International Journal of General Medicine

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ORIGINAL RESEARCH

Portal Vein Thrombosis in Patients with Liver Cirrhosis: What Went Wrong?

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Purpose: This study aimed to explore inflammatory biomarkers, stool's functional bacterial groups and their possible link to portal vein thrombosis (PVT) in patients with liver cirrhosis (LC).

Materials and Methods: An observational study of 300 participants: 200 inhospital cirrhotic patients, who met inclusion criteria, equally assigned into two groups, based on the presence or absence of PVT and 100 healthy controls was carried out.

Results: The PVT group displayed significant differences related to older age, cigarettes smoking history, emergency admission, higher Child-Pugh score, metabolic related disorders and nonalcoholic fatty liver disease, as well as non-obstructive aspects, with chronic thrombi. The PVT group exhibited significant differences related to biomarkers such as tumor necrosis factor (TNF)-alpha, C-reactive protein (CRP), D-dimers (D-D), as well as gut overall dysbiosis (DB) and alteration of different functional bacterial groups of the gut microbiota. Strong positive correlations were observed between PVT severity, and TNF-alpha, CRP, D-D as well as lipopolysaccharide (LPS) positive bacteria. Esophageal varices, age and abdominal pain were independent predictors for PVT severity as well as CRP, TNF-alpha and D-D.

Conclusion: Patients with LC and PVT displayed elevation of TNF-alpha, CRP, D-D alterations of the functional gut microbiota, as well as several morphological and clinical particularities. Although the LPS positive gut microbiota was linked to inflammatory biomarkers and PVT severity, it was not proven to be an independent predictor of the PVT severity like CRP, TNF-alpha and D-D.

Keywords: portal vein obstruction, end stage liver disease, biomarkers, gut functional bacteria

Introduction

The natural history of liver cirrhosis (LC) may be marked by thrombotic events, either in the peripheral circulation as deep vein thrombosis or in the portal vein circulation, which eventually worsen the prognosis. Portal vein thrombosis (PVT) is still considered a rare and challenging condition, that often requires a multidisciplinary approach to treat. In some patients, due to silent clinical presentation, diagnosis is either incidental within an imaging study or postmortem. The real incidence and prevalence of PVT is still unknown. Given the diagnostic approach, the prevalence of PVT in epidemiological studies varies from a general incidence rate of 1.1% according to a large population study, based on consecutive necropsies, to 1.7% according to imaging studies.[1,2] Since Virchow postulated the triad that includes vascular stasis, hypercoagulability and endothelial injury for the development of PVT, there has been a growing body of research in this area.[3,4] Many thrombogenic factors either local or general, inherited or acquired are considered to be involved in the initiation and development of venous thrombosis, including PVT.[5] When it comes to LC there are some

Received: 26 April 2023
Accepted: 10 August 2023
Published: 28 August 2023

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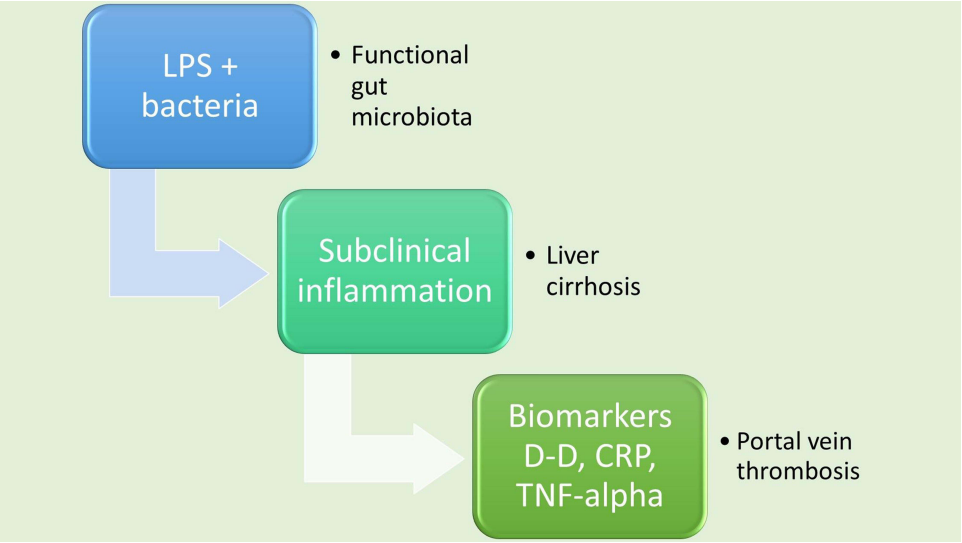
Graphical Abstract



particularities that should be pointed out. LC is known to be a risk factor for PVT development and many studies report up to 40% of PVT incidence in followed-up patients.[6] The probability of PVT occurrence increases proportionally with the severity of the liver disease. This situation is linked to the failure of protein synthesis responsible for coagulation/anticoagulation processes due to hepatocyte insufficiency and the decrease of velocities in portal vein territory secondary to severe liver fibrosis, as well as to inflammation secondary to multiple triggers related to bacterial gut dysbiosis (DB), translocation and endotoxemia.[7–10] The decrease of the portal vein velocity under 15 cm/sec represents the cutoffs for the risk of PVT and any velocities below this come with a greater risk for PVT and related mortality.[11] The PVT diagnosis has improved over recent years, as a consequence of high accuracy imaging methods. There are no specific signs and symptoms in patients with PVT and LC, and many patients present the so called "silent" clinical forms of the disease, that are often hidden by the primary liver condition. These patients are incidentally diagnosed within imaging investigations performed by systematic follow-up for LC, especially in those waiting for liver transplantation (LT).[12,13] Although the PVT diagnosis has gained in time a rational basis and current guidelines assure it, the PVT classification remains however a problem to debate.[14,15] There are currently many available classifications of PVT, but none of them has succeeded so far to overcome all issues related to this topic. One classification that uses time as criteria, divided PVT into acute and chronic, depending on the moment of onset, but this is not applicable in the clinical area and therefore is not used on regular basis.[16,17] Thus, the morphological aspects related to the thrombus localization and its characteristics, proportion of the venous occlusion, as well as local consequences, not to mention the liver status are given more credit and have been taken into account in order to set a classification as accurate as possible and to provide a highly efficient tool for clinicians. According to Ponziani et al, from clinical point of view, the PVT extension is the main characteristic to be taken into account, in terms of operability and outcome, resulting in four situations, as follows: (1) confined to the portal vein; (2) extension to the superior mesenteric vein, but with patency preserved; (3) extension to the entire splanchnic venous system, with associated large collaterals; and (4) extension to the entire splanchnic venous system, with only fine collaterals.[18] The Baveno VI consensus focused more on the anatomic aspects of PVT with obstruction of the extrahepatic portal vein, with or without involvement of the intrahepatic portal vein territories or other segments of the splanchnic venous axis. Isolated thrombosis of splenic or superior mesenteric veins is not included in this classification.[19] While PVT was for many years controversial regarding indication and procedural difficulties related to LT, a novel classification of non-malignant PVT has been recently released, providing hepatologists and surgeons with useful guides when it comes to taking important decisions.[20] The therapeutic options are besides "watch and see" with

close monitoring, to a less or more invasive approach depending on the clinician opinions and the specific goals to achieve.[21] The natural history and the outcome of PVT is still difficult to define, due to variability of etiology and onset, grade of vein obstruction, several underlying conditions, as well as multiple possible comorbidities. The most important factors that could significantly alter the outcome of PVT in general populations seem to be malignancies and advanced stages of liver disease.[22] Many studies highlighted the relation between gut microbiota and liver pathology from different perspectives. Recent data associated high levels of circulating lipopolysaccharides (LPSs) in the portal vein territory in patients with LC to PVT events, highlighting once again the role of the gut-liver axis. The release of the outer membrane's glycolipids from gram-negative bacteria and the subsequent endotoxemia could be the link between the gut microbiota DB and the increased coagulability.[23] Other researchers recently reported interesting results in animal model in which some probiotics based on *Lactobacillus delbrueckii* seemed to decrease both the mouse thrombosis and the liver oxidative stress.[24]

The primary aim of this study was to assess the particularities of the PVT in patients with LC in terms of serum inflammatory biomarkers, stool microbiology, morphological and clinical related spectrum. The secondary aim was to explore the possible link between gut microbiota DB, inflammation biomarkers and the PVT severity.

Materials and Methods

Study Design

A cross-sectional, exploratory study of cirrhotic patients was carried out. A consecutive sample recruitment of 200 patients with LC was performed during hospitalization in an Internal Medicine Department, University Hospital, between 2019–2022. Participants of the study were represented by 200 patients with LC, assigned into two groups: 100 patients with PVT (group 1), 100 patients without PVT (group 2) and 100 healthy controls. Controls were recruited from hospital administration personal and matched for age and gender. Exclusion criteria: patients with severe organ failure (respiratory, cardiac, renal), cancer including hepatocellular carcinoma, sepsis, coma, stroke, trauma, recent surgical procedures, spontaneous bacterial peritonitis, hemorrhagic conditions, systemic inflammatory conditions, hematologic and thyroid autoimmune conditions, deep vein thrombosis, history of severe acute respiratory syndrome - coronavirus 2 (SARS-COV2) infection, ongoing anticoagulant, antibiotic or probiotic treatment. The study complies with the Declaration of Helsinki, was approved by the Ethical Committee of Scientific Research of the University of Medicine and Pharmacy "Victor Babes" from Timisoara (approval number 10/02.03.2018) and written informed consent was obtained from all participants.

Clinical Approach of Research Participants

Thorough clinical examination was performed in patients and controls, including body mass index (BMI) assessment and blood pressure measurement. Venous blood samples were collected in special vacutainers, early in the morning in fasting patients. Complete blood count (CBC), erythrocyte sedimentation rate (ESR), international normalized ratio (INR), transaminases: (ALAT), total bilirubin (TB), plasma protein electrophoresis with albumin (alb) dosage, creatinine (creat), fast blood glucose (FBG), HBs antigen, Delta antigen, anti HCV antibodies, plasma iron and copper, alpha 1 antitrypsin, antinuclear antibodies (ANA), antimitochondrial antibodies (AMA), using routine standardized laboratory methods. Serum biomarkers were performed in all research participants, using standardized, accredited methods in European Community. Of these, inflammatory molecular biomarkers such as: C-reactive protein (CRP) were determined by latex–immunoturbidimetry methods and tumor necrosis factor (TNF)-alpha by chemiluminescent immunoassay (CLIA). Other studied molecular biomarkers were alpha fetoprotein (AFP) L3%, as standard serum biomarker for hepatocellular carcinoma, that was determined by isotachophoresis with laser induced fluorescence and D-dimers (D-D,) the serum biomarker for hypercoagulability, that was determined by latex-automatic agglutination with photometric detection method.

Thrombophilia panel, lupus anticoagulant, and anti-phospholipid antibodies were not performed on a routine ground, but only in selected cases. Patients included in present study in pandemic years were tested negative for SARS-COV 2 infection. Stool microbiology with DB assessment after identification of cultural species using MALDI-TOF method was

also performed, in all participants. Severity of gut microbiota was scored as: 1 = mild, 2 = moderate, 3 = severe.[25] 13-RNA next generation sequencing (NGS) was performed in patients with gut DB[26] Abdominal duplex ultrasound examination with Acusson S-3000, Siemens, high resolution ultrasound equipment with 3.5–5 MHz, convex array probes, contrast enhanced ultrasonography (CEUS), as well as abdominal CT and MRI were performed. Uniform protocols of abdominal ultrasound examination were used for each research participants, consisting in Duplex exams performed early in the morning, after 8 hours of fasting. The diagnostic of end stage liver disease, respective severe fibrosis (F4) was established based on noninvasive measurement of liver stiffness using point shear wave elastography (p SWE). The cutoffs for staging significant fibrosis, severe fibrosis and cirrhosis were 1.34m/s, 1.55m/s and 1.8m/s.[27,28] Child-Pugh score was used to assess severity of liver disease, as follows: A = 5–6 points, B = 7–9 points and C≥ 10 points.[29,30] Ascites was graded on a scale from 0 (absence) to 3 (massive) according to semiquantitative evaluation based on imaging methods.[31] The paracentesis was performed in order to rule out a spontaneous bacterial peritonitis or in patients with large volume/refractory ascites. Upper and lower digestive video-endoscopies (Olympus video endoscopes) were performed; esophageal varices were graded according to modified Paquet classification from 0 (absence) to 3 (large) and Forrest classification was used to assess the severity of upper digestive bleeding.[32,33] Encephalopathy was assessed according to West Haven criteria, based on clinical examination from 0 (no overt encephalopathy) to 4 (severe).[34] The abdominal pain was scored from absent (0) to severe (3). The short outcome during hospitalization ranged from 1 to 4, as follows: 1 = good outcome with improvement of clinical/biological status, 2 = maintaining the same state (neither improving, nor aggravation), 3 = bad outcome (state deterioration or complications ± surgical interventions), and 4 = death. The liver tissue samples were obtained either intra-vitam, during invasive procedures or in postmortem. The tissue samples protocol of pathological examination consisted of fixation with 10% formalin and embedding in melted paraffin. The obtained blocks were mounted on a microtome and sliced into thin sections. Usual hematoxylin-eosin (HE) staining of rehydrated sections was further performed and consecutive examination by optical microscopy (OM) was realized.[35]

Definition and Diagnosis of PVT

The imaging data (abdominal ultrasound: Duplex and CEUS, contrast CT/MRI exams) provided information related to the localization of the thrombus, the patency of the affected vessel (obstructive vs. nonobstructive thrombi) and permitted to classify the thrombi into acute and chronic with or without cavernous transformation. The assessment of PVT severity was based mainly on the morphologic description of the thrombus extension, as follows: 1 = thrombosis restricted to intrahepatic branches or main portal vein, 2 = thrombosis affecting both branches and main portal vein, and 3 = extension of thrombosis to splenic or mesenteric veins ± collaterals development.[36–38]

The Flow Chart of Research Participants Inclusion

Six hundred and twenty patients with confirmed diagnosis of LC, hospitalized in an Internal Medicine Department, University Hospital were checked for eligibility between 01.03.2019 and 01.11.2022. Of these patients, 200 cases met the inclusion criteria and joined this study. Figure 1 displays the inclusion flow chart's main steps in order to achieve the study group. Intermediate cohort I had 363 participants, because 85 patients had severe organ failure, 23 patients had oncologic conditions, 32 patients had hepatocellular carcinoma, 11 patients had spontaneous bacterial peritonitis, 14 patients had recent surgery, 18 patients had inflammatory bowel disease and 74 patients have been recently diagnosed with SARS- COV 2 infection. Intermediate cohort II was represented by 211 patients, while 35 patients already received anticoagulants, 65 patients had recent antibiotic therapy and 52 patients were under probiotics. The final cohort of patients enrolled in the present study reached 200 patients, while 11 patients did not agree to join the study.

Statistical Analysis

The Graph Pad Prism 9.5.1 (733) software (Graph Pad Software, Inc., La Jolla, CA, USA) was used in order to perform the statistical analysis. The variables were expressed as follows: quantitative as mean values (MV) ± standard deviation (SD) and categorical data as percentages. Chi-squared test was used to compare groups, when categorical variables were analyzed. The unpaired *t*-test was calculated, with confidence interval CI = 95%, $p \leq 0.05$ being considered statistically significant. The nonparametric Pearson's correlation test was performed with the calculation of "r" coefficient in order to

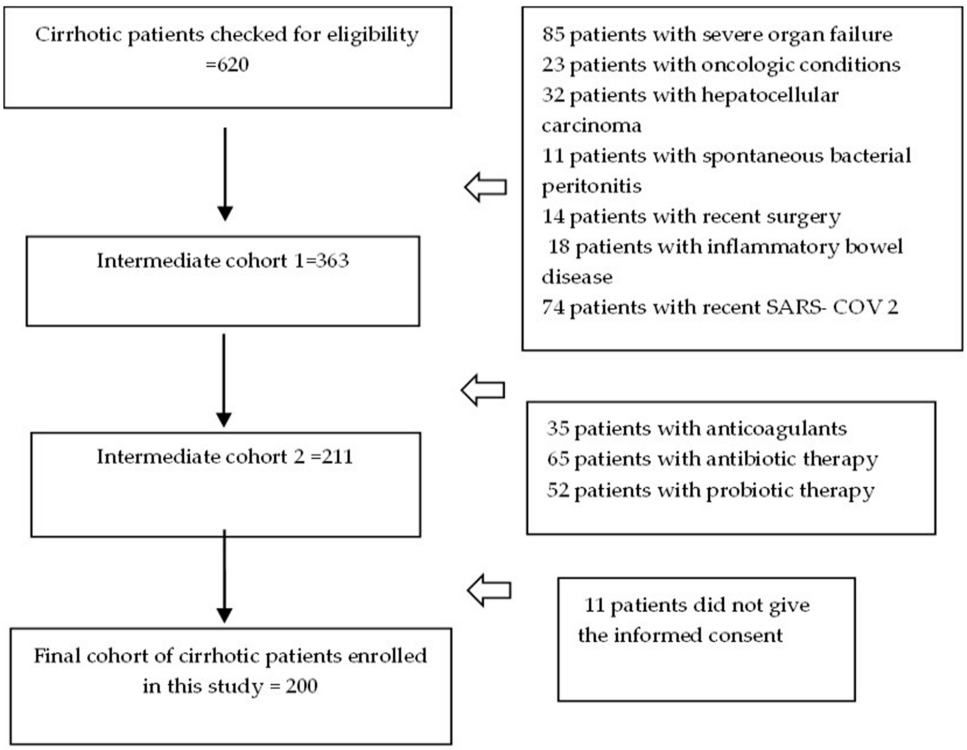


Figure 1 The study participants inclusion flow-chart.
Abbreviation: SARS-COV2, severe acute respiratory syndrome-coronavirus 2.

assess the magnitude and direction of correlations. The relationship between variables was expressed as an equation of linear regression and graphs were consecutively drawn. Multivariate analysis was consecutively used to estimate the relationship between some independent variables either clinical data or biological parameters and one dependent variable represented by PVT.

Results

This is an observational, exploratory study performed on 300 research participants: 200 consecutive inpatients with LC, divided into even groups, based on the presence or absence of PVT, as follows: the study group: PVT (+), the comparison group: PVT (-) and 100 healthy controls. The baseline demographic and biologic aspects of study population, patients and controls, such as: CBC, ALT, TNF-alpha, CRP, creatinine (creat), FBG, D-D, alb, TB, INR, AFP and overall gut DB are illustrated in Table 1.

As seen in Table 1, the PVT+ group presented statistically significant differences related to age, elevation of leukocytes, TNF-alpha, CRP, D-D, INR, DB range, LPS+ bacteria and decreased albuminemia, when compared to PVT- group, as well as to controls. Both groups (PVT+ and PVT-) displayed similarities of gender and residence distribution, with high incidence of male gender and urban location.

The most suggestive clinical data related to cigarettes smoking, BMI, admission, signs and symptoms, etiology of liver disease, complications, comorbidities, treatment, as well as inhospital outcome are illustrated in Table 2.

As observed in Table 2, patients with PVT displayed a lot of significant clinical differences related to smoking history, admission by ER for abdominal pain, variceal bleeding and overt encephalopathy, cirrhosis severity, complicated GSD, metabolic conditions such as: obesity and NAFLD related LC, T2DM/IGT, endoscopic treatment, statins, oral antidiabetics and anticoagulant therapy, as well as outcome.

Table 3 displays various aspects related to the alteration of stool's microbiome in dysbiotic patients.

As illustrated in Table 3, more than 60% of dysbiotic patients displayed decreased F/B ratio, 88% of patients presented enterotypes1I and 2 and only 12% enterotype 3, 76% exhibited decreased H index of microbiota biodiversity.

Table 1 Demographic and Biologic Characteristics in Research Participants

Variables	PVT +	PVT -	CON	P_1 PVT+ vs. CON	P_2 PVT- vs. CON	P_3PVT+ vs. PVT-
Age (years)	61.48±10.73	57.18±8.05	56.52±5.12	**<0.0001**	0.4899	**0.0256**
Gender ratio M/W	76%/24%	64%/36%	61%/39%	**0.0027**	0.6621	0.3594
U/R residence	68%/32%	68%/32%	78%/18%	0.1121	0.1121	1
Hb (g/dl)	10.75±1.49	10.89±2.38	12.56±1.02	**<0.0001**	**<0.0001**	0.7148
L/mm^3	7.33x10^3±2.94x10^3	6.30 × 10^3±1.84 × 10^3	5.8 × 10^3±0.9 x10^3	**<0.0001**	**0.0155**	**0.0395**
Plt/mm^3	196.32x10^3±152.92x10^3	240.10 × 10^3±104.18 × 10^3	285.50 × 10^3± 48.30 × 10^3	**<0.0001**	**0.0051**	0.0975
Plt<150x10^3	44%	36%	0%	**<0.0001**	**<0.0001**	0.4166
ALT (IU)	60.98±87.80	60.20±18.12	18. 78±5.22	**<0.0001**	**<0.0001**	0.9511
CRP (mg/dl)	1.68±1.15	0.52±0.11	0.21±0.03	**<0.0001**	**<0.0001**	**<0.0001**
TNF-alpha (pg/ml^2)	9.77±1.48	8.32±0.77	3.43±0.44	**<0.0001**	**<0.0001**	**<0.0001**
Creat (mg/dl)	1.17±0.78	1.03±0.40	0.71±0.09	**<0.0001**	**<0.0001**	0.2395
FPG (mg/dl)	102.10±23.97	97.80±8.55	87.21±5.67	**<0.0001**	**<0.0001**	0.2416
D-D (µg/mL)	0.72±1.27	0.24±0.05	0.13±0.014	**<0.0001**	**<0.0001**	**0.0093**
Alb (g/l)	3.31±0.54	3.61±0.36	4.81±0.25	**<0.0001**	**<0.0001**	**0.0024**
TB (mg/dl)	2.13±2.65	1.80±0.88	0.77±0.02	**<0.0001**	**<0.0001**	0.41
INR	1.678±1.448	1.138±0.199	0.92±0.05	**<0.0001**	**<0.0001**	**0.0104**
AFP (µg/mL)	7.455±4.768	7.222±3.993	2.54±0.49	**<0.0001**	**<0.0001**	0.7083
Overall DB	2.01±0.89	1.48±0.73	0.31±0.44	**<0.0001**	**<0.0001**	**<0.0001**
Increased LPS + bacteria (%)	53%	26%	0%	**<0.0001**	**<0.0001**	**0.0001**

Note: Bold p values indicate statistically significant.

Abbreviations: PVT, portal vein thrombosis; CON, controls; M/W, Men/Women; U/R, Urban/Rural; Hb, hemoglobin; L, leukocytes; Plt, platelets; ALT, alanine-aminotransferase; CRP, C-reactive protein; create, creatinine; TNF, tumor necrosis factor; FPG, fast plasma glucose; D-D, D-dimers; alb, albumin; TB, total bilirubin; INR, international normalized ratio; AFP, alpha-fetoprotein; DB, dysbiosis; LPS, lipopolysaccharides; g/dl, grams/deciliter; mm, millimeter; IU, international units; mg/dl, milligrams/deciliter; µg/mL, micrograms/milliliter; pg/mL, picograms/milliliter; ng/mL, nanograms/milliliter.

Table 2 Clinical Aspects in Research Population

Clinical Data	PVT (+)	PVT (-)	p
Smoking history	60%	32%	**0.0052**
BMI>30kg/m2	32%	10%	**0.0072**
Admission by ER	52%	24%	**0.04**
Variceal bleeding	48%	20%	**0.03**
Abdominal pain	1.48±0.86	0.66±0.52	**<0.0001**
Ascites	1.36±1.01	0.7±0.65	**0.0002**
Overt Encephalopathy	68%	48%	**0.0438**
Jaundice	38%	32%	0.5315
Child-Pugh score	10.06±2.50	8.68±2.22	**0.0044**
Viral etiology	24%	28%	0.6501
Toxic etiology (alcohol)	20%	28%	0.3514
Mixed toxic and viral etiology	24%	32%	0.3754
NAFLD related cirrhosis	30%	11%	**0.0009**
Miscellaneous etiology	2%	1%	0.5617
G-I associated conditions	52%	48%	0.6906

(*Continued*)

Table 2 (Continued).

Clinical Data	PVT (+)	PVT (-)	p
Complicated GSD	18%	0%	**0.0018**
Pancreatitis	4%	0%	0.1567
T2DM/IGT	48%	28%	**0.0404**
CKD	32%	28%	0.6641
C-V conditions	56	52	0.6897
Endoscopic Treatment	38%	12%	**0.0028**
Bleeding recurrence	4%	0%	0.1567
ACT	48%	0%	**<0.0001**
Insulin	8%	3%	0.1219
OAD Metformin	2%	10%	**0.0175**
Statins	11%	22%	**0.0366**
Inpatient mortality	8%	4%	0.4021
Outcome scoring	1.98±0.82	1.56±0.76	**0.0093**

Note: Bold p values indicate statistically significant.
Abbreviations: PVT, portal vein thrombosis; BMI, body mass index; NAFLD, nonalcoholic fatty liver disease; LC, liver cirrhosis; G-I, gastro-intestinal; GSD, gallstone disease; T2DM, type 2 diabetes mellitus; IGT, impaired glucose tolerance; CKD, chronic kidney disease; C-V, cardio-vascular; ACT, anticoagulant treatment; OAD, oral antidiabetics.

Table 3 Stool's Microbiota Alterations in Study Group

Variables	Dysbiotic Patients with PVT
Overall DB score	2.31±0.52
F/B	2.77±0.68
Decreased F/B	64%
Enterotype 1	45%
Enterotype 2	43%
Enterotype 3	12%
Biodiversity Shannon-Wiener H index	2.68±0.51
Decreased Shannon-Wiener H index	76%
Increased LPS (+) bacteria	63%
Decreased mucin degrading bacteria	42%
Decreased mucosa protective bacteria	38%
Increased neuroactive bacteria	44%

Abbreviations: PVT, portal vein thrombosis; F/B, Firmicutes/Bacteroidetes; DB, dysbiosis; LPS, lipopolysaccharides.

Table 4 Morphologic Particularities of PVT in Study Group

Variables	Incidence
Occlusive	8%
Non occlusive	92%
Main portal vein thrombosis	78%
PV branches	
Left	16%
Right	4%
Both	8%
Association main trunk - portal branches	16%
Mesenteric veins extension	4%
Acute	12%
Chronic without portal cavernoma	72%
Chronic with portal cavernoma	16%
Portal biliopathy	12%

Abbreviation: PV, portal veins.

Related to functional microbiota: 63% of patients presented increased LPS+ bacteria, 42% displayed decreased mucin degrading bacteria, 38% presented decreased mucosa protective bacteria and 44% had increased neuroactive bacteria.

Table 4 presents morphological aspects of PVT found in patients with LC. As illustrated below, occlusive PVT was observed in only 8% of cases. The localization of PVT was represented by the main portal trunk in 78%, the rest of cases presented intrahepatic branch thrombosis, association of portal trunk to branch thrombosis or extension to mesenteric veins. Acute PVT was diagnosed in 12% of these cases, whereas chronic thrombosis represented 88%, most of them without portal cavernoma. Portal biliopathy was observed in 12% of cases.

Study of the distribution of the various functional gut microbiota, in PVT+ patients vs. PVT- patients presented in Figures 2–4 revealed that both studied groups exhibited alterations of the functional microbiota. No significant

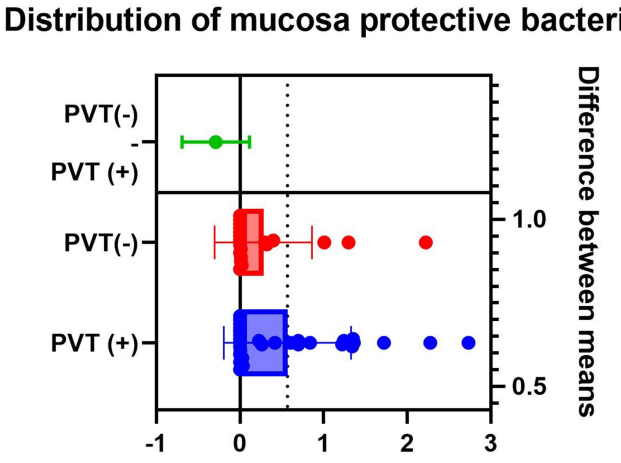


Figure 2 Distribution of mucosa protective bacteria in patients with LC.
Abbreviation: PVT, portal vein thrombosis.

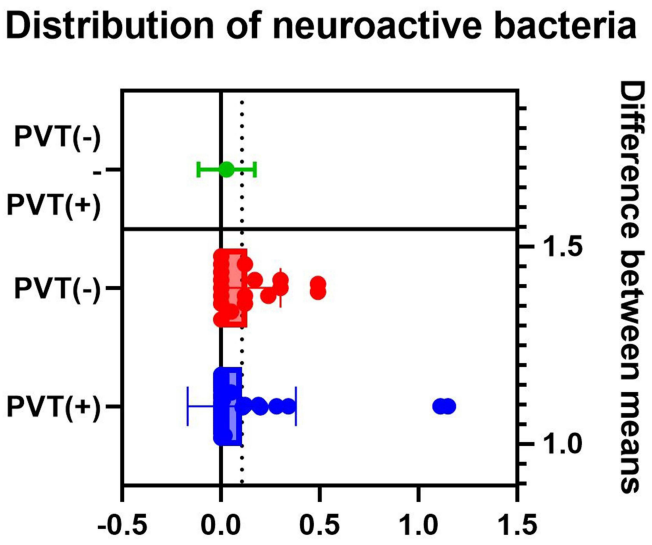


Figure 3 Distribution of neuroactive bacteria in patients with LC.
Abbreviation: PVT, portal vein thrombosis.

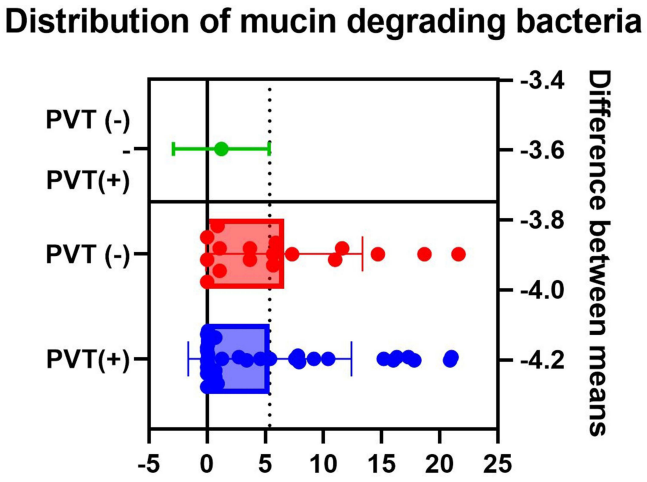


Figure 4 Distribution of mucin degrading bacteria in patients with LC.
Abbreviation: PVT, portal vein thrombosis.

differences were noted: for mucosa protective bacteria: p = 0.1562, for neuroactive bacteria: p = 0.6926 or for mucin degrading bacteria: p = 0.5494.

However, significant differences were observed related to LPS + bacteria in favor of cirrhotic patients with PVT (p = 0.0398), as illustrated in Figure 5.

The results of the Pearson's analysis of correlations of PVT severity to several biologic variables are illustrated in Figure 6. The severity of PVT positively strong correlated to CRP, D-D, TNF-alpha.

Regarding certain clinical aspects, such as: age, short inhospital outcome, esophageal varices, abdominal pain and overt encephalopathy, we have noted strong positive correlations to PVT severity, as illustrated in Figure 7.

Considering that significant differences were observed in terms of functional bacteria only in the case of LPS+ bacteria, further correlations studies were performed, as illustrated in Figure 8.

As observed in Figure 8, LPS+ bacteria positive strongly correlated to the biomarkers level, such as: D-D, CRP and TNF-alpha, as well as to the severity of the PVT. No significant correlations were noted between LPS+ bacteria and level of AFP.

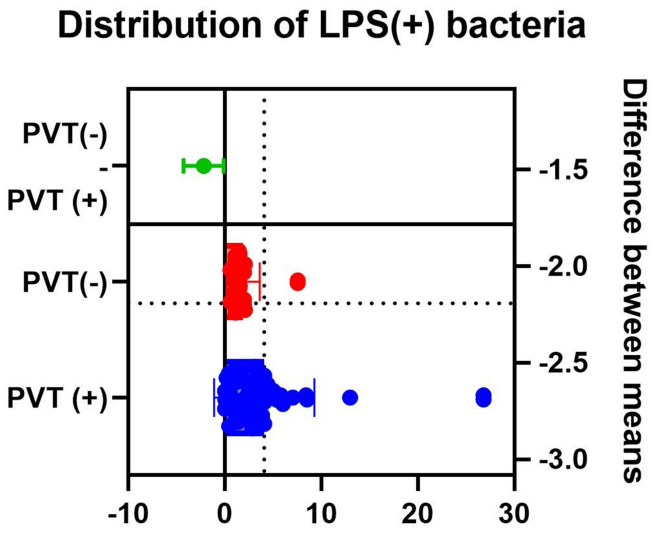


Figure 5 Distribution of LPS (+) bacteria in patients with LC.
Abbreviations: LPS, lipopolysaccharide; PVT, portal vein thrombosis.

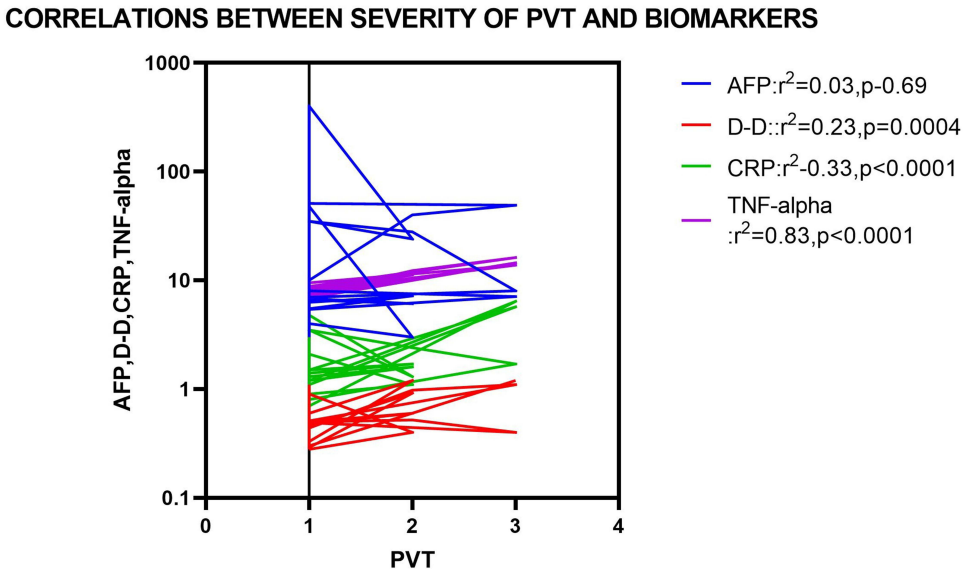


Figure 6 Correlations of PVT severity to biomarkers.
Abbreviations: PVT, portal vein thrombosis; AFP, alpha-fetoprotein; D-D, D-dimers; CRP, C-reactive protein; TNF, tumor necrosis factor.

Only the clinical variables that correlated well with the PVT severity were selected for multivariate analysis. As seen in Table 5 the multiple regression analysis revealed that from the variables included in analysis, the esophageal varices (95% CI: −2.065 to −0.2452; p<0.0001), the age (95% CI: 0.01337 to 0.03989; p = 0.0001) and the abdominal pain (95% CI: 0.008791 to 0.2197; p = 0.0341) were independent risk predictors for the PVT severity.

As illustrated in Table 6, several biological variables that strong correlated with the PVT severity were selected and included in a model of multiple regression analysis, as follows: D-D, CRP, TNF-alpha and LPS. Results of multiple regression equation emphasized that CRP (95% CI: 0.07887 to 0.2199; p<0.0001) TNF-alpha (95% CI: 0.1484 to 0.1899; p<0.0001) and D-D (95% CI: 0.04894 to 0.3400, p = 0.0094) were independent risk factors predicting severity of PVT.

Discussion

Many risk factors are involved in PVT development in patients with LC; in fact pathophysiology of PVT in cirrhotic patients being multifactorial.[39] Some are general factors, such as systemic inflammatory factors, as a prospective study

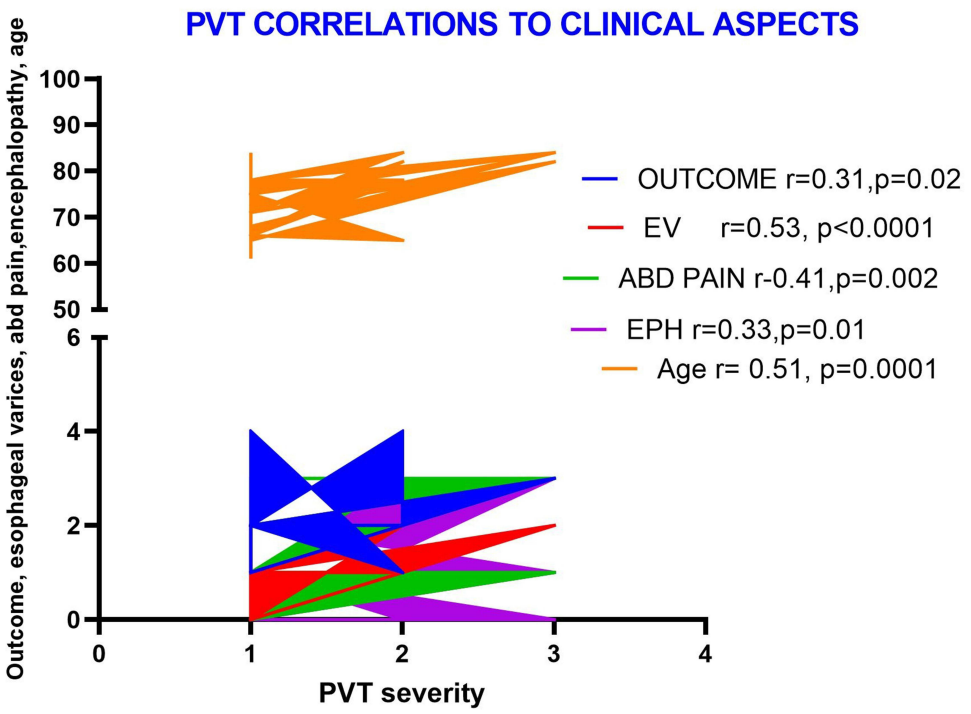


Figure 7 Correlations of PVT severity to various clinical aspects.
Abbreviations: PVT, portal vein thrombosis; EV, esophageal varices; ABD, abdominal; EPH, encephalopathy.

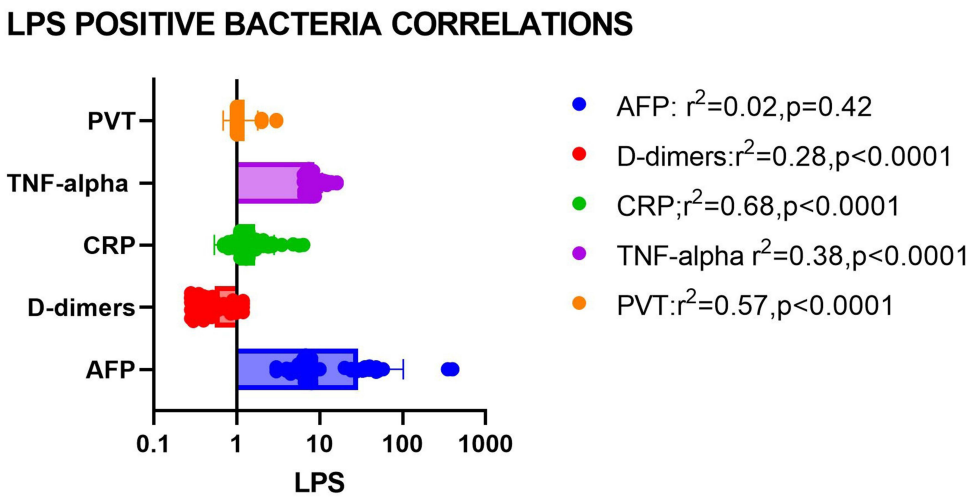


Figure 8 LPS positive bacteria correlations in PVT group.
Abbreviations: LPS, lipopolysaccharide; PVT, portal vein thrombosis; AFP, alpha-fetoprotein; CRP, C-reactive protein; TNF, tumor necrosis factor.

recently demonstrated, which reported apart from a greater incidence of PVT in higher Child scores, significantly higher levels of interleukin (IL)-6 and TNF-alpha in cirrhotic patients who presented PVT.[40] As many studies reported, local factors related to portal hemodynamics in advanced stage liver disease, with important decrease of velocities of portal flow are involved in PVT development. Risk of PVT in LC is elevated in patients waiting for LT and prevalence of PVT could rise up to 25%[41] However, half of patients with LC and PVT have more than one risk factor involved.[42] The clinical particularities displayed in the study group (PVT+), were characterized by several thrombogenic risk factors, often concurrent at the same patient. We have recorded an increased incidence of the smoking history, obesity, T2DM. NAFLD-related LC, as thrombotic risk factors, these aspects observed in the present study, were also reported by others,

Table 5 The Multivariate Analysis of the Clinical Variables

Table Analyzed	**Multiple Regression of Clinical Variables**						
Dependent variable	**PVT**						
Regression type	Least squares						
Model							
Analysis of Variance	SS	DF	MS	F (DFn, DFd)	P value		
Regression	14.47	4	3.616	F (4, 95) = 21.78	P<0.0001		
EV	3.546	1	3.546	F (1, 95) = 21.36	P<0.0001		
Abd pain	0.7681	1	0.7681	F (1, 95) = 4.626	P=0.0340		
E	0.3170	1	0.3170	F (1, 95) = 1.909	P = 0.1703		
Age	2.640	1	2.640	F (1, 95) = 15.90	P = 0.0001		
Residual	15.77	95	0.1661				
Total	30.24	99					
Parameter estimates	Variable	Estimate	Standard error	95% CI (asymptotic)	|t|	P value	P value summary
β0	Intercept	−1.155	0.4583	−2.065 to −0.2452	2.520	0.0134	*
β1	**EV**	0.2851	0.06169	0.1626 to 0.4076	4.621	<0.0001	****
β2	**Abd PAIN**	0.1143	0.05312	0.008791 to 0.2197	2.151	0.0340	*
β3	**E**	0.07720	0.05587	−0.03372 to 0.1881	1.382	0.1703	ns
β4	**Age**	0.02663	0.006679	0.01337 to 0.03989	3.987	0.0001	***
Goodness of Fit							
Degrees of Freedom	95						
R squared	0.4783						

Notes: *Statistically significant, ***High statistical significance, ****Very high statistical significance.
Abbreviations: PVT, portal vein thrombosis; EV, esophageal varices; Abd, abdominal; E, encephalopathy; ns, not statistically significant.

Table 6 The Multivariate Analysis of the Biological Variables

Table Analyzed	**Multiple Regression of Biological Variables**						
Dependent variable	**PVT**						
Regression type	Least squares						
Model							
Analysis of Variance	SS	DF	MS	F (DFn, DFd)	P value		
Regression	27.74	4	6.934	F (4, 95) = 263.1	P<0.0001		
D-dimers	0.1855	1	0.1855	F (1, 95) = 7.038	P = 0.0094		
CRP	0.4661	1	0.4661	F (1, 95) = 17.69	P<0.0001		
TNF-alpha	6.900	1	6.900	F (1, 95) = 261.8	P<0.0001		
LPS	0.01681	1	0.01681	F (1, 95) = 0.6378	P = 0.4265		
Residual	2.504	95	0.02635				
Total	30.24	99					
Parameter estimates	Variable	Estimate	Standard error	95% CI (asymptotic)	|t|	P value	P value summary
β0	Intercept	−0.5908	0.08784	−0.7652 to −0.4164	6.726	<0.0001	****
β1	**D-dimers**	0.1945	0.07330	0.04894 to 0.3400	2.653	0.0094	**
β2	**CRP**	0.1494	0.03552	0.07887 to 0.2199	4.206	<0.0001	****
β3	**TNF-alpha**	0.1691	0.01045	0.1484 to 0.1899	16.18	<0.0001	****
β4	**LPS**	0.007304	0.009146	−0.01085 to 0.02546	0.7986	0.4265	ns
Goodness of Fit							
Degrees of Freedom	95						
R squared	0.9172						

Notes: **Medium/high statistical significance, ****Very high statistical significance.
Abbreviations: PVT, portal vein thrombosis; CRP, C-reactive protein; TNF, tumor necrosis factor; LPS, lipopolysaccharides; ns, not statistically significant.

in patients with advanced stages of fibrosis. It is however not clear whether thrombosis associated to NAFLD is a primary or secondary issue.[43]

As others previously reported, we have also noted a higher prevalence of older age (over 61 years) in PVT+ group when compared both to cirrhotic patients without PVT and to controls.[44] Moreover, the correlations study revealed that age was positively strong correlated to PVT severity and the multivariate analysis set the age as an independent predictor for the PVT severity.

Over past decades, D-D were extensively used in deep vein thrombosis (DVT) and pulmonary thromboembolism (PE) as reliable diagnosis biomarkers.[45] This is why the study of D-D role in PVT seemed perfectly reasonable. A prospective study that investigated the value of D-D and plasma protein S in LC demonstrated that PVT can be excluded when D-D were low and protein S is elevated and vice versa, elevated levels of D-D should prompt imagistic studies in order to detect a PVT.[46] A meta-analysis including 21 studies addressing PVT in LC concluded that D-D could be considered as valuable diagnostic biomarkers.[47] The present study demonstrated that patients with PVT exhibited significantly higher level of D-D and moreover strong correlations were found between the level of D-D and the severity of PVT. The multivariate analysis in a logistic regression model set the D-D as independent predictors for PVT in patients with LC.

A gut microbial metabolite such as trimethylamine N-oxide (TMAO) was associated to increase the platelet reactivity and thrombi development in humans.[48] Gut DB and bacterial translocation was implicated in various complications of cirrhosis including PVT.[49,50] In accordance with previous studies our observations showed increased incidence of overall DB in cirrhotic patients with PVT, highlighting that gut DB could associate an elevated risk for venous thrombosis of the portal vein system. The discovery of the expression of the LPS r toll like receptor (TLR) 4 generated numerous studies aiming to highlight the capacity of LPSs to promote platelet activation, resulting in a prothrombotic phenotype probably associated to an inflammatory one.[51,52] CRP and adenosine diphosphate (ADP) seem to be stimulated secondary to TLR4 activation mediated by LPSs that eventually enhance the platelet stimulation, altogether with an important alteration of the platelet mitochondrial respiration.[53] There is a lot of experimental as well as clinical evidence that LPSs may trigger thrombogenesis by building up thrombin secondary to upregulation of tissue factor.[23] It seems that LPSs through TNF-alpha and nicotinamide adenine dinucleotide phosphate (NADPH) participate to acceleration of the oxidative stress at the platelet level, which is an important part of the thrombogenic process[54,55] The results of the present study have shown that patients from the PVT+ group exhibited an important decrease of the H index of microbiota biodiversity as well as of the F/B ratio. Functional bacteria presented important alterations in patients, with PVT. Of those, LPS+ bacteria were significantly increased in cirrhotic patients with PVT and subsequently correlation studies demonstrated a close link between LPS+ bacteria and several biomarkers such as: D-D, CRP and TNF-alpha, as well as the severity of the PVT. However, the result of the multivariate analysis in a logistic regression model of selected biological variables did not set the LPS+ bacteria as an independent predictor for the PVT severity.

A particular clinical tableau pictured the PVT+ group, where the patients with LC exhibited significantly increased incidence of admission by ER due to abdominal pain, upper digestive bleeding or overt encephalopathy. Some of these aspects, such as esophageal varices and abdominal pain were strongly correlated to PVT severity and our multivariate analysis placed them in the category of independent predictors for the PVT severity. In a recently large cohort study about the risk for PVT and venous thromboembolism in hospitalized cirrhotic patients, authors reported that esophageal varices, diabetes and invasive abdominal procedures were independent predictors for the PVT severity.[56] Digestive bleeding by variceal effraction was noted in significantly higher percentage in the PVT group, as a presentation complaint, however of mild importance. Endoscopic treatment was successful in controlling the digestive bleeding in the majority of cases, bleeding recurrence being low. Only 1 patient (2%) admitted with severe bleeding died on the second day after admission, by cardiac decompensation due to a myocardial infarction. However, more severe digestive bleedings were reported by others, in occlusive forms with extra hepatic location of PVT, that required multiple concurrent procedures in order to stabilize the patients.[57] Complicated GSD with acute cholecystitis or cholangitis could sometimes associate a particular form of PVT, known as pylephlebitis, as we rarely also observed in the present study.[58–61]

The morphology of PVT could interfere with symptoms, diagnosis, decision of anticoagulant therapy (ACT), outcome and patient prognosis. Many cases with chronic, nonobstructive PVT had an insidious clinical course, being hidden by

liver disease symptoms and are therefore incidentally discovered, whether during follow-up before LT, or at necropsy.[62] Cases of acute or recent PVT that complicate with extension to mesenteric territory, or develop digestive bleeding, as well as refractory ascites will become clinically symptomatic and diagnosis will be set earlier. However, these cases have a worse prognosis, especially those that require surgical therapeutical approach.[17] Related to PVT morphological characteristics, we noted that the vast majority of patients were diagnosed with chronic, non-obstructive forms, with or without portal cavernoma, confined to main portal vein or intrahepatic branches with good outcome. A minor proportion of PVT had acute onset, with total vein obstruction and extension to the mesenteric veins that prompted emergency surgery.

There are still a lot of controversies when it comes to treatment in PVT.[63] Some studies that evaluated natural history of PVT in cirrhotic patients reported that more than half of patients will display in time a spontaneous diminution of thrombus size, with venous patency reassessment.[64–66] Others describing risk factors, clinical presentation, complications and treatment of PVT have found that PV recanalization was present mostly in patients who received ACT.[67,68] However, a substantial experience in the area related to ACT in cirrhotic patients is still lacking.[69,70] Interesting studies related to effects of natural compounds, such as pentacyclic triterpenes highlighted their antithrombotic potential similar to prostaglandin (PG)I_2 receptor agonists and shed a new light upon betulinic acid, already known for many other beneficial effects in liver diseases including NAFLD.[71,72] The fact that only a half of PVT patients of the present study received ACT during hospitalization did not reflect neither in short term outcome, nor in higher rates of in hospital complications or death of the other half. Given the vast majority of PVT+ patients had chronic, non-obstructive PVT forms could at least explain why even without ACT, a low rate of complications and in hospital deaths was observed. Outcome of LC patients with PVT will principally depend on rapidity of onset, severity and etiology of thrombotic process, severity of liver disease, gravity of digestive bleeding, age and comorbidities.[73] Various situations could occur. Some of them are life threatening, such as intestinal ischemia, a complication of PVT with extension to mesenteric veins, which prompts surgical intervention in fragile patients, with high anesthetic and operatory risk, elevating the mortality rate up to 60%.[74] There is also a high risk of severe digestive bleeding due to the variceal effraction that requires a complex therapy with association of endoscopic procedures to pharmacologic treatment.[75,76] Many studies advocated the anti-inflammatory role of statins and also a possible protective influence over DVT and PE.[77] However, only recently researchers reported a relationship between statins and gut microbiome, some of them hypothesizing that statins could be implicated even in the modulation of the gut microbiome[78–80] We have observed that patients with LC complicated by PVT were less treated with statins and displayed more prominent alterations of the gut functional microbiota.

Over the past years there is more and more evidence that metformin may protect against DVT and PE.[81,82] Our observations from the present study revealed that patients from the PVT+ group that also displayed more severe gut DB received treatment with metformin less frequently. Moreover, as suggested by some authors, metformin treatment is associate with a particular composition of gut microbiota with abundance of mucin-degrading bacteria, *Escherichia* sp. and decrease of *Intestinibacter* sp., as well as *Bifidobacterium adolescentis* that seemed to negatively correlate to glycosylated hemoglobin (HbA1c).[83,84]

The natural history of bland, nonobstructive thrombosis is characterized by high rates of regression, as reported by some authors.[85] The present study has shown that PVT+ group exhibited mostly chronic, non-occlusive forms of PVT, with good response to therapy and favorable short-term outcome. As other studies reported, even before LT, patients with advanced liver diseases and PVT may not necessarily have a worse outcome, except for malignant thrombosis.[86,87] An important role in the natural history of PVT is also played by the systemic inflammation. Some studies recently investigated the link between the inflammatory cytokines such as TNF-alpha and IL-6 and the risk for PVT, setting these biomarkers as independent predictors for the PVT. A longitudinal study performed in cirrhotic patients, recently reported that markers of systemic inflammation, such as IL-6 and lymphopenia may independently predict the PVT. The present study investigated CRP and TNF-alpha, as systemic inflammatory biomarkers in cirrhotic patients, emphasizing that these biomarkers not only strongly correlated to PVT severity, but also proved to be independent predictors for PVT severity.[40,88]

Inhospital mortality in cirrhotic patients enrolled in this study, either PVT positive or negative was basically associated to fatal cardio-vascular events. That is why the assessment of cardio-vascular risk in these elderly patients with associated co-morbidities could become of critical importance.[89]

This observational study has however some limitations. Sample size of the study population was relatively small, triggering potential statistical draw-backs. Other limitations could be related to potential confounders and to other possible statistical biases that could have also intervened, related to information data or selection bias, given the cross-sectional study design.

Conclusion

Patients with LC and PVT displayed elevation of TNF-alpha, CRP, D-D, alterations of the functional gut microbiota, as well as several morphological and clinical particularities. Although the LPS positive gut microbiota was linked to inflammatory biomarkers and PVT severity, it was not proven to be an independent predictor of the PVT severity like CRP, TNF-alpha and D-D.

Acknowledgments

The authors would like to express their gratitude to "Bioclinica" Laboratories from Timisoara, Romania, for technical support.

Disclosure

The authors report no conflicts of interest in this work.

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