ABSTRACT

The development of spontaneous bacterial peritonitis (SBP) is a serious and life-threatening condition in patients with cirrhosis and ascites. The aim of this study was to determine the diagnostic potential of calprotectin in ascites for SBP in patients with liver cirrhosis and ascites before and after antibiotic treatment and to compare the mean values of calprotectin in ascites in patients with and without SBP. This prospective-observational study was comprised of 70 patients with cirrhosis and ascites, divided into two groups, the SBP and the non-SBP group. Quantitative measurements of calprotectin in ascites was completed with the Quantum Blue Calprotectin Ascites test (LF-ASC25), using the Quantum Blue Reader. The average value of calprotectin in the SBP group was 1.5 ± 0.40 µg / mL, and in the non-SBP group it was lower (0.4 ± 0.30). The difference between the mean values was statistically significant with p <0.05. The mean value of calprotectin in ascites before therapy among the SBP group was 1.5 ± 0.4, and after antibiotic therapy, the value decreased significantly to 1.0 ± 0.6; the difference between the mean values was statistically significant with p <0.05. ROC analysis indicated that calprotectin contributed to the diagnosis of SBP with a 94.3% sensitivity rating (to correctly identify positives), and the specificity was 62.5%, which corresponded to the value of 0.275. Our research confirmed that ascitic calprotectin was a good predictor, and is significantly associated with the occurrence of SBP in patients with liver cirrhosis. By monitoring the value of calprotectin in ascites on the 7th day of antibiotic treatment, the effectiveness of antibiotic treatment in patients with SBP can be determined.

Keywords: spontaneous bacterial peritonitis (SBP), calprotectin, PMNC, liver cirrhosis

INTRODUCTION

The development of spontaneous bacterial peritonitis (SBP) is a serious and life-threatening condition in patients with cirrhosis and ascites. [1–3] The gold standard for the diagnosis of SBP is a polymorphonuclear cells (PMNC) number greater than or equal to 250 in 1 mL of ascites fluid. The PMNC count in ascites can be determined in two ways: by hematological methods with a microscope and a manual counting chamber or by an automatic cell counter. Microscopic cell counting takes several hours and carries the risk of errors that depend on the observer. On the other hand, automatic cell counters give fast results in minutes; but errors can occur here as well, especially...
in ascites with relatively low levels of neutrophils in ascites. Also, during transport to the laboratory, PMNC can be dissociated and can return false negative results. Ascites in patients with cirrhosis of the liver contains other components that may affect the outcome (dense viscous ascites). [4–7] For these reasons, several authors have suggested other alternative biomarkers for rapid diagnosis of SBP. [8, 9]

OBJECTIVE

To determine the diagnostic potential of calprotectin in ascites, for SBP in patients with liver cirrhosis and ascites before and after antibiotic treatment and to compare the mean values of ascitic calprotectin in patients with and without SBP.

MATERIAL AND METHODS

In this prospective-analytical-observational study which was conducted at the University Clinic for Gastroenterohepatology in Skopje, 70 patients with cirrhosis and ascites were included, and they were divided into two groups. The division into groups was made according to the number of PMNC in the ascites. The first group included 35 patients with PMNC ≥ 250 in 1 ml of ascites fluid (SBP group) and the second group included 35 patients with PMNC < 250 in 1 ml ascites fluid (non-SBP group).

Patients included in the study were aged between 18 and 70 years. Exclusion criteria from the study were: acute liver failure, abdominal surgery in the last 3 months, infectious pleural effusion, peritoneal carcinomatosis, hemorrhagic ascites, hepatocellular carcinoma, and patients receiving antibiotics at least 2 weeks before enrollment.

After prior detailed information about the structure, content, and purpose of the study, patients were asked to sign the informed consent. The protocol of the study was in accordance with the ethical principles of the Declaration of Helsinki, and it was submitted, reviewed, and approved by the Ethics Commission of the Faculty of Medicine at the Ss. Cyril and Methodius University in Skopje.

Paracentesis was performed under aseptic conditions in a patient placed in a supine position and a puncture was made in the left or right lower quadrant of the abdomen with ultrasound imaging (none of the patients had complications associated with diagnostic paracentesis).

All diagnostic test specimens were immediately referred to the Central Clinical Laboratory. Out of a total of 20 mL of ascites, 5 mL was used for automatic PMNC counting, 5 mL for quantitative determination of calprotectin and 5 mL for biochemical analysis of ascites. At the same time, for the needs of biochemical blood tests, a venipuncture of 10 mL of blood was made. The PMNC number was determined directly from the non-centrifuged part of the ascites. 3 ml of fluid was placed in an EDTA test tube to assess the total number of cells and PMN cells, and the counting was made by using the Sysmex KxN 21 automatic cell counter model.

Quantitative measurement of calprotectin in ascites was made with the Quantum Blue Calprotectin Ascites test (LF-ASC25), using the Quantum Blue Reader. Test principle: The test is designed to selectively measure the calprotectin antigen (MRP8 / 14) by direct sandwich immunoassay. The test membrane is coated with the first monoclonal antibody (mAb), which is specific for capturing calprotectin. The second monoclonal antibody conjugates calprotectin to colloidal gold and then releases it into the reaction system after the addition of a dilute ascites sample. Anti/calprotectin-gold-conjugated calprotectin binds to membrane-coated anti-calprotectin antibodies (test line; test strip), and the remaining free gold-conjugated anti-calprotectin binds to goat anti-mouse coated test membrane (control line; control strip).

The ascites samples were diluted with Chase Buffer in a ratio of 1:5 and after 12 minutes of incubation at room temperature, the signal intensity of the test line and the control line were quantified with the BÜHLLMANN Quantum Blue®Reader. Procedure: Collected ascites samples were stored in sterile tubes in a refrigerator at a temperature of -20° C without any chemical or biological additives.

Scanning started automatically after 12 minutes (720 seconds). The range of calprotectin concentrations in ascites ranged from 0.18 μg / mL to 1.80 μg / mL. Criteria for STDs: clinical picture, PMNC number in ascites ≥250 / 1 mL and / or PMNC number <250 / in 1 mL ascites
fluid, with one bacterial species isolated in microbial culture (CNNA). The collected data were processed using the statistical program SPSS 20 and Statistica for Windows, version 10.

RESULTS

The average value of calprotectin in ascites in the SBP group was 1.5 ± 0.40, and in the non-SBP group it was lower (0.4 ± 0.30). The difference between the mean values was statistically significant with p <0.05 (t-test = 12.70849; p = 0.000000) (Tab.1 and Fig 1, 2).

<table>
<thead>
<tr>
<th>Average values of calprotectin in ascites in both groups and Student t-test, and calprotectin before and after therapy and Wilcoxon Matched Pairs test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>average</strong></td>
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<tr>
<td>calprotectin in ascites before therapy</td>
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<tr>
<td>calprotectin in ascites after therapy</td>
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<tr>
<td>Wilcoxon Matched Pairs-test</td>
</tr>
</tbody>
</table>

Table 1. Average values of calprotectin in ascites in both groups and Student t-test, and calprotectin before and after therapy and Wilcoxon Matched Pairs test

A positive, strong, statistically significant correlation between calprotectin and PMNC before therapy was registered during the analysis (Pearson’s linear correlation) (Figure 5).

The obtained value of Pearson's linear correlation coefficient (r) showed that calprotectin after therapy correlated positively with PMNC after therapy; the value of p as statistically sig-
Figure 2. Movement of calprotectin in patients individually in SBP before and after therapy.

Figure 3. Display of mean calprotectin values in SBP before and after therapy

Figure 4. ROC-curve of calprotectin as a predictor of SBP
significant confirmed the correlation. The correlation was positive and direct, meaning that the increase of calprotectin after therapy increased the number of PMNC after therapy, and vice versa (Figure 6).

Table 2. Area Under the Curve

<table>
<thead>
<tr>
<th>Area</th>
<th>Std. Error</th>
<th>Asymptomatic Sig.</th>
<th>Asymptomatic 95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.895</td>
<td>0.044</td>
<td>0.000</td>
<td>0.808-0.981</td>
</tr>
</tbody>
</table>

Figure 5. Pearson’s linear correlation between calprotectin and PMNC before therapy.

Figure 6. Pearson’s linear correlation between calprotectin and PMNC after therapy.
DISCUSSION

Over the last 20 years, the role of calprotectin as a non-invasive biomarker for the diagnosis of SBP has become increasingly important. The idea stems from its presence exclusively in neutrophils, which means that its value in body fluids is proportional to the influx of neutrophils. However, the number of studies addressing this issue is small despite promising and encouraging results, probably due to the relatively expensive method. Early publications by Homann et al. (10-12) examined the prognostic and diagnostic value of plasma calprotectin in patients with SBP. The authors showed that patients with alcoholic decompensated liver cirrhosis, who had higher concentrations of calprotectin in ascites, were at increased risk of mortality. At the same time, calprotectin was shown to be a good marker for predicting SBP relapses. Its prognostic value was demonstrated by a multivariate analysis, showing that calprotectin had a much higher prognostic value for SBP than albumin, international normalized ratio (INR), bilirubin, and ascites, and did not depend on the severity of liver disease.

Three independent studies (13–15) examined the role of fecal calprotectin (FC) and its association with cirrhosis complications, hepatic encephalopathy (HE), and SBP. The results in all three studies showed higher concentrations of FC in patients with SBP versus controls (P <0.001), as well as a positive correlation of FC with HP according to the West-Haven criteria (p <0.001). Burri et al. (16) in 2013 were the first who examined the correlation between calprotectin values in ascites and PMNC. In their prospective study, 130 samples of ascites were analyzed. Calprotectin was measured in 1 mL ascites by two biochemical methods: ELISA and point-of-care (POC) by Quantum Blue® Reader (Bühlmann Laboratories).

The results showed a positive correlation between PMNC and calprotectin for both tests (Spirman, r = 0.457 for ELISA, r = 0.473 for POC). Calprotectin concentrations detected by ELISA [median 0.43 μg / mL, (IQR) 0.23-1.23 (range 0.10-14.93)] were similar to those of POC [median 0.38 μg / mL, IQR 0.38–0.56 (range 0.38–13.31)]. Using the optimal cross-sectional value for ELISA (0.63 μg / mL), calprotectin in ascites had a sensitivity of 94.8%, specificity of 89.2%, positive and negative probability rates of 8.76 and 0.06, positive and negative predictive value of 60.0% and 99.0% and a total accuracy of 90.0%, while using the optimal cross-sectional value for POC (0.51 μg / mL), the corresponding values were 100.0%, 84.7%, 6.53%, 0.00%, 52.8%, 100% and 87.7%.

The author concluded that calprotectin in ascites can reliably predict PMNC > 250 / μL, and can be used in the diagnosis of SBP. The same study showed a lower mean value of calprotectin in ascites compared to the mean value of calprotectin in our study. However, it should be noted that this study was composed of inhomogeneous groups (11 patients with malignant ascites and 4 patients with SBP) and it cannot be concluded that the published mean value is realistic for the diagnosis of SBP. Similar results were reported in a study by Abdel-Razik A et al. (9) with an average calprotectin ascites value of 0.445 μg / ml.

Another prospective study [17] quantitatively measured calprotectin in ascites using POC with Bühlmann®. The results of the study presented an optimal cut off value of calprotectin above 1.57 μg / ml with high sensitivity (87.8%), specificity (97.9%) and positive (97.3%) and negative (90.2%) predictive values for diagnosing SBP.

A recent prospective study by Weil D. et al. [18] from 2018 evaluated the diagnostic significance of calprotectin concentration in ascites in SBP patients using the same laboratory method as we used in our study. The study registered a positive correlation between the values of calprotectin and PMNC (r = 0.57; p <0.001). The optimal calprotectin threshold for the diagnosis of SBP was 1.51 μg / mL (with sensitivity, specificity, and positive and negative predictive values of 86.1%, 92.0%, 65.9%, and 97.3%, respectively). Our analysis recorded a strong, positive, statistically significant correlation between the value of calprotectin in ascites and PMNC (Pearson linear correlation r = 0.7740 p = 0.000).
The average value of calprotectin was 1.50 μg/mL, which was consistent with the value demonstrated in the study by Heikl AA et al. [19].

The level of calprotectin in ascites fluid was significantly correlated with PMNC and was higher in patients with SBP (P <0.001), with a cut-off value for SBP at 783 ng/ml, with sensitivity, specificity, positive predictive value and negative predictive value and accuracy of 90%, 1005, 100%, 80% and 92.9%, respectively. Similar results were presented in the studies of Fayrouz and Ali [20,21]. Our study also recorded a statistically significant difference between the mean values of calprotectin in ascites in patients with SBP versus non-SBP (1.5 ± 0.40 vs. 0.4 ± 0.30; t-test = 12.70849; p = 0.00000).

An analysis of individual variables confirmed that PMNC was a significant predictor of calprotectin levels in patients with SBP. For PMNC values, the partial regression coefficient was 0.68, and the t-test showed that the PMNC effect of calprotectin was statistically significant at p = 0.000000. If PMNC increases by one unit of measure, the value of calprotectin concentration increases by 0.687187, on average. ROC analysis indicated that calprotectin is an excellent predictor for diagnosing SBP with 89.5% (p = 0.044) (excellent predictor), closer to the ideal value of 1.0 and above the worst value of 0.5. According to the coordinates of the ROC curve for calprotectin, the sensitivity was 94.3% (to correctly identify positive), the specificity was 62.5%, which corresponded to the value of 0.275 (cut off value).

Our multiple regression analysis recorded an association between calprotectin (dependent criterion variable) and a system of variables – total protein in serum and ascites, serum and ascites CRP, PMNC, Child-Turcotte-Pugh II score, MELD score in patients when the multiple regression coefficient (R) was 0.799. The coefficient of determination (R2) was 0.64 and indicated that all independent variables together affected calprotectin by 64%, while 36% were influenced by other factors.

The significance of the multiple regression coefficient test, based on the F-distribution, showed that the influence of the system of variables on calprotectin (dependent variable) in this group of patients with SBP was statistically significant for p = 0.00000. Other studies with multiple regression analysis showed a positive correlation between the value of calprotectin in ascites with PMNC, C-reactive protein (CRP) and total proteins in ascites (P <0.001, P = 0.036 and P <0.001), while a negative correlation was registered with age, etiology, sex, Child–Pugh and MELD scores as well as bacterial culture (P = 0.84; P = 0.41; P = 0.10, P = 0.86, P = 0.49 and P = 0.10). [20, 21, 22]

A pilot study [23] was published in 2020 to assess the probable factors predicting recurrence of SBP in patients recovering from the first episode of SBP, including patients with cirrhosis who did not receive secondary antibiotic prophylaxis for SBP. The study evaluated the values of the interferon-induced protein (IP-10), calprotectin, IL-6, and TNF-α. A multivariate analysis showed IP-10 (≥1220 pg/ml), calprotectin (50550 ng/ml), serum albumin (<2.5 g/dl), non-use of β-blockers, and use of a proton pump inhibitor (PPI) to be independent variables that can predict recurrence of SBP. The same author, in his retrospective cohort study, proposes a new, non-invasive scoring system (Mansoura) to confirm or exclude SBP. This system includes 4 variables: age (at least 55), mean Tr volume MPV (at least 8.5 fl), neutrophil-to-lymphocyte ratio NLR (at least 2.5), and CRP (at least 40 mg/l). The scoring system is as follows: age, MPV and NLR are scored with 1 point each, while CRP with 2 points. Thus, a specificity of 98.2% is achieved with a positive predictive value for diagnosing SBP of 88.1%. [24]

Previous studies have evaluated the efficacy and optimal duration of therapy in patients with SBP according to clinical criteria and by reducing the number of PMNC in ascites on the second day of therapy. [1, 25–28] However, a small number of studies have indicated possible errors (false low PMNC), resulting in higher mortality and higher recurrence rates of SBP. There is also evidence that not all SBP patients improve their clinical picture despite a reduced number of PMNC. [29–32] Therefore, we determined, in our study, the value of calprotectin in ascites before and after therapy. To our knowledge, there are no publications that determine the values of calprotectin in ascites before and after antibiotic treatment.

In our study, the average value of calprotectin in ascites before antibiotic therapy was 1.5 ± 0.4, and after antibiotic therapy a statistically significant decrease in value of 1.0 ± 0.6 was registered (Wilcoxon Matched Pairs test, T = 5, 00000, Z = 4.594930, p = 0.000004). The
obtained value of Pearson’s linear correlation coefficient \( r = 0.8894, p = 0.000 \) showed that calprotectin after therapy correlated positively with the number of PMNC per therapy; the value of \( p \) as statistically significant confirmed the correlation. The correlation was positive, i.e., direct, which means that the increase in calprotectin after therapy increased the number of PMNC after therapy, and vice versa.

**CONCLUSION**

Calprotectin in ascites can reliably predict PMNC > 250/μL, and can be used as an alternative to other conventional methods for diagnosing SBP. By monitoring the value of calprotectin in ascites on the 7th day of antibiotic treatment, the effectiveness of antibiotic treatment in patients with SBP can be determined.

**REFERENCES**


Резиме

ДИЈАГНОСТИЧКИ ПОТЕНЦИЈАЛ НА КАЛПРОТЕКТИНОТ ЗА СПОНТАН БАКТЕРСКИ ПЕРИТОНИТИС КАЈ ПАЦИЕНТИТЕ СО ЦРНОДРОБНА ЦИРОЗА И АСЦИТ

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Развиważнето на спонтанот бактерски перитонитис (СБП) кај пациентите со црнодробна цироза и асцит е сериозна и животозагрозувачка состојба.

Целта на оваа студија беше да се одреди дијагностичкиот потенцијал на калпротектинот во асцит, за СБП кают пациентите со црнодробна цироза и асцит пред антибиотискиот третман и по него и да се споредат средните вредности на калпротектинот во асцитот кают пациентите со СБП и не-СБП. Оваа проспективно-опсерваториска студија беше составена од 70 пациенти со црнодробна цироза и асцит, поделени во две групи, СБП и не-СБП. Квантитативните мерења на калпротектинот во асцитот беа одредувани со Quantum Blue Calprotectin Ascites test (LF-ASC25), со помош на Quantum Blue Reader. Просечната вредност на калпротектинот во групата СБП беше 1,5 ± 0,40 µg / mL, а во групата не-СБП беше помала (0,4 ± 0,30). Разликата меѓу средните вредности на калпротектинот пред терапијата беше 1,5 ± 0,4, а по терапијата со антибиотици, вредноста значително се намали на 1,0 ± 0,6; разликата меѓу средните вредности на калпротектинот пред и после терапијата кај групата СБП беше 0,5 ± 0,3. Рангирањето на калпротектинот во групата СБП беше значително над средната вредност на калпротектинот во групата не-СБП. ROC-анализата покажа дека калпротектинот придонел за дијагноза на СБП со чувствителност од 94,3 %, специфичност од 62,5 %, што одговара на вредноста од 0,275.

Откриениот извод бил дека одредувањето на калпротектинот во асцит е добар предиктор и сигнифицира асоциран со прикажана на пациентите со црнодробна цироза. Следењето на вредностите на калпротектинот во асцитот седмиот ден од почетокот на третманот, може да ја утврди ефикасноста на антибиотискиот третман кают пациентите со СБП.

Ключни зборови: спонтан бактерски перитонитис (СБП), калпротектин, ПМНК, црнодробна цироза