Markers of immunity and bacterial translocation in cirrhosis

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2. Mortensen C, Jensen JS, Hobolth L, Dam-Larsen S, Madsen BS, Andersen O, Møller S, Bendtsen F. Association of markers of bacterial translocation to immune activation in decompensated cirrhosis. Submitted.
3. Mortensen C, Karlsen S, Grønbæk H, Nielsen DT, Frevert S, Clemmesen JO, Møller S, Jensen JS, Bendtsen F. No difference in portal and hepatic venous bacterial DNA in patients with cirrhosis undergoing transjugular intrahepatic portosystemic shunt insertion. Accepted Liver Int.

INTRODUCTION

Patients with decompensated cirrhosis are characterized by an incompetent immune system that is both functionally deficient and chronically activated(1-3). This state of immunity leaves the patient vulnerable to infections(4;5) and may contribute to development of circulatory abnormalities that are associated with severe complications and reduced survival(6;7).

It has been hypothesized, that bacterial antigens of gut origin are the main source of immune activation(8). Enteric bacteria migrate to the mesenteric lymph nodes, a process termed bacterial translocation (BT)(9). Alternatively, bacterial lipopolysaccharide (LPS) may enter the portal vein through the intestinal capillary system and then bypass the liver through pathological shunts, described as endotoxemia(10). This hypothesis is substantiated by the fact that the use of antibiotics improves the circulatory changes in some patients, even in the absence of clinical infection(11-13). In addition it is of clinical relevance that commonly used therapies in the treatment of circulatory complications such as non-specific beta-blockers (NSBB)(14;15) and insertion of a transjugular intrahepatic portosystemic shunts (TIPS)(16) may reduce BT and augment endotoxaemia.

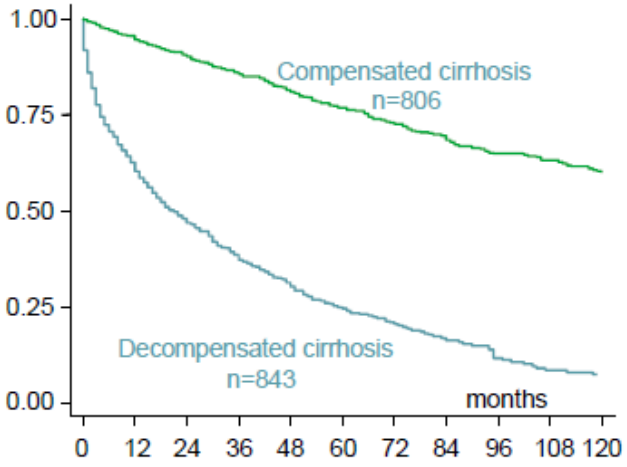
The present thesis encompasses three studies investigating immune activation and BT in patients with cirrhosis. In addition, the development of an assay for the detection of bacterial DNA (bDNA), a marker of BT is presented.

Background

Cirrhosis is considered the end-stage of a variety of different chronic liver diseases, resulting in a disease course characterised by complications and a reduced life expectancy(17). Cirrhosis is a global disease, although the specific causes of the individual etiologies for the disease show considerable regional variation. In the US, the National Center for Health Statistics ranked chronic liver disease as the 12th most common cause of death in 2006, responsible for more than 27,000 deaths(18).In Denmark, alcohol consumption is the leading cause of cirrhosis, and alcoholic cirrhosis, which is associated with a particularly poor prognosis, has a prevalence of approximately 10,000 patients(19). Progressive fibrosis and the formation of regeneration nodules in the liver lead to a destruction of the normal architecture of the liver tissue, resulting in an increased vascular resistance. Inside the sinuisoidal vascular bed, a dynamic vasoconstriction contributes to further increases in the hepatic resistance. Portal hypertension gradually develops despite the formation and expansion of collateral vessels between the portal and systemic circulation. A hyperdynamic circulatory state, caused by a progressive peripheral and arterial splanchnic vasodilatation, and characterized by increased cardiac output, and expanded plasma volume results in increased flow into the portal circulation(20).

Portal hypertension is an important determinant of both prognosis and of most of the major complications of cirrhosis(21). In patients with cirrhosis, development of these complications is the major determinant of survival. Decompensation, defined as the presence of ascites, variceal bleeding, encephalopathy, or jaundice, is associated with a poor outcome. At the time of diagnosis, the presence of decompensation reduces median expected survival from more than 10 years to approximately two years (Figure 1).

Figure 1



Survival in patients according to presence of decompensation at the time of diagnosis(17)

Infection(22;23), and the systemic inflammatory response syndrome (SIRS) are also highly associated with a decreased survival in cirrhosis(22;24). Hospital admissions are often caused or complicated by a range of infections(6;25) including spontaneous bacterial peritonitis (SBP), a specific infection often seen in patients with cirrhosis and ascites. Because of the presence of a multifactorial immune incompetence, infections have a more severe course in patients with cirrhosis, resulting in more adverse outcomes compared to the general population(26;27). Infections and development of complications are intimately inter-related and related to prognosis. For example presence of ascites predispose to SBP and on the other hand, infections may aggravate renal dysfunction(28). Thus, SBP is the most common precipitating factor for the development of the hepatorenal syndrome (HRS) (29). Patients with cirrhosis and oesophageal varices admitted for upper gastrointestinal bleeding also suffer frequently from acute infections during the bleeding episode(30). Prophylactic antibiotics in this setting have been shown to increase survival(31) as well as to reduce rebleeding rates(32). Infections in patients with bleeding oesophageal varices are associated with failure to control bleeding, suggesting that the infection per se may affect the course of the bleeding(30;33). Spontaneous bacterial peritonitis (SBP) is an infection in ascitic fluid, defined by a polymorphonuclear count above 250 million cells per liter, with or without cultivable bacteria(34). It is a common infection in hospitalised cirrhotic patients with ascites, occurring in between 10% to 31%(35;36). Despite improvements in prognosis due to early diagnosis and treatment, it is associated with a considerable in-hospital mortality, the lowest reported being 10%(37). The prognosis is poorest in the approximately third of cases complicated by renal failure(38).

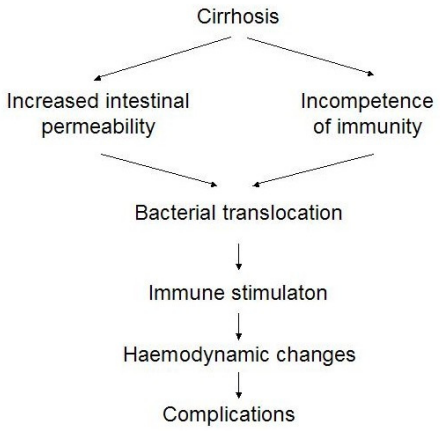
The most prevailing hypothesis of the pathophysiology of SBP is the migration of bacteria of gut origin into the systemic circulation and subsequently into the ascitic fluid(39). This hypothesis rests on the enteric bacteria cultured, the absence of intraperitoneal pathology as a source of infection, and the frequent E. coli bacteraemia in hospitalised patients with cirrhosis. A recent study investigated the link between SPB, haemodynamics and development of HRS in 23 patients with SBP. The degree of inflammation was assessed by plasma tumor necrosis factor-alpha (TNF-) and patients, who subsequently developed HRS had higher levels of TNF- and circulating vasoconstrictors at diagnosis(29). Thus, in SBP caused by enteric bacteria, systemic inflammation is associated with the development of circulatory complications.

Bacterial translocation

The term bacterial translocation (BT) was coined by Berg(9) who demonstrated by culture the presence of enteric bacteria in the mesenterial lymph nodes (MLN) of mice. In experimental cirrhosis, a number of studies have investigated BT and its relationship to pharmacological interventions(15;40-43). The main findings have been the frequent presence of BT (45%(40)- 78%(43)) in ascitic rats, and less frequent presence 4%(43) or absence(15;40) in control animals. The effect of several interventions has been investigated including prokinetic agents, beta-blockers, sympathectomy, and antibiotics. The prokinetic drug cisapride(44) and the non-specific betablocker propranolol(15) both reduced BT and intestinal bacterial overgrowth. The importance of sympathetic drive on BT was further assessed by chemical splanchnic sympathectomy, resulting in reduced e. coli BT but not by S. aureus(45) suggesting that sympathetic activation has differential immunological effects on the process of translocation. A number of studies have validated use of antibiotics in experimental cirrhosis and have found diverging results, some finding reduced BT(46;47) whereas others have been unable to demonstrate any effects(48).

The sum of the findings have supported the hypothesis that BT not only cause SBP induced inflammation and circulatory impairment but also by itself may elicit a response with immunological and haemodynamic consequences (7;49) (Figure 2).

Figure 2



The hypothesis of BT as a consequence of cirrhosis-related pathophysiology, causes immune stimulation and haemodynamic changes, leading to complications.

BT is most likely promoted by several different features related to the pathophysiology of cirrhosis. Increased gut permeablility in a high proportion of patients has been demonstrated by different methods in several studies(14;50-52). A complex incompetence of the immune system encompassing reduced neutrophil function(53), deficiency of complement(5), reduced hepatic reticuloendothelial function(4), and the presence of shunts bypassing the liver, may permit antigenic material to reach the liver and/or systemic circulation. Finally, recent studies have found presence of genetic polymorphisms in genes encoding receptors for innate pathogen recognition receptors NOD2(54;55) and TLR2(56) to be

associated with increased risk of SBP and increased mortality. A recent study, found both NOD2 and TLR2 carriers to have elevated levels of a lipolpolysaccharide binding protein (LPB), marker of BT, and of increased intestinal permeability(14). A variant of the gene encoding monocyte chemoattractant protein-1, a monocyte and macrophage cytokine chemotactic factor, was also found to confer risk of SPB in a subgroup of patients with alcoholic cirrhosis(57). Culture of mesenterial lymph nodes in human subjects has been attempted in only one major study. In that study, Cirera et al. compared 101 patients with cirrhosis and noncirrhotic controls undergoing general intraabdominal surgery. Although cultures were more frequently positive in Child-Pugh class C cirrhosis, (Child-Pugh class C: 31% positive MLN versus controls 9% positive MLN), no difference was observed when all patients with cirrhosis were compared to the general surgical population(58). There are several factors that limit the use of MLN in the assessing of human BT. These include the use of preoperative antibiotics and the often small number of lymph nodes obtainable in humans.

Accordingly, a number of direct and indirect markers of BT have been used to assess its clinical consequences in patients. Surrogate markers of bacterial translocation in clinical studies. Proposed markers of BT in clinical trials include direct markers (constituents of pathogens) and indirect (markers related to the immune response to such pathogens). BT can in its widest sense be defined as the passage of bacteria from the gut to the systemic circulation by different routes. BT has been assessed by bacterial LPS, LBP and the most recent marker, bDNA. LPS is a highly immunogenic constituent of the gram-negative cell membrane and causes release of proinflammatory markers and a marked haemodynamic response in healthy controls (59). In one study, a transhepatic concentration gradient favoring the portal vein(60), suggested a translocation of LPS via intestinal capillaries into the portal circulation, although this gradient was not discernible in a recent study(61). In addition, the elevated LPS has been found (62) associated with haemodynamic alterations in cirrhosis(12). However, in another study with a comparable population, LPS was entirely absent(63). LBP, the binding protein of LPS, has been investigated in several studies. In a large study, LBP was elevated in a subset of patients with ascites that was further characterised by elevated cytokines and severe haemodynamic alterations(13). In a recent study, LBP was related to portal hypertension and increased intestinal permeability, and was lowered by beta-blocker treatment(14). LBP, although increased by LPS, is also an acute-phase reactant, and may therefore be elevated unrelated to BT(64).

The most recently suggested marker of BT is bDNA . In a series of studies from the group of Such et al. (53;65-69), the presence of bDNA was demonstrated in ascites and serum of one-third of patients with cirrhosis but without signs of infection. A polymerase chain reaction-assay using primers specific for the gene encoding the 16S ribosomal subunit in eubacteria enabled the amplification of potentially any bacterial in the samples, and sequencing of the bands revealed the bacteria to be predominantly of gut origin. In controls, bDNA was absent(65). In a follow-up study, the relationship between bDNA and inflammation was demonstrated, and the bDNA delineated a group without elevated LPS(70). The magnitude of the inflammatory response to bDNA resembled that of samples with SBP(68). A multicenter study found that the bDNA-status predicted survival, although bDNA positive patients were not more likely to develop SBP(67). In a later study, bDNA-postitive patients exhibited more marked peripheral circulatory changes and increased hepatic endothelial dysfunction compared to bDNA-negative patients, although splanchnic haemodynamics and portal pressure was similar(71). In the most recent clinical study analysing bDNA in plasma, however, bDNA was unrelated to the primary focus of that study, neutrofile oxidative burst and toll-like receptor expression(53).

Application of bDNA seems in several ways to avoid the shortcomings of other markers; It is a direct marker, demonstrating the presence of components of the bacteria itself rather than the elicited immune response. It is not limited to determine the presence of gram negative bacteria. bDNA is immunogenic and stimulates toll-like receptor 9 in a range of immune cells(72), leading to the production of proinflammatory cytokines, and its apparent relation to both inflammation and prognosis fits within the framework of the hypothesis of BT.

HYPOTHESIS AND AIMS

The overall hypothesis of the present thesis is that in patients with cirrhosis, BT as assessed by bDNA, is associated with an inflammatory response that may affect haemodynamics and lead to a poorer prognosis.

Accordingly, we aimed:

1. To investigate the possible relationship between markers of inflammation, haemodynamics and prognosis (Study 1).
2. To develop a PCR assay for the detection of bDNA in blood and ascites (Appendix).
3. To use the assay to quantity bDNA in blood and ascites of patients with cirrhosis, and investigate the relation of bDNA to markers of inflammation (Study 2).
4. To describe markers of BT and inflammation in portal and hepatic veins (Study 3).

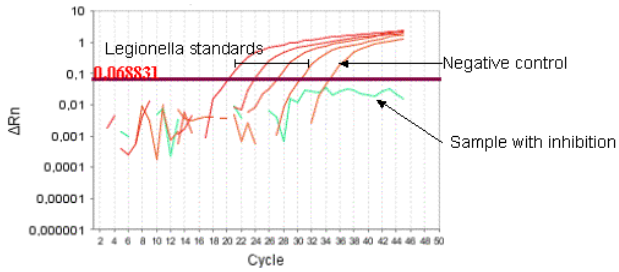
COMMON METHODS

16S rDNA quantitative PCR

16S rDNA polymerase chain reaction (16S PCR) is a PCR method targeting the gene encoding a ribosomal RNA sub-unit found solely in bacteria genomes. The gene, expressed in variable numbers, is present in all known prokaryotes. Containing both conserved and highly variable regions, primers flanking regions containing both conserved and variable regions permits both the amplification of DNA from any bacteria and subsequent determination of bacterial species. The quantification of target product can be enabled by the use of so-called real-time reporters. Reporters are molecules that allow the quantification DNA within the reaction mixture during the PCR process, by fluorescence. During optimal conditions, the increase in fluorescence as a function of a PCR cycle is exponential. The Cycle threshold (or Ct) is the cycle where the fluorescence generated by reporters significantly exceeds the background signal. By plotting increases in fluorescence from reporters by cycle number, and comparing the “cycle threshold” to the flurorescence of standards with known concentrations of DNA, it is possible to estimate the target concentration. The ability of 16S PCR to amplify DNA of any bacteria exacerbates the risk of contamination during preanalytical and analytical handling. A particular concern is the amplification of fragments of DNA contaminating the reagents of the PCR process. The nature of the sample may also complicate the reaction. Blood, for instance, contains various substances that cause unspecific inhibition of the PCR process. In samples demonstrating proces inhibition as evidenced by low PCR curves, the PCR was

rerun with Legionella DNA equivalent until the lowest positive control spiked to the samples. In all cases, the addition of Legionella DNA raised the PCR product to the level of the lowest positive control with finding of identical Ct to the lowest postitive control, indicating no additional DNA and were therefore classified as negative. In these samples, however, quantification was not possible. An example of inhibition is shown in Figure 3.

Figure 3



Amplification plot of increase in fluorescence plotted against cycle threshold, showing a sample demonstrating marked inhibition.

The development and a detailed description of 16S PCR assay used in Study 2 and Study 3 are presented in the appendix. Luminex multiplex cytokine assay We used an assay based on a multiplex cytokine analysis technology in all three protocols for the analysis of plasma (study 1-3) and ascites (study 2). This technology allows the simultaneous quantification of multiple substances of interest in small sample volumes. The assay uses distinctly coloured beads coated with antibodies specific to the analyses and a fluorescent reporter. Flow cytometry enables the excitation beads and the reporter by separate lasers, and photo detectors measure the resulting excitation. The quantity of coloured beads specific for an analyte and their excitation enables their quantification by comparison to a linear regression line generated by standards. All samples were run in duplicate. Coefficients of variation (CV) were typically less than 10% and measurements with CV > 20% or <50 beads counted per sample were discarded from analysis(73).

In cases where sample concentration was above the effective linear range, samples were rerun in a higher dilution. If the concentration of one duplicative measurement was below the lowe level of quantification (LLQ); the concentration of the measurement below LLQ was set to the LLQ for the relevant biomarker.

STUDY 1 "HIGH SENSITIVITY C-REACTIVE PROTEIN LEVELS PREDICT SURVIVAL AND ARE RELATED TO HAEMODYNAMICS IN ALCOHOLIC CIRRHOSIS"

The purpose of Study 1 was to investigate the possible relationship between markers of inflammation, haemodynamics anc prognosis. We included 45 patients (Child-Pugh stage A (n=16) Child-Pugh stage B (n=19) Child-Pugh stage C (n=10) and twelve age-matched healthy controls. Samples were analysed with regards to a panel of markers chosen to reflect different immune functions. Patients were stable outpatients without clinical signs of infection. Antibiotic-use within one week of sampling was an exclusion criterion. Patients had undergone extensive haemodynamic investigations, and the outcomes in patients were assessed by patient file review.

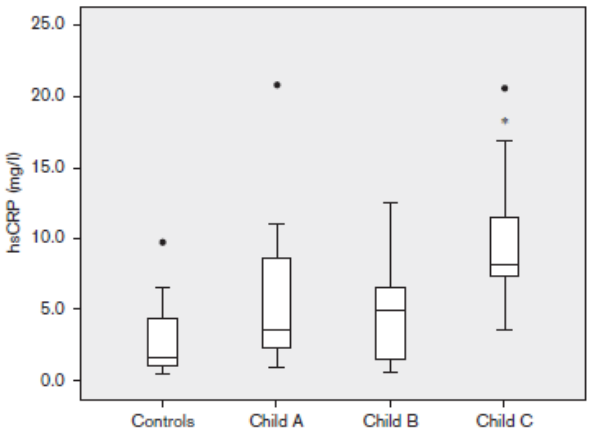
METHODS

Liver vein catherization and haemodynamic investigations. Liver vein catherization is an invasive method for indirect assessment of portal hypertension. The portal pressure is assessed as the difference between wedged and free pressure in a hepatic vein(74). Indications for measurement of portal pressure is classification of portal hypertension(75), assessment of risk of variceal bleeding and guidance for prophylactic treatment of patients with oesophageal varices(76), and for prognostic classification of patients with cirrhosis(21;77). All participants underwent investigations after an overnight fast. In local anaesthesia, by the Seldinger-method, catheters were placed in the right femoral artery and vein. Under fluoroscopic guidance, a catheter was advanced to the hepatic vein, where the hepatic venous pressure gradient (HVPG), an estimate of portal pressure, was determined using a Swan-Ganz balloon-tip catheter size 7F. The hepatic circulation was further characterized by assessment of the hepatic vascular resistance, and the hepatic blood flow by constant infusion technique using indocyanine green clearance, as previously described(78). The systemic circulation was characterized by cardiac output, determined by the indicator dilution technique, the systemic vascular resistance (SVR), and by direct measurement of arterial blood pressure. SVR was calculated as (mean arterial pressure - right atrial pressure)/cardiac output(79)

RESULTS

Hs-CRP, and Interleukin-8 were significantly higher in patients compared to controls (hsCRP: median 8.2 versus 1.6 mg/l, p<0.05, IL-8: 94.8 versus 18 pg/ml p<0.05.). In contrast, monocyte chemoattractant protein-1 (MCP-1) was lower in patients (460 versus 174 pg/ml, p<0.001). The TNF- , IL-6 and VEGF did not differ significantly between patients and controls. The patients with Child Pugh C-stage cirrhosis had higher median levels of HsCRP (8.2 mg/l) compared to Child-class B (5.0 mg/l, (p<0.05) and controls (1.6 mg/l, (p<0.05)) as shown in figure 4, and significantly lower levels of MCP-1 compared to Child class A and controls (174 pg/ml versus 220 pg/ml (p<0.05) and 460 pg/ml (p<0.05).

Figure 4

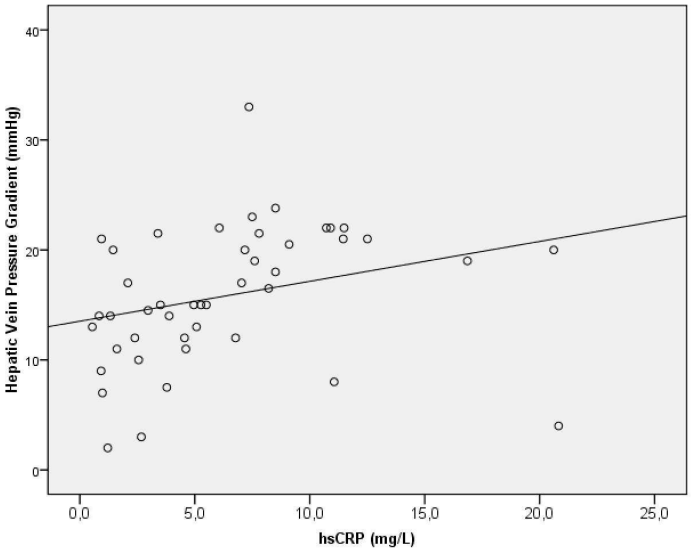


Concentrations of hsCRP in patients and controls.

Child Pugh class C patients had significantly higher levels of hsCRP compared to Child Pugh class B patients and controls. Inflammatory markers were generally not related to complications, with the exception of MCP-1, which was lower in patients

with ascites and VEGF, which was higher in patients with the hepatopulmonary syndrome. HsCRP was significantly correlated to portal pressure assessed by the HVPG (r=0.47, p=0.001, Figure 5) but not to other systemic and splanchnic hemodynamic variables.

Figure 5



Spearman's Correlation between hsCRP(mg/l) and hepatic venous pressure gradient (mmHg). Sperman's rho = 0.47, p=0.001

hsCRP and MELD scores were the only variables in an univariate analysis significantly associated with mortality. In a subsequent multivariate analysis eliminating the impact of MELD-score, hsCRP retained statistical significance (p<0.05), as shown in Table 1.

Table 1

Univariate analysis				Multivariate Analysis			
	Hazard Ratio	CI(95%)	p-value		Hazard Ratio	CI(95%)	p-value
hsCRP (mg/L)	1.084	1.015-1.158	0.017	hsCRP (mg/L)	1.074	1.001-1.153	0.046
MELD score	1.228	1.064-1.418	0.005	MELD score	1.211	1.047-1.403	0.010
Child Pugh score	1.123	0.907-1.390	0.286				

Table 1. Cox-regression analysis of relation between hs-CRP levels, prognostic scores and survival. Hazard ratios are for death.

STUDY 2 "DETECTION OF MARKERS OF BACTERIAL TRANSLOCATION AND IMMUNE ACTIVATION IN DECOMPENSATED CIRRHOSIS"

Study 2 aimed to detect the presence and quantity of bDNA in blood and ascites and potential relations to markers of inflammation. Thirty-eight hospitalized patients or outpatients with cirrhosis and ascites were included in the study to assess the frequency and quantity of BT and its relation to inflammation. Recent treatment with antibiotics excluded the patients from participation. Using bDNA and LBP as markers of BT and a panel of markers of inflammation, we assessed the potential relationship between BT and inflammation in both blood and ascites. In a small group of patients (n=6) with SBP, we also assessed PCR to diagnose culture-positive and -negative SBP.

METHODS

Paracentesis

In this study, the participants underwent paracentesis for diagnostic or therapeutic purposes. Paracentesis includes diagnostic paracentesis and large-volume paracentesis (LVP). In patients undergoing diagnostic paracentesis, puncture was performed by sterile technique in the lower left abdominal quadrant, after repeated skin disinfection. An intramuscular cannula mounted on a 20 ml sterile syringe was advanced through the abdominal wall under aspiration until ascites was visible. In patients undergoing therapeutic paracentesis, after skin disinfection, a small incision through the skin allowed the insertion of a pigtail catheter or intravenous catheter into the abdominal cavity. Immediately after free flow of ascites had been observed, aspiration of ascites into a sterile syringe was performed. The samples for bDNA analysis were immediately frozen and no samples rethawed until analysis. Ascites was analysed by automated cell-count and protein-quantification. Blood culture bottles, whose membranes were disinfected twice before inoculation, were sent for culturing.

Results

In patients without signs of SBP infection assessed by culture and cell-counts (n=32), bDNA was detected in ascites in 52% of patients, compared to 22% in blood. In a high proportion of blood samples (n=19/32, 59%) and in one ascites sample, bDNA was not quantifiable due to inhibition. bDNA did not differ between patients with Child-Pugh B or C, (Chi-square 3.3, p=0.07) although a tendency towards higher levels of bDNA, in Child-Pugh stage B was apparent. Levels of LBP were not significantly different when comparing bDNA-positive and negative patients (p=0.684). Plasma markers of inflammation were not significantly different between Child B and Child C cirrhosis, although a trend towards higher values in Child C for IL-8 (p=0.053) and hsCRP (p=0.059) could be discerned. VEGF alone differed between bDNA positive and negative patients, being higher in ascites(139.1 pg/ml versus 76.2 pg/ml p=0.03), but lower in plasma of bDNA positives (70.0 pg/ml versus 165.5 pg/ml, p=0.017). DNA performed poorly in the diagnosis of SBP, being negative in 2/6 cases.

Even in culture postitive SBP samples, (Table 2), bacterial content varied widely. The only patient with positive blood culture was PCR negative. In all culture-positive or bDNA-positive samples sequencing were attempted. All attempts of sequencing failed.

Table 2

Table 2: Bacterial DNA, PMN-count, and culture in SBP samples.

Sample	Ascites Cell-count (PMNs, millions/L)	Culture	BDNA Positive?	Bacterial quantity
1	650	E. Coli	Yes	276
2	545	E.Coli	Yes	5,1
3	616	neg	Yes	4,7
4	9900	E.coli	Yes	3,4
5	462	neg	No	0,0
6	468	neg	No	0,0

Bacterial DNA, PMN-count, and culture in SBP samples.

STUDY 3 "NO DIFFERENCE IN PORTAL AND HEPATIC VENOUS BACTERIAL DNA IN PATIENTS WITH CIRRHOSIS UNDERGOING TRANSJUGULAR INTRAHEPATIC PORTOSYSTEMIC SHUNT (TIPS) INSERTION"

This study aimed to assess markers of BT and inflammation in portal and hepatic veins in TIPS patients. Twenty-eight patients with cirrhosis (Child A/B/C: n=1/n=14/n=13) who underwent a TIPS-insertion were included. The indications for TIPS-insertion were refractory ascites (n=19) or variceal bleeding (n=9). Soluble urokinase plasminogen activator receptor (suPAR), recently described to be associated with disease stage and prognosis in a variety of liver diseases (80), was also measured.

METHODS

Transjugular Intrahepatic Portosystemic Shunt (TIPS)

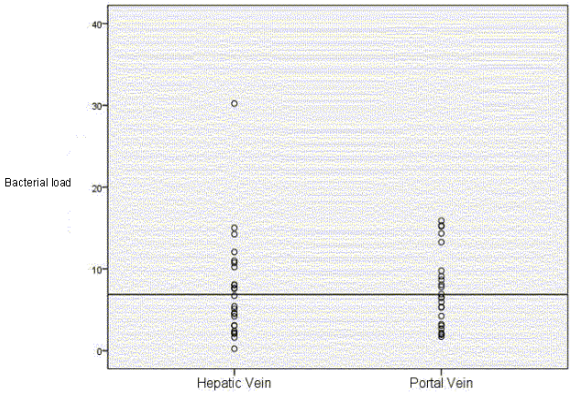
TIPS-placement is an intervention intended to reduce portal hypertension and its associated complications. The main indications are refractory ascites and variceal bleeding(81).

TIPS placement may reduce mortality in patients with recurrent variceal bleeding despite drug and endoscopic therapy, and early TIPS in patients with variceal bleeding improve survival(82). In patients with refractory ascites, results indicate better control of ascites, but impact on survival is unclear(83). The procedure was conducted under general anesthesia. By transjugular access, a catheter is advanced under fluoroscopic guidance into a hepatic vein. Puncturing a portal vein branch, a guide-wire enables the placement and subsequent expansion by a balloon and a stent, which reduces hepatic vascular resistance and increases flow from the portal circulation into the hepatic vein, thus reducing portal pressure. Samples from the hepatic vein and the portal vein were collected as follows: After transjugular access, with the catheter advanced into the hepatic vein, samples were collected by aspiration through the catheter and sterile transfer to the test tubes. Immediately after puncture of the portal vein, and before TIPS placement, sample collection from the portal vein was conducted.

RESULTS

bDNA levels were detectable in 11/28 patients (39%) of the portal vein samples and in 12/28 (43%) of the hepatic vein samples(p=0.126). Comparing bDNA levels in portal and hepatic venous blood, we found no significant difference (p=0.182). Markers of inflammation were not significantly different between the vascular territories, with the exception of suPAR (portal vein: 11.9 vs ng/ml, hepatic vein 13.7 ng/ml) and VEGF (68.8 pg/ml versus 91.3 pg/ml), both significantly higher in the hepatic vein (p=0.031 and 0.003, respectively). Markers of inflammation did not differ according to bDNA-presence, with the exception of portal vein suPAR, which was higher in bDNA positive patients (13.67 versus 6.86 ng/ml, p=0.021). Patients positive for bDNA in at least one of the vascular territories had similar pre-TIPS portal pressure gradients (16 mmHg, IQR 11-20) as patients without bDNA (13 mmHg, IQR 10-18, p=0.354). In 7 samples, bDNA was not quantifiable. In patients with quantifiable samples, levels correlated across the vascular territories (r=0.62, p=0.002). Levels of bDNA are shown in Figure 6.

Figure 6



bDNA levels in the hepatic and portal veins. The horisontal line denotes the background level of 6.81 bacteria. An outlier at 230 bacteria in the portal vein is omitted for graphical purposes.

DISCUSSION

The present thesis is based on three studies investigating presence of BT and its relation to alterations in the immune system, and the relation between immune activation and

The main findings of the present studies were;

- Stable cirrhotic patients show signs of low grade inflammation reflected by an increased serum hsCRP, especially in decompensated patients with relation to haemodynamics and survival;
- Presence of bDNA in ascites and blood was not associated to activation of cytokines, and was not found valuable as a diagnostic tool for the assessment of SBP;
- There is no degradation of bDNA across the liver and presence of bDNA is not related to portal pressure.

A number of markers that potentially reflect BT have been introduced within the last decade or reevaluated(10;65). At the time when the present study was planned, bDNA had rather recently been introduced as a promising candidate marker of BT wherefore we decided to develop an assay for its analysis.

Such et al. pioneered the application of bDNA as a marker of BT in cirrhosis, yielding a number of findings with clinical impact for the patient with decompensated cirrhosis, and in many cases confirming findings from the experi mental cirrhosis. This group demonstrated a near-perfect concordance between PCR findings in sterile ascites and in the serum of patients with cirrhosis, and with a complete absence of bDNA in healthy controls(65). Serum/ascites bDNA was closely linked to inflammation even in the absence of LPS(70), and retained its concordance in a multicenter study, which also linked the presence of bDNA to prognosis(67).

Although some corroboration of the initial findings emerged(84;85), a number of later studies found markedly different results(86-90). Although comparisons between individual studies are difficult to accomplish, due to different setups and sample materials, the number of patients demonstrating DNA have varied from 0 percent (plasma(90), to 60 percent (sterile ascites DNA)(88). The internal consistency of the results by Such et al. with regard to findings of the same bacterial species in ascites and serum, could be interpreted as validation of their methods. However, Appenrodt et al. compared bDNA in ascites

and blood and found in non-SBP patients presence of bDNA in ascites in 12%, presence of bDNA in blood in 1%, and bDNA both in ascites and blood in only one patient(87). Thus, the results of this study do not support the assumption that bDNA may represent a reliable marker of BT. The study by Soriano et al., (88) assessed the presence of bDNA in SPB and non-SBP samples in ascites only, by a real-time PCR methodology similar to ours. In non-SPB samples, bDNA was positive in 60%, the sequencing positive in only half of the cases. Bruns et al. also investigating bDNA presence in culture-negative non-neutrocytic ascites, and found 14% positive of bDNA. Clearly, our findings together with those of other groups challenge the findings of both frequency and perfect consistency of bDNA in blood and acites as assessed by the PCR methodology.

Another striking feature of the bDNA in the hands of the Such group is the close relation to immune activity(68;69). They found, that patients defined by presence of bDNA had elevated markers of inflammation, indistinguishable from the marked inflammatory response observed in SBP-patients(68). We observed TNF- levels markedly (approximately 20-fold) lower (Study 2, table 2) irregardless of bDNA status(68). The TNF levels we observed were similar to the levels in both stable outpatients and healthy controls investigated in study 1, and to levels reported by others in cirrhosis patients(13;63). Our studies, using a number of markers to assess various functions of the immune system, failed to find support for the notion of bDNA eliciting an immune response. Apart from the work of Such et al., only two studies have combined measurement of markers of inflammation and bDNA. The first, from Egypt, largely reproduced the findings of Such et al(84), whereas the second, previously mentioned study did not find any relationship between bDNA, leukocyte count, and CRP(87). Furthermore, in a recent study with Such as coauthor(53), levels of cytokines measured by multiplex technology were of a similar magnitude to ours, and showed no relationship to bDNA. In study 3, we investigated BT and inflammation and constantly found bDNA largely unrelated to markers of inflammation and without relation to the direct measurement of portal pressure. We interpret the absence of a transhepatic gradient as evidence of a lack of major elimination of bDNA by the liver, and moreover our findings speak do not suggest a marked translocation of bacteria into the portal vein as one study had proposed for LPS(60). The absence of a relationship of BT to portal pressure is not in agreement with a study of Albillos et al, who in patients with high LBP found correlation to the HVPG and with the recent findings of Reiberger(14), who demonstrated LBP levels to be markedly elevated in patients with HVPG above 20 mmHg. Another interesting finding was the absence of impact of antibiotics on bDNA presence in Study 3, where the proportion of patients under antibiotics treatment were no less likely to be bDNA-positive than patients without antibiotic treatment, contrary to the findings by Such et al, who observed that patients treated with norfloxacin did not present with bacterial DNA(68). The finding that antibiotics did not in all cases eliminate the presence of bDNA is not entirely surprising, however, given that diagnostics by PCR has been advocated as being particularly relevant in antibiotics-treated patients(91). This finding strengthens the suggestion, that bDNA amplified by 16S PCR may not be demonstrating viable pathogens, but may in fact be fragments of organisms from previous translocation episodes as suggested by Soriano(88). Another finding is the absence of relation between presence of bDNA, and Child-Pugh stage. In fact in study 2, a trend towards higher frequency in Child Pugh B was noticeable. No difference was apparent in the studies by Such (67), in contrast to the MNL study by Cirera et al, BT was more frequent in Child Pugh C(58).

To what extent may the PCR methodology used explain our observations? The assay we used was developed for the specific purpose of these studies and their application tested in simulated trials beforehand. Our method was developed in a laboratory accredited for diagnostic PCR, that has for many years worked with the development of diagnostic PCR techniques in other settings and in variety of sample materials(92-94).
The development process revealed a number of challenges during development of measurement of bDNA by 16S PCR in blood and ascites and although apparently resolved, two of these did in fact reappear during the analysis of samples in studies 2 and 3. PCR inhibition made the quantification of bDNA samples impossible in a number of blood samples, reducing the number of samples for quantitative analyses. Although a number of components of the blood may affect the PCR process, no characteristics of patients were found that explained the differences. In the study 3, paired blood samples from the same patient were inhibitory in one sample, but not in another. The number of quantification failures differed markedly between the two studies, being
higher in the study 2. Pre-analytical handling of samples differed due to different settings between the two studies, and delay in processing may in some cases have occurred in protocol 2, where the samples were processed at several sites, and may have subjected to various processing times. Although present in samples from all facilities, the majority of inhibitory samples were from Hvidovre Hospital. Although we could determine the absence of bDNA in all inhibitory samples as described in the methods section and appendix, we could not quantify these samples.
The other issue is the failure to sequence the amplified PCR products. Sequencing was generally successful during development with spiked E.coli samples (appendix, Table #).

For successful sequencing the presence of a certain quantity of a single species source is necessary. Bacterial load as assessed by 16S PCR was in many samples, close to the content of sterile molecular grade water, and low bDNA contents may have contributed to sequencing failures. Despite the sensitivity of PCR, culture has the distinct advantage of large volume of sample material, and in case of low numbers of pathogens, PCR may fail due to an by simply missing the few pathogens present. This may explain our failure to sequence even culture-positive samples. Furthermore as suggested by Soriano et al., the presence of polyclonal DNA, possibly fragments of earlier translocation episodes, may have contributed to sequencing failures. An unexpected discrepancy is the difference in proportion of bDNA-positive patients in the blood of the two studies. In the second study, the proportion of bDNA positive patients was 22% in peripheral venous blood whereas in the third study the proportion of bDNA-positives was 39% and 43% in the two vascular territories. The patients included in Study 3, comprised patients with variceal bleeding, and antibiotic treatment for known or suspected infections, may account for increased bDNA in this group. No previous study using bDNA as marker of BT has investigated levels in central vessels. The difference in sampling material, i.e. central versus peripheral blood, may also explain this difference. We hypothesized the frequent presence of bDNA in cirrhosis to be associated with marked immunological derangement. However, the absence of this association, in combination with the immunological abnormalities observed in Study 1 in cirrhotic patients compared to healthy controls, raise the question of the presence of bDNA in the blood of healthy would demonstrate bDNA. During development of the assay we used blood of a healthy volun-

teer member of our group, and found it to have bDNA quantities similar to water controls. We did not, however, include a control group systematically assessing bDNA levels in healthy controls. In studies assessing bDNA in healthy volunteers, results have been diverging, some suggesting its presence(95), others including Such's group(65), finding none(96). The use of LBP as an alternate marker in study 2 and 3 was based on previous finding of LBP as a likely marker of BT being related to immune activation and haemodynamics(13). Accordingly, we assessed LBPs relation to bDNA and found none. This finding supports the missing relation of bDNA to markers of inflammation and haemodynamics, speaking against bDNA as a clinically relevant marker of BT.

CONCLUSIONS

We have demonstrated abnormalities in the immune system of patients with cirrhosis pointing towards both activation as well as impairment of the immune system, and a relation to progression of the disease and prognosis. By understanding the source of inflammation, and the pathophysiological mechanisms that link the inflammatory proces to prognosis, it may be possible to identify patients who can benefit from interventions including antibiotics or change in current therapies such as beta-blockers.

Our results do not support the assumption that bDNA, as assayed in the present studies, is a relevant marker of BT, as it seems unrelated to inflammation and haemodynamics. Several other studies have also been unable to reproduce the findings of Such et al., and recent findings from this group are less encouraging than their initial reports. Although the identification of an ideal marker of BT in clinical research would be of great value, an alternate approach, interventional studies using antibiotics in patients at risk seems justified by the present and growing amount of direct and indirect evidence supporting the role of BT in the course and prognosis of cirrhosis. Such studies might address the impact of antibiotics on markers of BT, immune activity and haemodynamics, shedding light on both the pathophysiology and clinical impact of bacterial antigens, immunity, and haemodynamics in cirrhosis.

SUMMARY

Bacterial translocation (BT), the migration of enteric bacteria to extraintestinal sites, is related to immune stimulation and haemodynamic changes in experimental cirrhosis. These changes may be highly relevant to patients with cirrhosis, where changes in the circulation cause serious complications. The optimal surrogate marker of BT in patients with cirrhosis, however, is a matter of controversy. In the first study, we investigated the relationship between markers of inflammation, haemodynamics and prognosis in 45 patients and 12 controls. We found high-sensitive C-reactive protein to be correlated to portal hypertension, a clinically relevant haemodynamic alteration, and appeared to be associated with increased mortality. To assess the consequences of BT on immunity, we developed an assay for the detection of bacterial DNA (bDNA), a novel marker of BT.

Using the assay in the second study, in 38 patients with ascites, we found no association between bDNA and immunity, in contrast to some previous findings. In the final paper, exploring one possible translocation route, we hypothesized a difference in bDNA levels between the blood from the veins draining the gut on one hand and the liver on the other. Collecting samples during the insertion of a shunt between the two vessels in 28 patients, our finding did not suggest marked differences in bDNA, but conversely to expectations, suggested marked hepatic production of two markers of inflammation.

The main results of the present thesis support some concepts of current thinking on cirrhosis pathophysiology, including the relationship of markers of inflammation to haemodynamics, disease stage and prognosis. Our results also add to a growing body of evidence suggesting that bDNA is not a clinically relevant marker of BT.

Litterature

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