate" use of PPIs had no effect on serum bacterial DNA and other bacterial products, or on the cytokine profile of patients with cirrhosis.

In cirrhosis, the increased susceptibility to bacterial infections is related to a deficiency of bactericidal and opsonic activity, impaired monocyte function, depressed phagocytic activity of the reticuloendothelial system, defective chemotaxis, and low levels of complement in serum [36]. The clearance of translocated bacteria by the liver is inadequate because of the impaired reticuloendothelial cell phagocytosis and the increased collateral blood flow. Urinary tract infections, SBP,

respiratory tract infections, and bacteremia are the most frequent bacterial infections seen in this setting.

Patients with liver cirrhosis rarely have normal endoscopic findings on upper gastrointestinal endoscopy [35]. In everyday clinical practice, there are several reasons for clinicians to consider the administration of PPIs in patients with liver cirrhosis, including abdominal discomfort related to ascites, dyspeptic symptoms associated with impaired small bowel motility, esophageal motor disorders, and finally, the misperception that gastroesophageal reflux contributes to variceal bleeding [27]. In a study by Tzathas *et al* [37], the authors suggested that *H. pylori* eradication does not protect all cirrhotics from ulcer recurrence and the majority of ulcers recur in *H. pylori* negative patients; therefore, omeprazole maintenance treatment is mandatory, irrespectively of *H. pylori* status. In addition there are data to support acid suppression therapy in the treatment of post-banding ulcers [8,38].

Overuse of PPIs in this setting is well documented [27]. In the study of Bajaj *et al* [9], 47% of patients receiving PPIs had no documented indication. In two other recent studies, the use of PPIs was inappropriate in 63% and 39.6% of cirrhotic patients [6], while in another prospective evaluation of intravenous PPI use, 56% of patients had no acceptable indication for their use [39]. In the Goel *et al* study, no documented indication was found in two thirds of the patients [13]. Finally, in another cohort of cirrhotic patients, PPIs were the most frequently prescribed drugs (67%), often without a clear indication (59%) [20]. In the same cohort, the use of β -blockers by cirrhotic patients had beneficial effects, reducing the prevalence of infections and improving the patient's outcome when an infection occurred [20]. Moreover, other studies indicate that the reduction of hepatic venous pressure gradient was independently associated with a decreased risk of SBP or bacteremia [40,41]. In the current study, nine patients were on β -blockers and none of them discontinued their administration before inclusion in the study. In this study we used paired samples; thus, propranolol administration does not impair our results.

Use of PPIs has been related to infections because of hypochlorhydria, impaired gastrointestinal motility [17] and neutrophil functioning [22], decreased granulocyte and monocyte oxidative burst [42], increased intestinal paracellular permeability [43] and direct effects on the immune system [9]. Small intestinal bacterial overgrowth following PPI use has been reported in both animal and human studies [44]. However, there are methodological issues, because some were single-center studies that had small sample sizes [9,11,13] and mainly included hospitalized patients with ascites [9,11,22]. In addition, most published studies did not report the duration of exposure to PPIs, so the positive association could be related to possible bias [14]. The abovementioned limitations make the interpretation of these results difficult.

In the present study, none of our patients developed an overt infection during the follow-up period. Most importantly, no differences in the bacterial load and the cytokine profile were observed over time, implying that none developed a subclinical infection. Cirrhosis-associated immune dysfunction syndrome is related to both immunodeficiency and production of proinflammatory cytokines. Immunodeficiency affects both the innate and the adaptive arm of the immune system [5]. Systemic inflammatory response syndrome has been described in 57-70% of infected patients with liver cirrhosis [45]. Bacterial-derived products, such as peptidoglycans and lipopolysaccharides, bind to toll-like receptors and stimulate the release of NOx and proinflammatory cytokines such as TNF- α , IL-1 and IL-6. In systemic inflammatory response syndrome, anti-inflammatory cytokines are unable to counterbalance the increased production of proinflammatory cytokines [1].

We measured LBP, a protein with a relatively long half-life synthesized by the liver in response to bacteremia and bacterial DNA [46], considering the various limitations that accompany the use of endotoxin for the detection of infection in cirrhosis. We observed no differences in the concentrations of the proinflammatory cytokines TNF- α , IL-1, IL-6, IL-8 and IL-12p70, the anti-inflammatory cytokines IL-10 and TGF- β , endotoxin, LBP and NOx, before and after PPI administration. These results indicate an absence of subclinical infections over time in our cohort.

In addition, we did not detect bacterial DNA in our patients. These results are in disagreement with previous studies that reported the presence of bacterial DNA in both ascitic fluid and blood of 30-40% of hospitalized patients with cirrhosis and culture-negative non-neutrocytic ascites [47]. To date, detectable bacterial DNA has been reported as an indicator of poor prognosis [48]. However, our data is in line with a study by Vlachogiannakos *et al* [34], where bacterial DNA was not detected in the blood samples of 30 patients with cirrhosis who had ascites, even though endotoxemia was common in this cohort of patients and correlated with the severity of liver disease. In addition, in the Sersté *et al* study [49], 31 asymptomatic outpatients with cirrhosis and non-neutrocytic ascites (54.8% Child-Pugh C), who underwent therapeutic paracentesis in an outpatient clinic (18 patients had several consecutive paracenteses), were tested for the presence of bacterial DNA. The test was negative in the majority of the

patients (28/31). The authors concluded that bacterial DNA is rarely detected in serum and ascitic fluid of outpatients with cirrhosis and non-neutrocytic ascites, and the strength of this observation is that a number of patients underwent repeated paracentesis. Discrepancies between the results in different reports may be due to the inclusion of patients who were admitted to hospital for complications of cirrhosis, possibly associated with enhanced bacterial translocation [50]. Another possible explanation for not detecting bacterial DNA in our patients is that transient episodes of bacterial translocation could have been missed, as we only monitored twice for bacterial DNA. Lastly, we included only 18% patients with ascites, as some report that serum bacterial DNA is present only in cirrhotics with ascites [51]. However, our results seem to be consistent with the low prevalence of SBP reported in outpatient-based series [50].

The non-randomized design and the small sample size are limitations of this study. However, we prospectively followed our patients with or without ascites and we extensively [46] investigated the presence of either an overt or a subclinical infection.

In conclusion, in this cohort, short-term PPI administration had no effect either on cytokine concentrations or bacterial

Summary Box

What is already known:

- Bacterial infections influence hemodynamics and coagulation status and constitute one of the most important reasons for the progression of liver failure, development of liver-related complications and mortality in patients with cirrhosis
- There are data suggesting that the use of proton pump inhibitors (PPIs) is associated with bacterial infections in patients with liver cirrhosis

What the new findings are:

- There is a positive correlation between Model for End-stage Liver Disease score and baseline lipopolysaccharide binding protein (LBP) levels in patients with liver cirrhosis
- Short-term PPI administration does not affect the concentrations of the proinflammatory cytokines tumor necrosis factor- α , interleukin -1, -6, -8 and -12p70, or the anti-inflammatory cytokines IL-10 and transforming growth factor- β , or the levels of endotoxin, LBP and nitric oxide
- Short-term PPI administration has no effect on the bacterial load of cirrhotic patients
- There is no association between "appropriate" PPI treatment and the development of subclinical infection in cirrhotic patients

load in patients with cirrhosis, suggesting that the “appropriate use” of PPIs is safe in this setting. However, larger studies with longer follow up are needed to confirm the safety of the long-term use of PPIs, especially in patients with advanced liver disease.

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