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BACTERIAL TRANSLOCATION, INFECTION AND COAGULATION IN CIRRHOSIS AND PORTAL HYPERTENSION

S.S.D. MED12

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INTRODUCTION

Introduction

In cirrhotic patients there is an increased susceptibility to bacterial infection, related to the degree of liver dysfunction (1-7) leading to several abnormalities of defense mechanisms, all of which increase the susceptibility to infection, including deficiency of bactericidal and opsonic activities (8;9), impaired monocyte function (10), depressed phagocytic activity of the reticuloendothelial system (RES) (11;12), defective chemotaxis (13), and low levels of complement in serum (14).

A particularly important role is played by the reduced RES activity, which is due to the presence of extrahepatic shunts and intrahepatic shunts through sinusoids without Kupffer cells, a reduced number of Kupffer cells, and impaired Kupffer cell function (15). Thus cirrhotics with impaired RES phagocytic activity (as assessed by the elimination of 99m technetium-sulfur colloid) develop acute bacterial infections more frequently than cirrhotics with normal RES phagocytic activity (12).

Both community (4) and hospital acquired (16) bacterial infections are frequently diagnosed in cirrhotics (17), the most frequent being spontaneous bacterial peritonitis (SBP), urinary tract infections, pneumonia and skin infections (4), their incidence increasing with the severity of liver dysfunction (4;18-20). Importantly, half of these episodes may be asymptomatic (3).

Recently bacterial infections and/or endotoxaemia have been associated with failure to control variceal bleeding (21), more early variceal rebleeding (22), abnormalities in coagulation (23;24), vasodilatation of the systemic vasculature (25) and worsening of liver function (26). There is an increased recognition that bacterial infections are involved in several pathophysiological abnormalities in cirrhosis, so that in this introduction we aim to evaluate

the potential mechanisms and the clinical evidence illustrating the pivotal role of bacterial infection. This could lead to new treatment strategies.

Endotoxaemia in cirrhosis

Endotoxaemia in liver disease was first described in 1970 in patients with biliary obstruction (27) and it is frequently found in cirrhotics (28-34), even in the absence of any signs of sepsis (28). Thus there are higher endotoxin concentrations in peripheral blood of cirrhotics than in normal subjects (32;33) with a statistically significant gradient between portal and peripheral blood (33), highlighting the role of the bowel as the source of endotoxin. Both peripheral and portal levels of endotoxaemia are correlated with the severity of liver disease (29;32;33;35) which is more important than porto-systemic shunting or portal hypertension as a major factor associated with of plasma endotoxin concentration (28;32).

Bacterial overgrowth, altered intestinal motility and increased intestinal permeability

Alteration of small bowel motility, bacterial overgrowth in the small intestine, and increased intestinal permeability are all factors leading to increased endotoxaemia, the incidence of bacterial translocation and ultimately the risk of bacterial infection in cirrhotics.

In cirrhotic rats with ascites, bacterial overgrowth results from intestinal hypomotility, and bacterial translocation only occurs in presence of overgrowth and severe disruption of the gut barrier (36).

In humans with cirrhosis, bacterial overgrowth has been shown in one third of alcoholic cirrhotics, especially those with ascites and severe liver dysfunction (37), and is associated with reduced small bowel motility (38), hypochlorhydria (39), decrease in intraluminal immunoglobulins and reduced secretion of IgA (40). In addition, an abnormal colonization of the small bowel by colonic bacteria has been found in cirrhosis (41). The alteration of small bowel motility is due to a prolonged intestinal transit time (42) which is related to portal hypertension by stimulation of neurons through changes in the intestinal wall (43). The altered small bowel motility in cirrhosis worsens with severity of liver disease (44;45). A coordinated motor function of the bowel is probably the most important mechanism for preventing bacterial overgrowth (36).

Intestinal permeability is impaired by portal hypertension (46), particularly in patients who are Child grade C (47) or have an alcoholic aetiology (48), especially when there is ongoing alcohol intake (49). Mucosal abnormalities of the gastrointestinal tract (leading to increased permeability) secondary to portal hypertension, are also found in cirrhotic patients (40). Overproduction of nitric oxide (NO) is constantly present when there is portal hypertension, leading to disruption of the integrity of the intestinal epithelium (50). In one study, intestinal permeability was assessed in 80 cirrhotic patients (mostly alcoholic): an increase in permeability was found to be correlated to the occurrence of septic complications (47).

SBP is a haematogeneous infection localising in the ascitic fluid with the source of the infecting bacteria most likely being the gut through bacterial translocation. A history of SBP is associated with more severe intestinal dismotility and more frequent bacterial overgrowth (38;40). Bacterial overgrowth leads to bacterial translocation (51) and together with increased intestinal permeability leads to an increased risk of SBP and also worsening of hepatic encephalopathy (52-55).

Cisapride and antibiotics (such as norfloxacin and neomycin) improve both small intestine dysmotility and bacterial overgrowth in cirrhosis (56). Interestingly, propranolol, which lowers portal pressure, reduced the risk of post surgical infections in a cohort of 73 cirrhotic patients (15 % vs 42 %) (57). The mechanism may not only be by lowering portal pressure but also by increasing motility of the bowel by its sympatholytic action, thus indirectly decreasing microbial translocation to the systemic circulation. A recent retrospective study of 139 patients suggested that propranolol may prevent SBP (58). Thus propranolol could have another mode of action in reducing variceal rebleeding by preventing or reducing bacterial translocation, thus reducing the frequency of infection which may be a trigger for bleeding (59).

Bacterial translocation

The term bacterial translocation was first coined by Berg and Garlington (60), and was later defined as the passage of both viable and non-viable microbes and microbial products, such as endotoxin, from the intestinal lumen through the epithelial mucosa into the mesenteric lymph nodes (MLNs) and possibly other organs (50).

There are multiple routes by which an organism potentially could translocate from the gut to extra-intestinal sites (retrograde migration to the lungs, direct transmural migration across the intestinal wall, lymphatic migration via Peyer's patches, MLNs, the thoracic duct and the systemic circulation or via vascular channels to reach the portal system) (50), but in the setting of liver cirrhosis the intestinal macrophages seem to play a key role by carrying bacteria from the gut to MLNs (61).

The principal factors that normally prevent bacterial translocation are the balance of intestinal bacterial populations (essentially controlled by peristalsis), the integrity of the intestinal barrier and immunocompetence (17).

In addition decreased killing of bacteria may be responsible for increases in colony-forming units in the MLNs, rather than an increase in transepithelial penetration (50), as most microbes which breach the epithelial barrier are killed (62). Thus, for bacterial translocation to become clinically significant, a failure of local and/or systemic immune defence, as is commonly present in cirrhotic patients, is also required (50).

In experimental models of liver cirrhosis, bacterial translocation has been reported to occur with an incidence ranging from 37 to 83 % (50). Portal hypertension is one factor determining bacterial translocation, as it increases in cirrhotic rats after the development of ascites (63), but other factors also have a role, such as reduced defenses and bacterial overgrowth (64). A lower rate of translocation has been observed in portal vein ligated rats with chronic prehepatic portal hypertension but without parenchymal liver damage, which also have milder histological changes in the bowel (64;65). In human cirrhosis**,** the incidence of bacterial translocation is also related to the degree of portal hypertension and liver dysfunction, and is found in approximately 30 – 40 % of cirrhotics with ascites (36;63;66;67) and is more frequent in Child C compared to Child A and B cirrhosis (66).

Only a few types of intestinal bacteria are able to translocate into mesenteric lymph nodes: Escherichia coli, Klebsiella pneumoniae, other Enterobacteriaceae, Pseudomonas aeruginosa, Enterococci and some Streptococci (68). These bacteria constitute up to three quarters of microorganisms isolated in SBP (69). In cirrhotic rats, genotype identity is frequent between

the ileal flora and the bacteria colonizing mesenteric lymph nodes (70). A recent study found that 7 of 17 advanced cirrhotic patients with culture-negative, nonneutrocytic ascites had the simultaneous presence of bacterial DNA in blood and ascitic fluid at admission to hospital and which persisted in blood for 24-72 hours (71). Furthermore, nucleotide sequencing demonstrated that bacteria detected in the first sample were identical to those noted in subsequent detections over time, proving that bacterial translocation is a single-species, dynamic process (71). Cirrhotics are also predisposed to spontaneous infections other than in ascites (72-76) by intestinal bacteria (72;75-77) as a consequence of bacterial translocation (63). Selective intestinal decontamination with antibiotics reduces bacterial translocation (66) and decreases the risk of infections, especially those due to Gram negative bacteria (78;79). This again supports the role of intestinal bacteria as a source for infections in cirrhotics and may explain why prophylactic antibiotics both orally and intravenously are so effective in reducing infections during variceal bleeding (80).

Effects of endotoxaemia and infection

Chronic exposure to sublethal levels of endotoxin may prime liver parenchymal cells for production of NO when exposed to increased levels of endotoxin and/or TNF-α (81). NO itself may prime monocytes to endotoxin (82), as well as other biological mediators, released by endothelial cells when exposed to biomechanical stimuli such as shear stress in portal hypertension. In ascitic cirrhotics, monocytes are spontaneously activated mainly by enteric bacterial products to produce TNF-α and contribute in a major way to the elevated serum TNF-α (83). In response to endotoxin, in cirrhosis, there is an increased release of TNF-α, IL-1 and IL-6 and an increased expression of CD11b/CD18 on the surface of monocytes (82;84), not only due to the preactivation status of monomacrophages but also due to a reduced

catabolism of cytokines accompanying hepatic dysfunction (85-87). Cirrhotic patients with bacterial infection have very large concentrations of endotoxin in the systemic circulation and a marked and sustained increase in TNF-α and IL-6 concentrations (88). These remain high for a longer time as compared to non infected decompensated cirrhotics and non cirrhotic infected patients (88). Treatment with norfloxacin in a recent study in 60 alcoholic ascitic cirrhotic patients normalised the increased number of monocytes, reduced their activated phenotype and their ability to produce TNF-α (83).

As endotoxin is primarily removed by Kupffer cells (89), the hepatic RES modulates most of the effects of endotoxin on the liver (such as increase of ICAM-1 expression on hepatocytes, endothelial cells and macrophages) (90). Clinically, the increased endotoxin concentrations may lead to increased disturbance of the systemic and regional circulations, coagulopathy, encephalopathy, and lead to both renal and hepatic failure and ultimately death, in chronic as well as acute liver disease (28;34;91-95).

Influence of infection on haemodynamics

Arterial vasodilation, particularly in the splanchnic circulation, is a major causative factor in the pathogenesis of the hyperdynamic circulatory syndrome that is well documented in cirrhosis with portal hypertension (96). The impairment in vascular contractility with hyporeactivity to vasoconstrictors in cirrhosis (97;98) is due to overproduction of NO (97;99;100), the major source being endothelial nitric oxide synthase (NOS) (101), as well as other mechanisms (102).

Bacterial translocation, in the presence of portosystemic shunting and reduced hepatic clearance, leads to the presence of bacteria, endotoxins and cytokines in the systemic circulation (25;50), thus enhancing NO production (103-105). The latter occurs via a direct stimulatory effect of endotoxin, but also by endotoxin induced secretion of cytokines such as TNF-α and via GTP-cyclohydrolase I which increases tetrahydrobiopterin (BH_4) synthesis (an essential cofactor in synthesis of NO) (106). This increase in NO further aggravates arterial vasodilation in cirrhosis (17;25;107).

Cirrhotics with bacterial translocation have haemodynamic derangement (108). Systemic vacular resistance (SVR) is lower and cardiac output higher in cirrhotic patients with endotoxaemia than in those without (32); Moreau et al observed that cirrhotic patients with septic shock had lower vascular resistances than patients with septic shock without cirrhosis (109). Levels of lipopolysaccharide binding protein (LBP), a surrogate marker of endotoxaemia, are increased in 40 % of cirrhotics with ascites, but not in cirrhotics without ascites (108), suggesting that the increase in nitrate levels due to endotoxaemia and bacterial translocation might be related to worsening of portal hypertension and/or liver function. This is in agreement with the finding that serum nitrate levels not only correlate significantly with endotoxaemia (29) but are also increased in cirrhotic patients with ascites or kidney failure and are associated with high plasma renin activity, aldosterone and anti-diuretic hormone levels and low urinary sodium in cirrhotics (29). Very interestingly, selective intestinal decontamination can normalize LBP levels, reduce renin and nitrate levels and increase SVR in these patients, but it had no effect on patients with normal LBP (108). In another study, cirrhotic patients with high TNF-α concentrations in mesenteric lymph nodes (suggesting bacterial translocation) had a higher cardiac index and a higher portal pressure (110). In rats with portal hypertension, inhibition of TNF-α production is accompanied by amelioration of the hyperdynamic syndrome and even a decrease in portal pressure (111-113). These studies

suggest that endotoxaemia and bacterial infection can exacerbate the haemodynamic alterations in cirrhosis, thus leading to a further worsening of portal hypertension via activation of neurohumoral pathways and further fluid retention in response to systemic vasodilatation. The correlation between the presence of endotoxaemia or bacterial translocation and the worsening of the haemodynamic status is confirmed by the fact that intestinal decontamination reduces nitrate and endotoxin levels and vasodilation, both in cirrhotic patients (29) and in an experimental rat cirrhotic model (114). In the latter study intestinal decontamination also reduced the development of ascites (114). In a human study basal forearm blood flow and the vasoconstrictive effect of the NOS inhibitor, N^G-monomethyl-L-arginine was increased in cirrhotics compared to controls but returned to normal in cirrhotics after selective intestinal decontamination with norfloxacin (115). In a recent study in 14 patients with alcoholic cirrhosis (25), selective intestinal decontamination reduced serum endotoxin concentrations and partially reversed the chronic peripheral and systemic vasodilatation by increasing SVR and mean arterial pressure and decreasing cardiac output and forearm blood flow. Interestingly, HVPG was also decreased by a mean of 2.43 mm Hg (25), further strengthening the hypothesis that bacterial products contribute to the hyperdynamic circulation and portal hypertension in cirrhosis.

In addition to the direct effects of the release of vasoactive mediators such as NO with regard to the worsening of the haemodynamic derangement in infected cirrhotics, the release of inflammatory mediators may also play a role. Tumour necrosis factor alpha (TNF-α) is involved in the pathogenesis of the hyperdynamic syndrome in portal hypertension (111;112); the gut and its associated lymphoid tissue produce and release TNF-α in response to bacterial translocation even in the absence of portal or systemic spread of bacteria (116;117). Vascular reactivity in cirrhotic rats with bacterial translocation to the MLNs is more impaired than in

those without translocation and is most likely due to an increase in endothelial NOS activity mediated by TNF-α (103).

Renal failure in SBP occurs in the setting of an intense inflammatory response and is related to deterioration of circulatory dysfunction (as shown by higher aldosterone and norepinephrine levels and plasma renin activity) (118); it can be viewed as the result of the combined circulatory dysfunction of cirrhosis, and sepsis, thus leading to additional stimulation of the renin-angiotensin system. Cirrhotics with SBP who developed renal impairment had significantly higher ascitic fluid levels of neutrophils and both plasma and ascitic fluid levels of TNF-α and IL-6, than those without renal impairment (119).

Hepatorenal syndrome (HRS), which is accompanied by the most extreme haemodynamic derangement in cirrhosis, is associated with increased concentrations of IL-6 and TNF-α, as compared to patients with liver failure and normal renal function (120). After liver transplantation, IL-6 and TNF-α levels return to near normal**,** concomitant with improving renal function, suggesting that IL-6 and TNF-α emanating from the liver may play a role in HRS (120)**.** This could be either through TNF-α induced oxygen radical formation by mesangial cells (121) or through TNF-α related increase in NO by mesangial (122) or endothelial cells (123). Another link between inflammation and haemodynamic dysfunction is the finding that patients with gross ascites had increased levels of C-reactive protein (35) and that serum nitrate levels were correlated to levels of orosomucoid (a protein synthesized by the liver in response to cytokines) (124;125). Treatment with intravenous albumin in addition to antibiotics reduces the likelihood of renal impairment in SBP (126), not only by albumin expanding the decreased plasma volume but possibly also by binding of cytokines or NO (127-129) or its antioxidant effect in sepsis (128;130;131). Renal failure occurs frequently in

patients with variceal bleeding so that bacterial infection may contribute directly to this over and above any hypotension which can lead to acute tubular necrosis.

Endotoxin also stimulates the synthesis of endothelin (132), the renal production of thromboxane A2 (133) and increases the plasma levels of leukotrienes (134), all of which act as renal vasoconstrictors. This release of vasodilators and renal vasoconstrictors due to infection may be another factor contributing to the development of renal impairment in SBP (135).

The most consistent clinical data on the influence of bacterial infection on systemic haemodynamics in cirrhotic patients has been reported in the setting of SBP: renal impairment develops in one third of patients (135), especially in those with the highest degree of inflammation as expressed by higher concentrations of cytokines in plasma and ascites (118;119). SBP is associated with a worsening of the haemodynamic status as shown by the activation of the renin-angiotensin system (119) caused by the decrease in effective arterial blood volume as a result of infection (119;135). Treatment with cefotaxime and albumin as compared to cefotaxime alone leads to less renal impairment and lower mortality, and lower levels of plasma renin activity (126). Thus in SBP circulatory dysfunction develops which may be prevented by albumin treatment. Recently, in another study (118) WHVP and HVPG increased (from 19.0±2.6 to 23.6±2.6 mm Hg, $p<0.025$) in 8 cirrhotic patients with SBP and renal failure despite a decrease in effective arterial blood volume and the systemic vasodilatation. This emphasizes the differential effect that the same stimulus can have on the systemic and splanchnic circulations. When SBP is associated with systemic circulatory dysfunction there is an increase in HVPG, which is thought to be secondary to the action of endogenous vasoconstrictors on vascular smooth muscle cells in small venules and on activated hepatic stellate cells (myofibroblasts) (136). In the setting of portal hypertension

there is a relative deficiency in NO in the intrahepatic circulation, thus increasing the susceptibility to vasoconstriction (15).

Endothelin-1 (ET-1) is the most potent mediator of stellate cell contraction (137) and in the liver ET-1 receptors predominate in hepatic stellate cells (138), which have an important role in the regulation of intrahepatic portal hypertension in cirrhosis (139). Both endotoxin itself and the cytokines released in response to it, are potent stimuli for the production of ET-1 (59), which may act in combination with cyclooxygenase products to increase portal venous resistance during endotoxaemia (140). In the cirrhotic liver there is a greater induction of the vasoconstrictor (ET-1) over vasodilatory forces (NO, carbon monoxide) after lipopolysaccharide (LPS) injection (141). There is a compromised ability to upregulate sufficient vasodilatory forces to counterbalance the constrictive effect of ET-1 following increases in endotoxaemia, thus leading to increased intrahepatic resistance. This would result in an acute increase in portal pressure.

Other vasoconstrictors such as angiotensin II and norepinephrine can also lead to an increase in intrahepatic resistance in rats and in vitro models (136;142;143) and thus lead to an increase in portal pressure (118). These mediators are increased in cirrhosis and are further augmented in response to the systemic vasodilatation occurring in bacterial infection.

Thus, if infection has a causal role in precipitating variceal bleeding then it could be acting via an increase in portal and/or variceal pressure secondary to a rise in intrahepatic resistance as we have previously outlined as part of our hypothesis suggesting infection may be a trigger for variceal bleeding (21).

Influence of infection on coagulation

Using thromboelastography (TEG), 20 cirrhotic patients who had bled from varices, and then suffered early rebleeding were found to have a worse TEG trace on the day before rebleeding as compared to those who did not rebleed (144). TEG parameters have also been found to worsen in 84 decompensated cirrhotic patients in the presence of bacterial infection (145). A recent study found heparin activity in blood by using heparinase I-modified TEG in 28 of 30 patients with bacterial infection, but not in non-infected patients, and this effect disappeared after resolution of infection; other coagulation parameters were not modified significantly (23). This heparin activity was subsequently found to be associated with anti-Xa concentrations in many, although not all patients, suggesting that the heparinase effect is due to an endogenous low molecular weight heparin (24).

In a single study plasma heparan sulphate concentrations were significantly raised in patients with recent variceal bleeding as compared to those without bleeding or non cirrhotic patients (146). Endotoxin or cytokines could release heparinoids from endothelium in a dose-dependent manner (23) and mast cell activation due to bacterial infection could also release heparin. Mast cells and activated endothelial cells also are able to release tissue plasminogen activator (t-PA), which induces fibrinolysis (23).

Sepsis causes defects in platelet aggregation (147), and this may constitute another reason for an impairment of the haemostatic process in cirrhotic patients, either via a decrease in platelet aggregation due to NO (148) or due to the fact that endotoxin and ET-1 can impair platelet aggregation through the release of prostacyclin (149-151).

The production of cytokines in the presence of bacterial infection can lead to the activation of clotting factors and fibrinolysis (152;153). In 30 % of patients with advanced liver disease an accelerated intravascular coagulation and fibrinolysis has been shown, and these patients are prone to develop DIC if sepsis occurs (154).

Hyperfibrinolysis has been directly associated with gastrointestinal haemorrhage in cirrhotic patients (155-158); unfortunately presence of infection was not documented in these studies. Bleeding from oesophageal varices has some correlation with the circadian rhythm of fibrinolysis in cirrhotics (159)**.** Features of DIC have been reported in cirrhotic patients with variceal haemorrhage, especially in those who die of continued bleeding (160), where multiple transfusions may be a confounding factor. However there is little evidence for DIC occurring as a primary event in cirrhosis (161). Moreover, another study found a strong association in 41 cirrhotic patients between endotoxaemia and high plasma levels of prothrombin fragment F1+2 and D-dimer (a pattern suggestive of secondary fibrinolysis), which interestingly then returned to normal after administration of non absorbable antibiotics (162)**.** A recent report showed a lower protein C activity in cirrhotics with severe sepsis as compared to those with non severe sepsis or without sepsis; this deficiency in protein C activity is associated with significant inflammatory response and DIC (163;164).

Influence of infection on liver damage

Normal liver microcirculatory function is maintained by a balance of vasoconstrictors (ET-1) and vasodilators (NO, carbon monoxide) (141). Both endotoxaemia and cytokines such as TNF-α and IL-1 cause hepatic necrosis by disruption of the microcirculation (165-168), the alteration in microcirculation due to endotoxin being mediated via Kupffer cells (169).

Endotoxin leads to the activation of Kupffer cells and the subsequent release of cytokines that worsen liver failure in several experimental models of liver failure (170-172). This production of proinflammatory cytokines and prostaglandins leads to infiltration of leukocytes and then liver failure (93). LPS has also been found to induce a marked influx of activated neutrophils into the liver. These are a source of reactive oxygen species (173), which are also triggered by LPS itself, as well as nitrogen species (174) leading to lipid peroxidation in the liver and secondary damage to hepatocytes.

Kupffer cells play a pivotal role in endotoxin-induced hepatic injury (175-177). LPS triggers production of IL-1β, TNF-α, IL-12, IL-18 from Kupffer cells, IFN-γ from hepatic lymphocytes, and induces Fas-Ligand on the surface of NK cells, all leading to hepatic injury (178). TNF-α is hepatotoxic in itself, inducing the apoptosis of hepatocytes (179;180). LPS-induced liver failure in mice is prevented by inhibition of NF-kB, thereby decreasing cytokine production (181). In addition, hepatocytes have membrane receptors for both endotoxin and the lipid A component of LPS, thus offering another possible pathway for liver injury (172).

Antibiotic treatment prevents early liver injury caused by ethanol in rats (182) and both cisapride and antibiotics improve liver function in cirrhotic patients (56), strongly suggesting that bacterial products via translocation can worsen liver function.

Other clinical evidence also suggests that liver function may be worsened by bacterial infections. Indeed, infections can aggravate liver dysfunction in patients with cirrhosis (26;183) and severe liver failure occurs in most patients with cirrhosis and septic shock (109;184;185). Thus sepsis may affect liver function independent of its haemodynamic effects.

Clinical evidence linking bacterial infection to variceal haemorrhage

Bacterial infections are frequently associated with upper gastrointestinal (GI) bleeding in cirrhotic patients (18;183;186;187), developing in up to 66 % (20 % within the first 48 hours, 35-66 % within 2 weeks) (2;18;188;189). About two thirds of these infections are present at hospital admission, while the remaining third develops during admission (3). Moreover, bacterial infections are more common in cirrhotic patients with acute variceal bleeding than in those admitted to hospital with other forms of decompensation such as encephalopathy (3;190).

Our group showed that proven bacterial infection, or a surrogate of its presence, use of antibiotics, had the strongest independent association with failure to control bleeding in cirrhotic patients with variceal bleeding, even stronger than active bleeding at endoscopy and severity of liver disease (21). Recently a prospective survey of 1037 cirrhotics reported that the 297 with proven infection had a fourfold increase in the incidence of GI bleeding (8 % vs 2 %, $p<0.001$) compared to 346 known not to have bacterial infection, and that infection was independently associated with the occurrence of GI haemorrhage (191).

The strong association between infection and variceal bleeding in cirrhotics has been confirmed in several studies: association with failure to control bleeding (21;192), early rebleeding (2;191;193) and mortality (192).

Two meta-analyses have shown that antibiotic prophylaxis prevents infections in cirrhotic patients with gastrointestinal bleeding and significantly increases the short-term survival rate (80;187). The improvement in mortality is equivalent to that seen with terlipressin (194). In a very recent randomised study of 120 patients prophylactic ofloxacin compared to on demand antibiotics was shown to prevent early rebleeding (24 % vs 64 %, $p<0.01$) and to decrease the amount of blood transfused (1.40 ± 0.89 vs 2.81 ± 2.29 units, $p<0.05$), in addition to preventing bacterial infections (22). Therefore, since our hypothesis was published (59), there are now a

number of publications strongly suggesting a causal link between the presence of an infection and the initiation of acute variceal bleeding and its associated early rebleeding, and supporting our hypothesis.

Worsening of liver function is a recognized risk factor for first variceal bleeding (195), so that infection might contribute to this risk, or indeed be a trigger for variceal haemorrhage, particularly as the liver damage occurring in sepsis may itself contribute to an acute increase in portal hypertension.

The risk for portal hypertensive related bleeding in cirrhotic patients is related to the degree of portal hypertension, liver dysfunction and to the size and endoscopic appearance of varices (195). However trigger factors are not known. In the setting of portal vein thrombosis in non cirrhotic patients, where often there are oesophageal varices of the largest size with red signs, the incidence of bleeding is much lower than in cirrhotics with similar varices (196). Thus the bleeding rate in portal vein thrombosis has been documented as 12,5 episodes per 100 patient years (197;198), whereas the risk of first bleeding with Child grade A cirrhotics with large varices and moderate red signs is 24 % in 1 year and in Child grade C with small varices and no red signs it is 20 % (195).

The difference probably lies in the presence of liver disease (although the thrombophilic conditions often associated with portal vein thrombosis cannot be discounted). Cirrhosis predisposes to risk of infection, which is not reported in non-cirrhotic portal hypertension. In particular bacterial translocation is much less frequent in rats with portal vein ligation as compared to cirrhotic rats (64;65).

This increased release of endotoxin and viable bacteria into the portal and systemic circulation is closely related to liver cirrhosis, with portal hypertension and liver dysfunction influencing increased intestinal permeability and altered small bowel motility on the one hand and bacterial overgrowth on the other (Figure 1). The low grade endotoxaemia leads to the priming of monomacrophages and an increase of NO and TNF-α. Bacterial translocation causes a further increase in NO and TNF-α, a reduced response to vasoconstrictors and an increased risk of bacterial infection, with the associated risk of variceal bleeding, renal failure, hepatocellular injury, hepatic encephalopathy and mortality (Figure 2).

The possible causative role of bacterial infection in variceal haemorrhage, is less easily understood, but nevertheless is most intriguing (59). Indeed, the known risk factors for variceal bleeding (HVPG, liver function, size of varices and presence of red signs) do not readily explain why bleeding and early rebleeding occur unpredictably in patients with cirrhosis. Portal pressure rises significantly with daily meals as well as exercise (199;200), yet despite these marked daily changes bleeding episodes are relatively infrequent. Thus a merely mechanical understanding of variceal bleeding as a consequence of portal pressure and tension on the variceal wall does not in our view explain the pattern of variceal bleeding. Endotoxaemia secondary to bacterial infection may indeed be the critical trigger for variceal haemorrhage (59), since it produces a wide series of effects that may predispose the cirrhotic patient to bleeding: impairment of primary and secondary haemostasis, increase of portal pressure and worsening of liver function (Figure 3).

METHODOLOGICAL STUDIES REGARDING THROMBOELASTOGRAPHY

THE EFFECTS OF UNFRACTIONATED HEPARIN, LOW MOLECULAR WEIGHT HEPARIN AND DANAPAROID ON THE THROMBOELASTOGRAM (TEG): A COMPARISON OF STANDARD AND HEPARINASE MODIFIED TEG WITH CONVENTIONAL COAGULATION ASSAYS

Introduction

Thromboelastography, developed in 1948 (201), has been used extensively as a research tool and more recently has been employed during liver transplantation and cardiac surgery to assess coagulation and guide blood product replacement in the peri-operative period (202-204). The initial unpopularity of TEG as a coagulation test was in part due to the poor understanding of how variables in the coagulation system affecting the TEG, how these variables correlate with other more conventional clotting tests and how the procedure should be standardised (205-207). More recently, a number of studies re-investigating the properties of the TEG have been published, prompted by the increasing use of the TEG in clinical practice (208-214).

Thromboelastography provides a global and dynamic assessment of coagulation that is not addressed by most conventional laboratory coagulation tests (215). Conventional assays terminate with the formation of a fibrin clot, and hence possess a well-defined end point which is easily standardised. Different parts of the clotting cascade are artificially isolated in these assays, and therefore they tend not to reflect other haemostatic abnormalities that might interfere with the formation of a stable fibrin clot, such as for example impaired platelet function. In contrast, TEG starts to generate data at the point at which fibrin strands begin to form, and as such is a more dynamic and global investigation, with multiple measurable endpoints (216). This reflects the interaction between platelets, the clotting cascade and

fibrinolysis. TEG is sensitive to qualitative and some quantitative differences in platelets, proteins of the clotting cascade and fibrinolysis (217;218).

The TEG trace can be modified by several factors, but because of the increasing use of TEG during surgical procedures in which the administration of heparin is routine, the effects of heparin and heparin-related compounds have been the most widely studied (219-224). Commercially available TEG cuvettes coated with heparinase I can be used to inactivate heparin and other glycosaminoglycans (GAGs) such as heparan sulphate, for the assessment of TEG in patients who have received heparin (225-228). However, little data has been published regarding the correlation between the variables of the standard and heparinise-modified TEG with conventional clotting tests. The present *in vitro* study was divided into two parts: in the first part, the sensitivity of standard TEG parameters was compared with conventional assays of coagulation for the detection of unfractionated heparin (UFH), low molecular weight heparin (LMWH) and Danaparoid in whole blood, over the concentration range of 0.025 – 1 U/ml. In the second part, the sensitivity of heparinise-modified TEG parameters was compared with the standard (unmodified) TEG and conventional assays of coagulation, for the detection of very low concentrations of UFH, LMWH and Danaparoid (concentration range of 0.005 – 0.05 U/ml).

Materials and Methods

Heparins and Danaparoid

Unfractionated heparin (UFH) was supplied by CP Pharmaceuticals Ltd., Wrexham, UK. Low molecular weight heparin (LMWH) – Dalteparin (Fragmin®) was supplied by Pharmacia &

Upjohn, Milton Keynes, UK. Danaparoid (DPD) – Orgaran® was supplied by B & S Durbin Ltd., Middlesex, UK.

Subjects

This study was performed as part of a larger ongoing study of Thromboelastography in various medical disorders, which has been approved by the local research ethics committee (LREC) of the Royal Free Hospital. Blood samples were taken from ten healthy male volunteers for each part of the study. For all subjects, baseline haemoglobin, white cell count, platelet count, prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT), reptilase time, anti-Xa activity, standard TEG and heparinase I modified TEG were all within the reference ranges for these investigations.

Blood sampling

Blood samples were collected into plastic 10ml Sarstedt Monovette® vacuum tubes containing sodium citrate solution (final concentration 0.106 M), using a 19G needle and no tourniquet. In the first part of the study, a 10u/ml solution of LMWH, UFH or Danaparoid was diluted 1/10 with citrated whole blood, and serial dilutions were made using citrated blood to achieve final concentrations of 1.0, 0.5, 0.25, 0.1, 0.05 and 0.025 U/ml. Control samples were prepared for three subjects using an equal volume of 0.9% (isotonic) saline instead of heparin / Danaparoid and diluting with citrated blood using the same protocol. This was in order to exclude a dilutional effect on blood coagulability from the addition of these drugs, and to determine the stability of the sample over the time from phlebotomy to completion of all TEGs. In the second part of the study, serial dilutions of LMWH, UFH or Danaparoid were made to final concentrations of 0.05, 0.025, 0.010 and 0.005 U/ml. After phlebotomy, all samples were allowed to stabilise in citrate for at least one hour prior to TEG analysis (229;230).

Thromboelastography

Disposable standard and heparinase I coated plastic cuvettes and pins were supplied by Medicell UK (for Haemoscope Corp., Skokie, IL). Two Haemoscope Thromboelastograph® coagulation analysers were used for this study. 20µl of 30mmol calcium chloride were added to a 340µl aliquot of test blood (total volume 360µl) in the cuvette to reverse citrate chelation, and mixed by raising and lowering the TEG pin three times. A thin film of mineral oil was floated on the surface of the blood to prevent evaporation during analysis. The cuvettes were pre-heated to 37°C in the analyser.

In the first part of the study, TEGs on saline and heparin / Danaparoid spiked samples from the three control subjects were performed in parallel at each concentration. Standard and heparinise-modified TEGs for each sample dilution in the second part of the study were also performed in parallel. Tracings were recorded and analysis stopped once the tracing had reached the maximum amplitude, or after 60 minutes if a straight-line trace was produced. Clot retraction and fibrinolysis were not assessed in this study. TEG variables assessed were reaction time (R-time), clot formation time (K-time), clot formation rate (α-angle) and maximum amplitude (MA) (Figure 4). A value of 60 (minutes) for R-time and K-time, and 0 (degrees) for α-angle and 0 (mm) for MA were assigned arbitrarily to straight-line traces. Reference ranges for these variables were obtained from Medicell UK (for Haemoscope Corp., Skokie, IL, based on a normal population mean +/- 2 SD), and were confirmed by our own in-house assessment of 20 normal subjects from the local population.

Other coagulation assays

In addition to a TEG, the PT, aPTT, TT, and anti-Xa activity were determined for each sample. A reptilase time was also performed on samples from three control subjects to confirm that abnormalities of coagulation in the test samples were due to heparin and not an intrinsic abnormality of coagulation. Approximately 2.5ml blood from each sample dilution was centrifuged at 1200g for 12 minutes at 4□C, the plasma aspirated and re-centrifuged for a further 12 minutes. The supernatant plasma was frozen at -40°C until testing. Clotting times were assayed using standard techniques, with an IL-Futura® Coagulation Analyser from Instrumentation Laboratories, Warrington, UK. Anti-Xa activity was determined using a chromogenic assay (Sigma Diagnostics, UK).

Statistical analysis

Descriptive statistics for all TEG and conventional test parameters are expressed as the mean ± the standard error of the mean (SEM). The change in each TEG parameter vs. concentration (for both parts of the study) was analysed by calculating a summary slope of each parameter for each individual subject, and then assessing the mean of the summary slopes with a two-tailed t-test. The correlation between TEG parameters and conventional coagulation assays was evaluated by comparing means of summary slopes using Pearson's correlation coefficients.

For the second part of the study looking at low concentrations (0.005-0.05 U/ml) of heparins and Danaparoid, the effect of heparinase on each TEG parameter at each concentration was evaluated using a paired two-tailed t-test. In order to compare the sensitivity of heparinise-modified thromboelastography with the anti-Xa assay for detecting differences between concentrations of heparins and Danaparoid in the range 0.005-0.05 u/ml, two-tailed p-values were calculated using mean summary slopes for the difference between heparinase-modified

and standard TEG parameters vs. concentration, which were compared with p-values calculated from anti-Xa summary slopes. All calculations were performed with SAS for Windows version 8.02 and Microsoft Excel 2000.

Results

Tables 1a and 1b show the mean ± the standard error (SEM) for each TEG parameter and conventional assay performed in ten subjects for the first part of the study (concentrations of heparins and Danaparoid 0.025-1.0 U/ml). The mean ± SEM of standard and heparinise-modified TEG parameters and conventional tests performed in ten subjects from the second part of the study (concentrations of heparins and Danaparoid 0.005-0.05 U/ml) are presented in Table 2. Values outside the normal range are underlined in both tables. In the both parts of the study, the addition of UFH, LMWH or Danaparoid to citrated whole blood resulted in prolongation of the R-time and K-time, and reduction in the α-angle and MA of the standard TEG compared with control samples. The highest concentration of each anticoagulant (1.0 U/ml) produced a straight-line trace for up to 60 minutes, demonstrating complete inhibition of fibrin formation. TEG parameters were within the reference range for all saline control samples.

The results indicate that measurable differences in TEG parameters occurred at lower concentrations of UFH, LMWH and Danaparoid than standard PT, aPTT and TT assays were able to detect. In the first part of the study, concentrations of all three anticoagulants of less than 0.1 U/ml were undetectable by PT, aPTT or TT assays, but caused a reduction in the MA of the TEG for all three anticoagulants (Tables 1A and 1B). The K-time was prolonged by LMWH at 0.025 U/ml, but not by UFH or Danaparoid at this concentration. In the second part

of the study examining low concentrations (0.005-0.05 U/ml) of heparins and Danaparoid, the PT, aPTT and TT were within the reference range for all samples, but there was still a reduction (outside the reference range) in the MA of the TEG at concentrations of UFH of 0.025 U/ml, and concentrations of LMWH and Danaparoid down to 0.005 U/ml (Table 2). The K-time was prolonged by LMWH at concentrations of 0.005 U/ml, by Danaparoid at 0.01 U/ml, and UFH at 0.05 U/ml. Overall, the R-time and α-angle were the least sensitive of the TEG parameters in this study.

In the first part of the study, the variation in all four TEG parameters was concentration dependent over the range 0.025 to 1.0 U/ml, for all three anticoagulant drugs ($p<0.0001$) (Table 3A). In the second part of the study, the concentration dependent variation in all four TEG parameters due to UFH over the range 0.005 to 0.05 U/ml was still significant ($p<0.02$), but changes in TEG parameters caused by LMWH or Danaparoid were not significant at these low concentrations (Table 3B). Overall, there was no correlation between TEG parameters and the conventional clotting assays over the entire concentration range of 0.005 to 1.0 U/ml for any of the anticoagulants studied.

TEG parameters were within the reference range for all tests performed in heparinase I coated cuvettes in the second part of this study, suggesting that the enzyme had successfully neutralised the anticoagulant effect of all three drugs. 'Heparinase-modified' TEG parameters were calculated as the difference between TEG parameters recorded from aliquots of the same blood sample tested in standard and heparinase I coated TEG cuvettes in parallel. All four heparinise-modified TEG parameters were significant at the lowest concentration of LMWH and Danaparoid studied (0.005 U/ml; $p<0.05$) (Table 2). In contrast, only the heparinise-modified K-time and α-angle were significantly sensitive to UFH at this concentration.

Heparinase-modified TEG parameters were further compared with the sensitivity of the anti-Xa assay for distinguishing between concentrations of all three anticoagulants over the range 0.005 to 0.05 U/ml. In addition to significantly detecting the lowest concentration of UFH studied (0.005 U/ml), the heparinise-modified K-time and α-angle of TEG were found to be more sensitive at distinguishing between concentrations of UFH across the range 0.005 to 0.05 U/ml than the anti-Xa assay ($p<0.01$ vs. $p=0.43$). In contrast, the opposite was observed with LMWH and Danaparoid over this concentration range ($p<0.05$ for anti-Xa vs. $p>0.1$ for heparinise-modified TEG) (Table 4). The results suggest that at low concentrations of UFH, heparinise-modified thromboelastography may be more sensitive than the anti-Xa assay for detecting differences between small amounts of UFH in blood samples.

The clotting activity of citrated blood samples did not deteriorate significantly with storage for several hours prior to analysis, as demonstrated by the preservation of TEG parameters within the reference range over time for saline control samples. These controls also confirmed that observed effects on TEG or coagulation assays were not due to haemodilution from the addition of heparin / Danaparoid to blood samples. Reptilase times performed in three control subjects were within the reference range (results not shown), confirming that changes in TEG and coagulation parameters in this study were due to the addition of heparin / Danaparoid.

Finally, it is of note that UFH, LMWH and Danaparoid significantly prolonged the aPTT at concentrations similar to those expected *in vivo* when prophylactic doses are administered subcutaneously (i.e. approximately 0.3 u/ml). Unfractionated heparin given subcutaneously might prolong the aPTT at this concentration, but prophylactic doses of LMWH should have little or no effect on the aPTT. This discrepancy probably results from the *in vitro* addition of these drugs to blood samples in what would be equivalent to an intravenous route of

administration *in vivo*, and increased relative bioavailability of each anticoagulant due to the absence of endothelial cell binding.

Discussion

The TEG is increasingly used in surgery to detect global coagulation changes and guide blood product replacement peri-operatively. Most experience to date has been achieved during hepatic and cardiac surgery (231-234), but the use of TEG in other settings is also increasing (235). The observation from the present study that TEG is extremely sensitive to very small quantities of UFH, LMWH and Danaparoid in whole blood is significant, because many patients undergoing surgery receive some form of heparin therapy or prophylaxis pre-operatively (236;237). Moreover, the implication from this and other studies is that even the minute quantities of heparin often used to flush intravenous catheters may also affect TEG (238;239). Therefore, the reliability of the standard TEG as a guide for blood product replacement during surgery is limited by the possibility of heparin contamination.

Heparinase I coated TEG cuvettes are commercially available, and it has been suggested that these should be used for all samples where there is a risk of heparin contamination (240). However, under conditions of endothelial stress such as surgery or sepsis, endogenous release of very small quantities of glycosaminoglycans (GAGs) such as Heparan Sulfate can occur in some patients (241). There is also evidence that these GAGs can influence TEG, an effect that is reversed by the use of heparinase I coated TEG cuvettes (242-245). These findings are supported by the present study, where TEG changes due to minute quantities of Danaparoid (a combination of Heparan Sulfate, Dermatan Sulfate and Chondroitin Sulfate) were completely reversed by heparinase I. Endogenously released GAGs may be clinically significant in terms

of an increased bleeding risk for some patients (246;247), therefore eliminating this effect on TEG with heparinase and disregarding it as heparin contamination may ignore some clinically important information regarding blood coagulation. These limitations of the standard and heparinise-modified TEG make it very difficult to standardise the assay or to produce reliable guidelines for blood product replacement during surgery. Therefore, both standard and heparinase modified TEGs should be performed in parallel in all patients where there is a risk of heparin or GAG contamination, but the results should be cautiously interpreted in the context of each individual clinical setting.

Inhibition of coagulation by UFH, LMWH and Danaparoid as measured by the TEG was shown to be concentration dependent over the range 0.025 to 1.0 U/ml, which is in agreement with the results of earlier studies (248;249). However, the current study is the first to assess concentrations of UFH, LMWH and Danaparoid below 0.1 U/ml in an attempt to define a lower limit for this effect. While standard TEG changes due to UFH were significantly concentration dependant between 0.005 and 0.05 U/ml, this was not true for LMWH and Danaparoid. In addition, heparinise-modified TEG parameters were shown to be more sensitive to minute quantities of UFH than anti Xa activity, but less sensitive to LMWH and Danaparoid. The variable sensitivity of TEG parameters and conventional coagulation assays to the presence of low concentrations of UFH, LMWH and Danaparoid may reflect the different composition and mechanisms of action of these anticoagulants. UFH acts via potentiation of antithrombin in the inhibition of thrombin and factor-Xa (250), whereas LMWH predominantly inhibits factor-Xa (251). Both UFH and LMWH are excreted via the kidneys, but unlike LMWH, UFH also binds non-specifically to plasma proteins and macrophages, accounting for the variable *in vivo* activity that is often observed after administration of UFH to patients (252). Danaparoid is a mixture of 84% Heparan Sulphate, 12% Dermatan Sulphate and 4% Chondroitin Sulphate. Whilst heparan sulphate acts via

antithrombin in the same way as UFH and LMWH (253), dermatan sulphate has a different mechanism of anticoagulation via potentiation of heparin cofactor II (254), and chondroitin sulphate is thought to be devoid of any anticoagulant activity (255).

Zmuda et al commented that concentrations of Enoxaparin and Danaparoid resulting in 'minor' prolongations of the aPTT (< 10s) markedly or completely inhibited coagulation as measured by TEG (256). The results of the current study support these findings, and similar observations have been made in a recent *in vitro* study of the effects of fondaparinux and enoxaparin on tissue factor-triggered thromboelastography (257). Since the generation of TEG is multifactorial and involves the entire blood coagulation system including platelets and fibrinogen (258;259), one may have expected TEG to be less sensitive to heparins and Danaparoid than the aPTT, because the aPTT analyses only a part of the coagulation cascade in isolation.

In the current study there was no significant correlation between concentration dependant changes observed in TEG parameters compared with conventional coagulation assays, which is in contrast to the findings of previous *in vivo* studies (260-262). The absence of a correlation in the current study may reflect the *in vitro* addition of anticoagulants to blood samples and the wide range of concentrations of heparins and Danaparoid that were used (0.005 to 1.0 units/ml), since changes in the TEG parameters observed at low concentrations of each anticoagulant were not detected by the PT, aPTT and TT assays.

Blood samples require 30-60 minutes standing time before processing in order to obtain reproducible TEG results (263;264). In addition, the large number of anticoagulant concentrations tested per subject in this study and the duration of each TEG (up to 60 minutes) resulted in storage of blood in citrate for a significant period of time. There is

conflicting data as to whether citrate storage significantly affects TEG. Most studies agree that citration of blood does influence TEG, but there is conflicting data as to whether these changes are stable and lead to reproducible results. Roche et al (265) compared TEG parameters obtained by native blood, citrated blood stored at room temperature and citrated blood that had been refrigerated. They found that citration and recalcification of blood 'pre-activated' the sample, and that refrigeration increased the magnitude of this effect. However, this study was performed on blood samples taken on different days from a very small number of subjects (five). Other studies performed on blood from patients with liver disease (266) or undergoing surgery (267), have demonstrated that blood stored in citrate remains stable and produces reliable TEG tracings between one and eight hours after phlebotomy. These findings are supported by our study, where TEG parameters were preserved within the reference range for citrated saline control samples stored for up to seven hours before analysis. However, such arguments are more important for standardisation of TEG for use in clinical practice than to the results of the present *in vitro* study, where like samples were compared with like.

In conclusion, the results demonstrate that measurable effects on variables of the standard TEG occur at much lower concentrations of UFH, LMWH and Danaparoid than conventional fibrin based PT, aPTT and TT assays are able to detect. Heparinase modification of TEG greatly increases the sensitivity of this assay for UFH, LMWH and Danaparoid, and was more sensitive to minute quantities of UFH than anti-Xa activity.

A COMPARISON OF KAOLIN- VERSUS NON KAOLIN-ACTIVATED THROMBOELASTOGRAPHY IN NATIVE AND CITRATED BLOOD

Introduction

Hartert first described thromboelastography (TEG) as a global test of blood coagulation in 1948 (268). It enables a global and dynamic assessment of haemostatic function, as it evaluates the interaction of platelets with the protein coagulation cascade, starting from the initial platelet-fibrin interaction, through platelet aggregation, clot strengthening and fibrin cross-linkage and eventually clot lysis (269).

This technique is increasingly used particularly in the setting of liver transplantation and cardiovascular surgery, as it allows for a rational guide to blood component replacement or treatment of fibrinolysis (270-275). However, the method has proven itself to be useful also in other settings such as trauma and obstetric emergencies (276-278).

In research, this method has been of great value in the assessment of the role of bacterial infection in impairing coagulation in cirrhotic patients, possibly triggering variceal bleeding (279-284).

TEG can be performed on native or citrated blood, the latter being a surrogate of native blood in healthy controls (285;286), surgical (287) and cirrhotic patients (288). However, because the generation of the thromboelastographic trace using calcium activated blood is slow, the addition of activators has been proposed to reduce the time to generate the trace, especially since the main clinical use of TEG is in an intraoperative or emergency setting and requires

rapid results. Kaolin is increasingly used as an activator for this purpose, substantially reducing the time to trace generation.

Despite its increased use, only two studies have formally assessed the use of kaolin activated thromboelastography. Both of these studies evaluated surgical patients undergoing aprotinin treatment and compared kaolin-activated with celite-activated blood (289) or with celite- and tissue factor-activated blood (290).

The aim of this study was to compare kaolin-activated thromboelastography with non kaolin-acitvated thromboelastography in both native and citrated blood in patients with liver disease, patients undergoing anticoagulant treatment with warfarin or low-molecular weight heparin and healthy volunteers. We also intended to compare non-kaolin activated thromboelastograohy in native and citrated blood in this range of patients.

Materials and methods

We studied TEG blood and coagulation parameters in 21 healthy volunteers (Group 1) and 50 patients, including 20 patients with liver cirrhosis with a non biliary aetiology, mainly alcohol and viral hepatitis (Group 2), 10 patients with primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) (Group 3), 10 patients taking warfarin (Group 4) and 10 patients receiving enoxaparin prophylaxis for medical and surgical conditions (Group 5). All patients were inpatients admitted to the Royal Free Hospital.

Informed consent was obtained, and the procedures followed in this study were in accordance with the regulations of the Local Research Ethics Committee of the Royal Free Hospital. The

selected subjects were not taking drugs known to affect coagulation parameters or platelet aggregation for at least 1 week before starting the study (with the exception of anticoagulants in Groups 4 and 5).

A blood sample was taken by a single peripheral venepuncture from the antecubital fossa using a 21 gauge butterfly needle and a light tourniquet to avoid stasis or platelet activation. Blood was collected into plain tubes (native) and into 3 mL polypropylene tubes containing 0.3 mL of 0.106M sodium citrate solution (citrated) (Sarstedt Monovette, Leicester,UK; 9NC/10 ml tubes, nine parts venous blood to one part trisodium citrate) and gently inverted three times.

The samples from all patients were evaluated by TEG using non kaolin-activated and kaolin-activated native blood and non kaolin-activated and kaolin-activated citrated blood. TEG was performed using a computerized thromboelastograph (Haemoscope Corp., Skokie, Illinois, USA) as recommended by the manufacturer: 360 μL of native whole blood were pipetted into the warmed TEG cuvette (37°C) within 4 minutes of venepuncture. Citrated whole blood was stored at room temperature for 90 minutes as previously advised (286). After this time, 340 μL of citrated blood were pipetted into the preheated cup containing 20 μL of 0.2M $CaCl_2$ as activator.

Kaolin (Haemoscope Corp, Skokie, Illinois, USA) was used as advised by the manufacturer. Kaolin vials were warmed to room temperature and 1 mL of the blood sample (native or citrated) was pipetted in to the vial. Blood and kaolin were mixed by inverting the vial five times. Subsequently, 360 μL of treated native blood were pipetted into the preheated cup. As for citrated blood, after 90 minutes 340 μL of treated citrated blood were pipetted into the preheated cup containing 20 μL of 0.2M $CaCl_2$ as activator.

The principal variables analysed by the TEG analyser are (Figure 4): the reaction time (*r*), which represents the rate of initial fibrin formation and is related to plasma clotting factors and circulating inhibitor activity; the clot formation time (*k*), which represents the time taken by the forming clot to reach a fixed degree of viscoelasticity and is related to the activity of the intrinsic clotting factors, fibrinogen and platelets; the alpha angle (α), which represents the rate of clot growth and is mainly a function of platelets, fibrinogen and plasma components residing on the platelet surface; and the maximum amplitude (*ma*), which is a reflection of the absolute strength of the fibrin clot and is a direct function of the maximum dynamic properties of fibrin and platelet number and function (269).

Statistics

Spearman's test was used for assessing correlation of the TEG variables inbetween the 4 different techniques with an r (correlation coefficient) value of ≥ 0.7 considered to be a good correlation; Wilcoxon's matched pairs test was used for assessing differences in absolute values between native and citrated blood (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). For analysis of differences between the groups the Kruskal-Wallis test was used. The Bland and Altman plot was used to assess the degree of agreement between the different techniques(291).

Results

We performed TEG on native and citrated blood, both with and without addition of kaolin as an activator, on 21 healthy volunteers and 50 patients. The healthy volunteers (13 males, 8

females) had a mean age ± S.D. of 31.8±4.7 years. Of the 50 patients, 28 were male and 22 were female, and the mean age ± S.D. was 53.0±12.4 years. Details are listed in Table 5. In Group 2, the Child-Pugh score (mean±S.D.) was 9±1.8. In Group 4, the INR (mean±S.D.) was 3.0±1.0 and in Group 5, the enoxaparin daily dose was 20 mg in 4 patients and 40 mg in 6 patients.

Correlation between native blood and kaolin-acitvated native blood (Table 6)

Considering both healthy volunteers and patients (Groups 1-5), there was a good correlation for the r time (r=0.70) and almost for the ma (r=0.69), but not for k time and α angle. Considering the single groups, in Group 1 (healthy volunteers) there was no good correlation with only the ma approaching an r value of 0.70 (r=0.69). In Group 2 (patients with non biliary cirrhosis), there was a good correlation only for the ma (r=0.77). In Group 3 (patients with primary biliary cirrhosis or primary sclerosing cholangitis) there was no correlation for any variable, with an almost inverse correlation for α angle (r=-0.68). In Group 4 (patients on warfarin treatment) there was good correlation for the r time (r=0.77) but not for the other variables. In Group 5 (patients on enoxaparin prophylaxis) there was good correlation for all variables: r time (r=0.90), k time (r=0.71), α angle (r=0.78) and ma (r=0.75).

Correlation between citrated blood and kaolin-activated citrated blood (Table 7)

Considering both healthy volunteers and patients (Groups 1-5), there was a good correlation only for ma (r=0.80) but not for the other variables. In Group 2 and 3, there was good correlation for ma (r=0.80 and r=0.77, respectively) but not for the other variables. In Group 4, there was no correlation for any of the variables. In Group 5, there was good correlation only for the r time (r=0.73), but not for the other variables.

Correlation between native blood and citrated blood (non kaolin-activated) (Table 8)

Considering both healthy volunteers and patients (Groups 1-5), there was a good correlation only for the ma (r=0.72), but not for the other variables. In Group 1 and Group 2, there was a good correlation only for the ma (r=0.71 and r=0.71, respectively). In Group 3, there was no correlation for any of the variables. In Group 4, there was good correlation only for the α angle (r=0.82). In Group 5, there was good correlation for all variables: r time (r 0.84), k time (r=0.90), α angle (r=0.82) and ma (r=0.75).

Comparing absolute values (Table 9), there was a significative difference between native and citrated blood only for ma ($p<0.05$) when considering both healthy volounteers and patients. For healthy volounteers (Group 1), there was a significant difference for all variables, while in Group 2 this was present only for ma and in Group 4 for r time and k time. In Groups 3 and 5 there was no difference between the two values for any variable.

Considering both healthy volunteers and patients together, for all four TEG variables the Bland & Altman plots (Figure 5) did not suggest a systematic error as the mean was not significantly different from zero. However, for all four TEG variables, the differences within the mean and 1.96 times the standard deviation (2 SD) were clinically important and thus the agreement was not good. For all four TEG variables, the differences increased with the mean between the two techniques, suggesting the agreement is worse with increasing magnitude of measurement.

Changes in TEG between the different groups (Table 10)

Considering *non kaolin-activated native blood*, the r time was not significantly different between groups, while k time and α angle was significantly different between groups 1 and 3 and ma between groups 1 and 3, 1 and 5, 2 and 3 and 2 and 5.

Considering *kaolin-activated native blood*, the r time was significantly different between groups 1 and 3, the k time and the α angle between groups 1 and 3, 1 and 5, 2 and 3, 2 and 5, and the MA between groups 1 and 5, 2 and 3, 2 and 4 and 2 and 5.

Considering *non kaolin-activated citrated blood*, the r time was significantly different between groups 1 and 4, 3 and 4 and 4 and 5, the k time between groups 1 and 4 and 3 and 4, the α angle between groups 3 and 4, and the ma between groups 1 and 5, 2 and 3, 2 and 4 and 2 and 5.

Discussion

We evaluated the correlation between kaolin-activated and non kaolin-activated thromboelastography (TEG) in a group of 21 healthy volunteers and a group of 50 patients with different types of altered haemostasis. Amongst these we considered patients with cirrhosis (who are known to have an impaired platelet function and number as well as an impaired coagulation (292)) and patients receiving anticoagulant treatment, either warfarin or low molecular weight heparin prophylaxis against deep vein thrombosis. We evaluated patients with primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) as a separate group from patients with cirrhosis of non biliary aetiology, as the former seem to have a hypercoagulable state (293).

The results of this study do not show good correlation between the kaolin-activated and non kaolin-activated TEG for either native or citrated blood in both healthy volunteers and the different patient groups considered. The only good correlations were that between native and kaolin-activated native blood for r-time in patients on anticoagulant treatment and the ma in all correlations with the exception of Groups 3 and 4. Overall, correlation between kaolin-activated and non kaolin-activated blood seemed to be better with native than with citrated blood.

The use of kaolin as an activator for TEG has only been reported in two studies. Avidan and coworkers (290) found a good correlation between kaolin- and celite-activated TEG with regard to r-time, α angle and ma. Pivalizza and coworkers (289) found no statistically significant difference in TEG parameters between kaolin- and celite-acitvated TEG. Neither study compared kaolin-activated with non kaolin-activated TEG using either native or citrated blood.

Surprisingly, the correlation between non kaolin-activated native and citrated blood was also unsatisfactory, with the exception of the group 5 and for the ma in all groups except the PBC/PSC and warfarin group. There was a statistically significant difference in the absolute values between native and citrated blood for all variables in healthy volounteers, for the ma in Group 2 and r-time and k-time in Group 4.

Similar results to ours were found in two studies, one in 8 healthy controls (285) and one in 90 surgical patients (287), which compared native and citrated blood samples. The first study (285) did not find any statistical difference comparing all the TEG variables between native and citrated blood. In the second study (287) TEG variables in citrated blood were different from those in native blood but remained stable between 1 and 8 hours. However, in this surgical study all subjects had both normal coagulation and platelet counts; citrated blood was used with celite, a potent activator of coagulation.

Another study, from our group (286), compared TEG parameters of native blood to those of recalcified citrated blood without celite in 10 healthy subjects. In this study, a significant difference was found between citrated and native blood between 60 and 120 minutes for r-time, k-time, α angle and ma. Our current findings in the group of healthy volunteers substantially confirm these previous results with the exception of a significant shortening of the r-time with citrated blood (Table 10).

Non kaolin-activated native blood was compared to non kaolin-activated citrated blood in patients with liver disease in a previous study from our group, involving 32 patients with cirrhosis or liver failure (288), with no significant difference found for all the variables. Again we found similar results in our current study, with the exception of a significantly greater ma in the citrated blood, confirming our previous findings in patients with cirrhosis of a non-biliary origin. Why TEG with native blood is different to TEG with citrated blood in healthy volounteers but apparently not in cirrhotics remains unexplained.

In the current study we evaluated changes of both kaolin- and non kaolin-activated blood in both native and citrated blood between the different groups in order to assess if the different techniques resulted in different changes in different clinical settings. *Non kaolin-activated native blood* results were different between healthy volunteers and PBC/PSC patients, the latter showing an expected hypercoagulability (293). *Kaolin-activated native blood* values were also different between healthy volunteers and PBC/PSC patients (again showing a hypercoagulable state in the latter), but there were also differences between healthy volunteers and patients on enoxaparin prophylaxis (surprisingly showing hypercoagulability in the enoxaparin group), between patients with cirrhosis and PBC/PSC patients (hypercoagulability in the latter), and lastly between patients with cirrhosis and patients on enoxaparin prophylaxis (again with hypercoagulability in the latter). Interestingly, *non kaolin-activated citrated blood* values were markedly different between patients on warfarin and healthy

volunteers (the former being less coagulable as expected) and PBC/PSC patients (the warfarin group again being less coagulable).

However, none of the methods gave significantly different values between healthy volunteers and patients with cirrhosis. Overall, the ma was the variable with the best discriminating potential. This was true especially for the kaolin-activated samples, in which the r-time values had little discriminative value between the groups. This may reflect the normal thrombin generation recently described and commented on in cirrhosis (294). With non kaolin-activated native blood healthy volounteers seemed to coagulate tendentially less than cirrhotics while with non kaolin-activated citrated blood the opposite seemed to be the case, even though the differences were not statistically significant. Our sample might have been too small to detect a statistically significant difference between these groups as patients with cirrhosis are very heterogeneous in terms of their haemostatic disturbances (alterations of platelet number and function, reduced synthesis of clotting factors, hyperfibrinolysis) and thus any differences might have been masked by the heterogeneity in our limited sample.

Again, TEG with non kaolin-activated citrated blood seems to be the only technique to detect a significantly impaired coagulation in the warfarin group as compared to healthy volunteers. The enoxaparin group also coagulates tendentially less only with this technique, even though again the differences were not statistically significant. Indeed, the apparent hypercoagulability of the enoxaparin group with the other two techniques is surprising, and might be due to the underlying medical conditions that led to the initiation of enoxaparin prophylaxis. Another possible explanation for the lack of hypocoagulability on TEG shown in the warfarin and enoxaparin group might be the age difference (31.8±4.7 in Group 1 and 55.2±17.5 and 55.8±15.9 in Groups 4 and 5, respectively), as increasing age seems to be associated with hypercoagulability (295).

One more interesting finding was the confirmation of the presence of hypercoagulability in the PBC/PSC group, with a markedly prolonged ma as compared to patients with non PBC/PSC cirrhosis, suggesting a difference in platelet function between these patients (296).

In conclusion, we found a poor correlation between kaolin-activated and non kaolin-activated thromboelastography in both healthy volunteers and patients with a wide range of conditions affecting coagulation. Moreover, there was poor correlation between native and citrated blood with non-kaolin activated blood. The ability of TEG with non kaolin-activated citrated blood to detect changes in patients receiving warfarin, and the ability of TEG with kaolin-activated blood to detect changes in patients having enoxaparin-induced anticoagulation deserves to be investigated further.

The lack of a standard reference in the literature for TEG and the results of this study do not give grounds as to which technique to use. It is clear that any TEG study should at least have its own internal control, e.g. if kaolin activation is used, then have non kaolin-activation. Kaolin-activated blood is extensively used in intensive care, but most published comparative data is only available for non-kaolin activated blood. Further studies investigating the correlation between these different techniques when used in different clinical settings are needed. Meanwhile, the TEG methodology should be carefully noted when comparing different studies.

CLINICAL STUDIES USING THROMBOELASTOGRAPHY

ENDOGENOUS HEPARINOIDS IN ACUTE VARICEAL BLEEDING

Introduction

Bleeding from gastrooesophageal varices is the most common source of bleeding in cirrhotics. The risk of variceal bleeding is associated with the severity of liver dysfunction, large varices, endoscopic red signs on varices and portal pressure. However, why bleeding occurs unpredictably and infrequently in individual patients considering the frequent daily changes in portal pressure is unknown.

Bacterial infections occur in 35-66 % of cirrhotics presenting with gastrointestinal bleeding (187). We have proposed a causal relationship between variceal bleeding and bacterial infection, the latter acting as a trigger (59). The possible pathophysiological basis is endotoxin-induced endothelin release and subsequent increase in intrahepatic resistance (via hepatic stellate cell contraction) and consequent rise in portal pressure, combined with impairment of haemostasis by inhibition of platelet aggregation due to endotoxin-induced nitric oxide and prostacyclin (59).

We have shown a heparin effect using heparinase I-modified thromboelastography (TEG) a highly sensitive method for detecting heparin-like activity, in infected cirrhotics (23), and in another study anti-Xa activity was detected in several but not all patients who had the heparin effect (297). The infected patients already had infection present for several hours, before presenting to the hospital when the TEG was performed.

We describe the first two cirrhotic patients with endoscopically confirmed variceal haemorrhage who bled whilst in hospital, studied immediately after the first episode of haematemesis.

Patients and methods

Patient 1 was a 66 year old caucasian male and patient 2 a 42 year old black female, both with alcoholic cirrhosis. Patient 1 had Child-Pugh grade C, patient 2 Child-Pugh Grade B cirrhosis. Both patients had endoscopic banding and intravenous terlipressin and received therapeutic doses of intravenous cefotaxime for prophylaxis of bacterial infections as currently recommended (187). Informed consent was obtained for taking multiple blood samples (with prior approval of the protocol by the Ethics Committee) for Heparinase I-modified TEG and anti-Xa activity assay. The baseline samples were taken before administration of colloid, blood products or antibiotic therapy, and then at subsequent times over 7 days. Heparinase I-modified TEG (Haemoscope Corp., Skokie, IL) was performed in one channel whilst simultaneously perfoming a standard TEG in the second channel using calcium-activated citrated blood from the same sample 90 minutes after venepuncture**,** as we have previously shown that citrated blood can substitute native blood in cirrhotic patients (288). Anti-Xa was assessed by chromogenic assay (Sigma Diagnostics, Poole, Dorset, UK) and by clotting assay (Diagnostic Reagents, Thame, Oxford, UK). A heparin effect was defined as an improvement of r time, k time and α angle occurring together in Heparinase I-modified TEG as compared to the standard TEG. Informed consent was obtained from each patient included in the study and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the local ethics committee.

Results

A heparin effect (Figure 6) was detected between 1 hour (patient 2) and 6 hours (patient 1) after the initial bleeding episode and persisted for 6 to 7 days, not corrected by administration of fresh frozen plasma and/or red blood cells (Figure 7). In patient 1 anti-Xa activity was positive during the same time span during which there was a heparin effect. Baseline urine, blood and ascites cultures were negative in both patients, and neither developed infections subsequently.

The TEG parameters worsened immediately after the bleeding episode: in patient 1, r time from 11 minutes (4 hours after the bleeding episode) to 18.8 minutes (10 hours after the bleeding episode), k time from 2.7 minutes to 7.3 minutes, α angle from 53.8 ° to 28.7 °. In patient 2 r time from 11.2 minutes (1 hour after the bleeding episode) to 33.8 minutes (3 hours after the bleeding episode), k time from 3.1 minutes to 10.7 minutes, α angle from 50.8 ° to 17.8 °. These values slowly returned to normal concomitantly with the disappearance of the heparin effect after 6 to 7 days: r time 11 minutes in patient 1 and 16.2 minutes in patient 2, k time 3.5 minutes in patient 1 and 4.4 minutes in patient 2 and α angle 43.6 ° in patient 1 and 42.2 ° in patient 2.

The worsening of the TEG parameters was only partially corrected by the administration of fresh frozen plasma and/or red blood cells (Figure 2). Both patients received all their blood products within the first 24 hours. The greater amount of blood products given in patient 1 (6 units of packed red cells and 4 units of fresh frozen plasma) probably prevented the worsening of TEG parameters to the degree seen in patient 2 (who only received 2 units of fresh frozen plasma), but in neither patient did the TEG parameters return to the baseline values nor the heparin effect disappear. The routine coagulation parameters (PT, aPTT) did not show any correlation with the worsening TEG parameters or the heparin effect.

Discussion

We have shown the presence of endogenous heparinoids, and their disappearance over several days for the first time in cirrhotic patients with acute variceal bleeding. There was no evidence of infection, but the antibiotic prophylaxis possibly prevented or treated infection. Neither patient experienced early rebleeding. The heparin effect was documented shortly after the beginning of the haemorrhage and disappeared over 5 days over the same time course of antibiotic therapy. This was also seen by Montalto et al (23). The absence of a heparin effect at the beginning of bleeding and appearance thereafter could suggest that bleeding is a cause of its manifestation, but in addition the heparin effect needs to be compared in native as well as citrated blood as the citrate may mask an initial heparin effect. The heparin effect could influence continued variceal bleeding or early rebleeding. It is possible that the heparin effect might be worse in the absence of antibiotics. This phenomenon deserves wider study, particularly as bacterial infection has been linked to failure to control variceal bleeding and early rebleeding, in a randomised study of prophylactic antibiotics (22).

HEPARIN EFFECT ON THROMBOELASTOGRAPHY AFTER TIPS PROCEDURE

Introduction

Bacterial infections are a common and severe complication in hospitalized patients with decompensated cirrhosis and are frequently associated with upper gastrointestinal (GI) bleeding and other complications of portal hypertension (298).

We have previously reported that proven bacterial infection, or a surrogate of its presence, use of antibiotics, had the strongest independent association with failure to control bleeding in cirrhotic patients with variceal bleeding (21). The strong association between infection and variceal bleeding in cirrhotics has been confirmed in several studies: association with failure to control bleeding (21;192), early rebleeding (21) and mortality (192). A recent meta-analysis confirms that antibiotic prophylaxis prevents infections in cirrhotic patients with gastrointestinal bleeding and significantly increases the short-term survival rate (80). In a recent randomised study of 120 patients prophylactic ofloxacin compared to on demand antibiotics was shown to prevent early rebleeding and to decrease the amount of blood transfused, in addition to preventing bacterial infections (22). This has been confirmed in another randomized study (299). Therefore, since our hypothesis was published (59), there are now a number of publications which support it, suggesting a causal link between infection and variceal bleeding.

How bacterial infection might worsen or be a trigger for variceal haemorrhage, is still under study (59). Using thromboelastography (TEG), we previously showed impaired coagulation in cirrhotic patients with bacterial infection (145) and the presence of a heparin effect on heparinase I-modified TEG in the same setting (23), which was subsequently found to be associated with the presence of anti-Xa activity (297) (suggesting the presence of endogenous

heparinoids). We also reported the presence of a heparin effect immediately after variceal bleeding in cirrhotic patients (300).

Endotoxin or cytokines could release heparinoids from endothelium in a dose-dependent manner (23) and mast cell activation due to bacterial infection could also release heparin.

Higher endotoxin concentrations are found in peripheral blood of cirrhotic patients than in normal subjects (32) with a statistically significant gradient between portal and peripheral blood (33), highlighting the role of the bowel as the source of endotoxin.

During a transjugular intrahepatic portosystemic shunt (TIPS) procedure, a shunt is created between the portal and the systemic circulation with an inflow of portal blood into the systemic circulation bypassing the liver.

The aim of our study was to assess the presence of a heparin effect on heparinase I-modified TEG in patients undergoing a TIPS procedure, both before and after the procedure. Our hypothesis was that given the presence of a portosystemic gradient of endotoxaemia, and the role of endotoxaemia and bacterial translocation on the release of heparinoids and the subsequent worsening of coagulation as shown by TEG, the inflow of portal blood unfiltered by the liver after a TIPS procedure might show the presence of the heparinoids in the portal blood through a heparin effect on TEG.

Materials and Methods

We recruited 10 consecutive patients admitted to the Liver Transplantation and Hepatobilitary Medicine Unit of the Royal Free Hospital who underwent a TIPS procedure. Exclusion criteria were refusal or inability to give consent. Informed consent was obtained, and the procedures followed in this study were in accordance with the regulations of the Local Research Ethics Committee of the Royal Free Hospital. The selected subjects were not taking

drugs known to affect coagulation parameters or platelet aggregation for at least 1 week before starting the study.

Blood samples were taken for TEG before the procedure, at 1 hour after the procedure, at 6 hours after the procedure and the morning after the procedure, with the possibility of taking further samples on a daily basis until any thromboelastographic changes had reverted to baseline.

Blood samples were taken by a single peripheral venepuncture from the antecubital fossa using a 21 gauge butterfly needle and a light tourniquet to avoid stasis or platelet activation. Blood was collected into 3 mL polypropylene tubes containing 0.3 mL of 0.106M sodium citrate solution (Sarstedt Monovette, Leicester,UK; 9NC/10 ml tubes, nine parts venous blood to one part trisodium citrate) and gently inverted three times.

TEG was performed using a computerized thromboelastograph (Haemoscope Corp., Skokie, Illinois, USA) as recommended by the manufacturer. The samples were stored at room temperature for 90 minutes as previously described by our group (286). After this time, 340 μL of citrated blood were pipetted into the preheated cup containing 20 μL of 0.2M $CaCl_2$ as activator. For each patient, a native (without heparinase) and a heparinase I-modified TEG was performed.

The principal variables analysed by the TEG analyser are (Figure 4): the reaction time (*r*), which represents the rate of initial fibrin formation and is related to plasma clotting factors and circulating inhibitor activity; the clot formation time (*k*), which represents the time taken by the forming clot to reach a fixed degree of viscoelasticity and is related to the activity of the intrinsic clotting factors, fibrinogen and platelets; the alpha angle (α), which represents the rate of clot growth and is mainly a function of platelets, fibrinogen and plasma components residing on the platelet surface; and the maximum amplitude (*ma*), which is a reflection of the absolute strength of the fibrin clot and is a direct function of the maximum dynamic properties of fibrin and platelet number and function (269).

A heparin effect was defined as an improvement of at least 20 % of a TEG variable after addition of heparinase I.

Results

The 10 patients in our study (Table 11) were 6 male and 4 female. Mean age was 48.8 years (range 16 to 64). Aetiology of liver disease was alcohol in 6 patients, Budd Chiari disease in 2, HCV related cirrhosis in 1 and cryptogenic cirrhosis in 1. The indication for TIPS placement was recurrent variceal bleeding in 4 patients, refractory ascites or hydrothorax in 4 and Budd Chiari disease in 2. Patients were mostly in Child class B and C (5 and 4, respectively) and mean Child score was 8.8 (range 6 to 12). In all patients the TIPS was successfully placed.

In 8 of 10 patients a heparin effect appeared on TEG after TIPS placement on the day of the procedure (7 patients) or day 1 post-procedure (1 patient) and disappeared within 24 to 48 hours (Table 12). This heparin effect was present on at least 2 of 4 TEG variables in 7 out of the 8 patients, and in 3 of 4 TEG variables in 5 out of the 8 patients. When present after TIPS placement, the heparin effect was almost always detected by k time (7 of 8 patients) or alpha angle (7 of 8 patients), while no heparin effect could be detected with the maximum amplitude (ma).

In only 2 of 10 patients (both with alcohol related cirrhosis, the indication for TIPS placement being refractory ascites in one and recurrent variceal bleeding in the other) was there a heparin effect before TIPS placement, and this was present in only 1 and 2 of the 4 TEG variables. One of these patients (Patient 4) never developed a heparin effect after TIPS placement, while the other patient (Patient 8) went on to develop a heparin effect after TIPS

placement in 2 TEG variables different from the single TEG variable where a heparin effect occurred before the procedure.

In only 1 patient (Patient 1) no heparin effect could be detected either before or after the TIPS placement.

Discussion

Our study has shown the presence of a transient heparin effect in blood after the placement of a transjugular intrahepatic portosystemic shunt (TIPS) in the great majority of patients who underwent this procedure for a different number of indications.

We have previously described the importance of bacterial infection in complicating cirrhosis and portal hypertension (298) and formulated the hypothesis that its presence may trigger variceal bleeding (59).

We reported that 20 cirrhotic patients who had bled from varices, and then suffered early rebleeding, had a worse TEG trace on the day before rebleeding as compared to those who did not rebleed (144). TEG parameters have also been found to worsen in 84 decompensated cirrhotic patients in the presence of bacterial infection (145). A recent study found heparin activity in blood by using heparinase I-modified TEG in 28 of 30 patients with bacterial infection, but not in non-infected patients, and this effect disappeared after resolution of infection (23). This heparin effect was subsequently found to be associated with anti-Xa concentrations in many, although not all patients, suggesting that the heparin effect is due to an endogenous low molecular weight heparin (297). Moreover, a heparin effect was reported immediately after acute variceal bleeding in cirrhotics and thus could contribute to failure to control acute bleeding and early rebleeding (300). In a single study plasma heparan sulphate

concentrations were significantly raised in patients with recent variceal bleeding as compared to those without bleeding or non cirrhotic patients (146).

Endotoxin or cytokines could release heparinoids from endothelium in a dose-dependent manner (23), and mast cell activation due to bacterial infection could also release heparin. There is statistically significant gradient of endotoxin concentrations between portal and peripheral blood (33), highlighting the role of the bowel as the source of endotoxin. Recently, bacterial translocation has been described as a common albeit transient event in patients with cirrhosis and ascites (71;301).

In a pilot study we had attempted to perform TEG on portal blood sampled directly from the portal vein during the TIPS procedure, comparing it with venous blood drawn at the same time from a peripheral vein. Unfortunately almost every sample of portal blood taken in this manner clotted almost immediately, probably through activation of the coagulation cascade by the catheter used for accessing the portal vein. Thus we have been limited to assaying systemic blood.

We did observe the presence of a heparin effect on systemic venous blood shortly after the creation of a significant shunt leading portal venous blood in the systemic circulation (and thus bypassing the hepatic filter) with a method known to be very sensitive for detection of heparin-like substances (302) such as TEG. Clearly the origin of the heparinoid effect could be from the splanchnic vasculature or due to liver trauma. In fact, we cannot entirely exclude that the presence of a heparin effect after TIPS placement might only be due to the amount of hepatic tissue damage during the TIPS procedure, with the subsequent release of hepainoid substances into the venous system. This would explain the transient nature of the heparin effect. However, the fact that not all patients presented the heparin effect despite the sensitivity of our method for heparinoid substances makes this hypothesis, in our view, less likely.

The presence of a heparin effect only after the TIPS procedure in the majority of patients suggests in our view a higher concentration of heparinoids in the portal venous system. The reason for two patients having a heparin effect before TIPS placement is not clear. It is certainly possible that these patients had a subclinical bacterial infection, which was subsequently treated (with disappearance of the heparin effect) by the prophylactic antibiotics administered to all patients undergoing TIPS placement. Also, it is not quite clear why both patients with Budd Chiari syndrome had a heparin effect after TIPS placement, as the presence of endogenous heparinoids has to our knowledge not yet been reported in these patients. However, the presence of portal hypertension in these patients might explain the same events of bacterial translocation and release of heparinoid substances as in the cirrhotic patients. The transient nature of the heparin effect after TIPS placement is also not entirely clear. It is possible that the endogenous heparinoid substances suddenly released into the systemic venous system after the procedure subsequently get diluted in the systemic venous system, and that heparinase I-modified TEG is not sensitive enough to detect the constant ongoing release after the procedure.

In conclusion, our study reports the appearance of a transient heparin effect in systemic venous blood after TIPS in patients with cirrhosis or Budd Chiari syndrome, suggesting the presence of heparinoid substances in the portal venous system in these patients. Further research is warranted on the role of bacterial translocation as a fundamental factor in the occurrence of complications in patients with liver disease.

STUDIES ON BACTERIAL TRANSLOCATION AND INTESTINAL FUNCTION

INTESTINAL FUNCTION AND PRESENCE OF BACTERIAL DNA IN SERUM AND ASCITES IN PATIENTS WITH CIRRHOSIS

Introduction

Bacterial infections are common in cirrhotic patients (3) and seem to play an important role in the development of the complications of cirrhosis (298), in particular with regards to variceal haemorrhage (21;23) and renal dysfunction (118;126).

Bacterial translocation seems to be the initial step preceding the occurrence of overt bacterial infection in the cirrhotic patient (298). Bacterial translocation has been defined as the passage of microbes or microbial products into the mesenteric lymph nodes (50); for obvious reasons this is difficult to study in the human subject. However, the presence of bacterial DNA (detected by PCR) in biological fluids such as blood and ascites has recently been reported to correlate well with the presence of bacterial translocation in rats (303).

Using this as a surrogate marker of bacterial translocation, Frances and coworkers reported in a recent study the presence of bacterial DNA in serum and ascites in 10 out of 17 patients with advanced cirrhosis without signs of infection from a minimum of 24 to a maximum of 72 hours (71).

On the other hand, it is also well known that patients with cirrhosis have an impairment of their intestinal function, involving increased permeability (47;304), reduced motility (45) and bacterial overgrowth (37;38).

Our aim was to search for bacterial DNA in a range of cirrhotic patients both with and without ascites, and to study its correlation with any abnormality of intestinal motility or permeability and the presence of bacterial overgrowth.

Materials and Methods

Patients

We recruited 8 consecutive patients with cirrhosis. Cirrhosis was diagnosed by histology or by clinical, laboratory and/or ultrasonographic findings. Exclusion criteria were age of less than 18 years, presence of the systemic inflammatory response syndrome (SIRS)(305) or overt infection, upper gastrointestinal bleeding or intake of antibiotics (either systemic or luminal) within the preceding two weeks, history of intestinal disorders including major abdominal surgery, conditions impairing small bowel motility (such as diabetes mellitus or scleroderma), intake of lactulose, beta blockers, prokinetics or any other drug with an effect on small bowel motility, presence of hepatocellular carcinoma or portal vein thrombosis, acute alcoholic hepatitis, pregnancy and refusal of consent. The Ethics committee of our hospital approved the study protocol, and all patients provided informed consent.

Blood and ascites samples were obtained by a single operator in aseptic conditions. On the day of recruitment blood was obtained for blood cultures (both aerobic and anaerobic, 10 mL each) and detection of bacterial DNA (in serum-gel bottles, 12 mL, Venosafe, Terumo, Leuven, Belgium), and ascites (if present) was obtained for culture in blood culture bottles (both aerobic and anaerobic, 10 mL each), standard neutral specimen bottles (20 mL) and detection of bacterial DNA (in serum-gel bottles, 12 mL, Venosafe, Terumo, Leuven, Belgium). On the following three days, blood was obtained in the morning for culture and bacterial DNA detection at 8 o'clock in the morning and for bacterial DNA detection at 2 o'clock in the afternoon.

Cultures

Ascitic fluid (AF) sampled from patients were examined for the presence of bacteria using standard culture-based methods including the enrichment of the sample by inoculation in

blood culture bottles and incubation at 35°C for eight days. Moreover, samples of blood of each patient, taken twice each day for 4 days, were examined using the standard culture method.

DNA extraction and purification

Samples of 0.5 ml AF were treated with 0.5 mg/ml proteinase K for 60 min to eliminate excess of proteins and were subsequently centrifuged at 1000 rpm for 5 min to eliminate red blood and other cells. Bacterial cells present in supernatants and in blood samples from the patients were broken in order to extract the DNA which was then purified using the kit Extragen (Nanogen) which has been shown to be very efficient in recovering nucleic acids from gram-negative and gram-positive species. For each sample, two internal controls consisted of 0.5 ml ascitic fluid added with 10^4 *E. faecalis* or *E. coli* cells were used. An AF sample demonstrated negative to the culture method and to DNA detection by PCR was also included as a negative control. Purified DNA was suspended in TE buffer and used for the PCR reaction.

Polymerase chain reaction and visualization of amplification products

The nucleotide sequences and the properties of the primers designed to detect the different groups or species of bacteria are listed in Table 13. The PCR reaction was performed on a Thermal Cycler (Perkin-Elmer, USA). Final concentrations of the different components in the PCR reaction were: 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 200 µM of each deoxynucleotide triphosphate, 100 pmol of each of the two primers of the pairs listed in Table 13 and 2 U Taq DNA polymerase (Boehringer Mannheim). PCR was performed twice on each sample in two separate experiments and also in a positive (*E.coli* + *E. faecalis* purified DNA) and a negative (water instead of DNA) control, with 30 cycles consisting of 30-sec

denaturation at 94°C, 30-sec annealing at the adequate temperature and a 30-sec extension period at 72°C. A sample of 10 AL of the PCR product was resolved by 1% agarose gel electrophoresis at 100 V for 30 min in 0.5-Tris-borate-EDTA (pH 8.0) buffer containing ethidium bromide, and the images were visualized and photographed.

PCR specificity and sensitivity test

The specificity of each pair of primer was checked using DNA obtained from suspension of different bacterial groups or species cultures (see Table 13). The sensitivity of the method was evaluated by inoculating ascitic fluid with bacterial suspensions (10^6 to 10 cells) prepared from *E. coli* and *E. faecalis* cultures and extracting the DNA from 500 µl of each of the suspensions.

Lactulose breath test (LBT)

All patients underwent LBT to evaluate intestinal transit time and to detect small intestine bacterial overgrowth (SIBO).

To minimize basal hydrogen (H_2) excretion, subjects were asked to have a carbohydrate-restricted dinner on the day before the test and to fast for at least 12 hours. Smoking and physical exercise were not allowed starting from 2 hours before testing. Before testing, patients had an antibacterial mouthwash with 20 mL of chlorhexidine 0.05%.

End-alveolar breath samples were collected immediately before lactulose ingestion. Subsequently, a dose of 10 g of lactulose in 200 mL of water was administered and samples were taken every 15 minutes for 3 hours. From these bags, breath samples were aspirated into a 20 mL plastic syringe. Samples were evaluated for H_2 using a gas chromatograph (Quintron microlyzer, Milwaukee, WI, USA). Results were expressed as parts per million (p.p.m.).

Orocaecal transit time was considered normal if a peak was observed within 90 minutes after the lactulose oral load. The peak was calculated as an increment of at least 20 ppm above the basal hydrogen value.

A test positive for SIBO required an elevated breath hydrogen concentration within 90 min and two distinct peaks, the first being within 90 minutes.

Intestinal permeability

The intestinal permeability test was performed after the patients had been fasting overnight and emptied their bladder in the early morning before starting the test. A urine sample was collected before drinking 100 mL of a solution containing 10 g of lactulose and 5 g of mannitol dissolved in water. Urine was collected for the next five hours. Patients were asked to remain fasting for one hour after the start of the test and were then allowed to drink water for the remainder of the 5 hours.

Total urine volume was measured and samples were centrifuged and kept at -20°C. 20 µL of the sample was analized using high performance liquid chromatography (HPLC; Agilent 1200, Agilent Technologies, Santa Clara, CA, USA) to determine the concentration of urinary lactulose and mannitol.

Intestinal permeability was expressed as the excretion ratio (R) of percentage of lactulose excreted in the urine (%L) to percentage of mannitol excreted in the urine (%M) (R=%L/%M).

We used a sample of 14 healthy volunteers to determine normal values of intestinal permeability using our system. The mean±S.D. of %L was 0.25±0.10, the mean±D.S. of %M was 14.99±3.59 and the mean±S.D. of the excretion ratio (R) was 0.02±0.005.

Results

Of the 8 patients we recruited, one was excluded from the study after day 1 as he became encephalopathic and had to be treated with antibiotics. Thus data was available on 7 patients, 6 males and a female. The mean age was 57.7 years (range 42 to 78 years, aetiology was alcohol in 3, HCV in 2, HBV and alcohol together with HCV in one patient each. Child-Pugh class was A in 4 patients and B in 3, and mean Child-Pugh score was 6.7 (range 5 to 9); ascites was present in 4 patients, oesophageal varices in 6 and encephalopathy in none (Table 14). Baseline laboratory and clinical parameters are shown in Table 15. The main findings are summarised in Table 16.

Intestinal transit time, permeability and bacterial overgrowth

The median intestinal transit time was 135 minutes. Six of the 7 patients had a delayed intestinal transit time and one patient presented a physiologic H_2 peak after 60 minutes. Two of the six patients without a peak within 90 minutes had no significant H_2 rise even at the end of the test (180 minutes). One patient had a LBT positive for SIBO.

Intestinal permeability was increased in all patients as compared to healthy controls, with the excretion ratio ranging from 0.027 to 0.072 (mean±S.D. 0.05±0.01) as compared to normal values of 0.02±0.005 in the healthy volunteers (Table 17).

Bacterial DNA

Of all the samples analysed, only one sample of ascitic fluid and 8 samples of serum (all taken from the same patient) were PCR positive using universal primers. In all the other samples from the remaining patients, PCR using universal primers was negative. The samples that were PCR positive for universal primers were also positives to PCR with EntB primers specific for Enterobacteriaceae (Figure 8). The same samples analyzed by PCR with primers

eco and pbp5 resulted negative, indicating that in these samples there was no DNA from *Escherichia coli* or *Enterococcus faecalis*.

Discussion

The importance of the sequence of events starting from bacterial translocation and leading up to bacterial infection in the cirrhotic patient has been increasingly recognised in recent years (59;298). Bacterial translocation and different intestinal dysfunctions (such as altered motility, permeability and bacterial overgrowth) in the setting of chronic liver disease have been described in several studies.

Altered small bowel motility leading to delayed intestinal transit has been shown in cirrhosis (43;45). with a direct relationship between these changes and the severity of liver disease (45). A significantly increased frequency of cluster of contractions and an increased duration of phase II of the migrating motor complex were seen in patients with liver cirrhosis (43;306); these changes have been reported to resolve after orthotopic liver transplantation (307). In patients with cirrhosis, bacterial overgrowth has been shown in one third of alcoholic cirrhotics, especially those with ascites and severe liver dysfunction (37). According to Perez-Paramo et al, a coordinated bowel motor function might be the most important mechanism for preventing bacterial overgrowth (36).

Intestinal permeability is impaired by portal hypertension, particularly with severe liver disease (47), especially if there is ongoing alcohol intake (48;49;304). Gastrointestinal mucosal abnormalities (leading to increased permeability) due to portal hypertension (40) can be caused by the overproduction of nitric oxide (NO), which leads to disruption of the integrity of the intestinal epithelium (50). Also, the oedema of the splanchnic tissues in portal hypertension may affect the permeability of the gut (46).

Bacterial translocation occurs with an incidence ranging from 37 to 83 % in animal cirrhotic models (50). In human cirrhosis**,** the incidence of bacterial translocation is also related to the degree of portal hypertension and liver dysfunction, being reported in approximately 30 – 40 % of cirrhotics with ascites (36;63;66) and more frequently in Child C than in Child A and B cirrhosis (66). In a recent study, 7 of 17 advanced cirrhotics with culture-negative, nonneutrocytic ascites had the simultaneous presence of bacterial DNA (a surrogate marker of bacterial translocation (303)) in blood and ascitic fluid at admission to hospital, which persisted in blood for 24-72 hours (71). Furthermore, nucleotide sequencing demonstrated that bacteria detected in the first sample were identical to those detected later, proving that bacterial translocation is a single-species, dynamic process (71).

In the 7 patients reported in this study, intestinal motility and permeability were impaired in virtually all patients, both with ascites and without. The presence of bacterial DNA in the ascitic fluid and serum, however, was found in only a single patient with ascites (14 %), as compared to 7 out 17 ascitic patients (41 %) reported by Frances and colleagues (71). Bacterial overgrowth was also present in only one patient of our group. Not surprisingly, the only patient with presence of bacterial DNA was also the one with the hightest intestinal permeability.

The main difference between our group of patients and the group reported by Frances and colleagues (71) is the severity of liver disease. While only four of our seven patients had ascites, this was present in all of their patients; moreover, while none of our patient was in Child-Pugh class C, none of their patients was in Child-Pugh class A. However, portal hypertension was present in almost all of our patients as confirmed by the presence of oesophageal varices.

In addition to the findings of Frances and colleagues (71), we report for the first time the relationship between the presence of bacterial DNA in biological fluids and alterations of intestinal dysfunction in the natural history of cirrhotic patients. Our findings suggest that

decreased intestinal motility and increased intestinal permeability are early events occurring even in patients with relatively preserved liver function, while both the presence of bacterial overgrowth and the appearance of bacterial DNA in serum or ascitic fluid seem to be related to increasing severity of liver disease, as already suggested by other authors (37;66).

Altered small bowel motility, bacterial overgrowth in the small intestine, and increased intestinal permeability all lead to increased endotoxaemia, increased bacterial translocation and ultimately the risk of bacterial infection in cirrhotics. In cirrhotic rats with ascites, bacterial overgrowth is promoted by intestinal hypomotility, and bacterial translocation only occurs in presence of overgrowth and severe disruption of the gut barrier (36). Bacterial translocation also increases in cirrhotic rats after the development of ascites (63).

The limitations of our study are the relatively small sample and the fact that we only monitored for the presence of bacterial DNA in biological fluids in a limited time frame of four days, thus potential missing episodes of bacterial translocation (since it is known that this is a transient occurrence in the cirrhotic patient (71)). However, the timeframe chosen was identical to the one used in the previous study (71). It is also unexpected that the only patient with bacterial overgrowth did not have ascites and had a good liver function with a Child-Pugh score of 5. Another possible limitation is that two of the patients with no physiological H_2 peak within 90 minutes had no peak even at the end of the test at 180 minutes. This is most likely to be explained by a severely delayed intestinal transit, however a lack of colonization by H_2 producing bacteria cannot be excluded. Also, while lactulose breath testing is an established method for the assessment of intestinal transit time, its accuracy regarding the detection of small bowel bacterial overgrowth (SIBO) is less than optimal, with a sensitivity of 68 % and a specificity of 44 % (308). The gold standard for assessing SIBO is culture of small bowel aspirate (308), which however is an invasive method; while xylose breath test seems to be performing better with regard to other non invasive methods. However, we decided to use the lactulose breath test as it allowed us to assess intestinal transit time and

SIBO with a single technique, thus avoiding the need for two different breath tests which might have prolonged the length of hospitalization.

Our findings are of importance as they contribute a further essential piece of information to the understanding of the natural history of cirrhosis and portal hypertension, in particular with the occurrence of bacterial translocation and infection leading to further complications, and also provide the scientific rationale for the development of new therapeutic approaches.

The circumstantial evidence associating bacterial translocation and infection with intestinal dysfunction in this setting has already been reviewed (298). An increase in permeability was correlated with the occurrence of septic complications in 80 patients with mainly alcoholic cirrhosis (47). A history of spontaneous bacterial peritonitis (SBP) (a haematogeneous infection localising in ascitic fluid, the source most likely being bacteria translocating from the gut) is associated with more severe intestinal dysmotility and more frequent bacterial overgrowth (38). Bacterial overgrowth leads to bacterial translocation and together with increased intestinal permeability leads to an increased risk of SBP and worsening of hepatic encephalopathy (55). Cisapride and antibiotics (such as norfloxacin and neomycin) improve both small intestine dysmotility and bacterial overgrowth in cirrhosis (56). Interestingly, propranolol, which lowers portal pressure, reduced the risk of post surgical infections from 42 % to 15 % in a cohort of 73 cirrhotics (57). This protection may be due to increasing bowel motility by its sympatholytic action, indirectly decreasing microbial translocation. A recent retrospective study of 139 patients suggested that propranolol also may prevent SBP (58;309). Selective intestinal decontamination with antibiotics reduces bacterial translocation (66) and decreases the risk of infections, especially those due to Gram negative bacteria (78;79). Moreover, the addition of cisapride (a prokinetic agent) to norfloxacin has been reported to further decrease the incidence of SBP in cirrhotic patients with ascites (310). This again supports the role of intestinal bacteria as a major infective source in cirrhotics and may

explain why prophylactic antibiotics both orally and intravenously are so effective in reducing infections during variceal bleeding (80).

In conclusion, our study shows the presence of impaired intestinal motility and permeability in a cohort of cirrhotic patients (both with ascites and without) with a relatively preserved synthetic liver function but with presence of portal hypertension, while both the occurrence of bacterial overgrowth and bacterial translocation as defined by the detection of bacterial DNA in biological fluids only occurred in one patient each. We suggest that reduced intestinal motility and increased permeability (which may at least in part be correlated to the presence of portal hypertension) precede the occurrence of bacterial overgrowth and bacterial translocation in cirrhotic patients. Further studies are needed to explore the natural history of intestinal dysfunction and bacterial translocation in cirrhosis and to study possible therapeutic interventions such as the use of probiotics, selective intestinal decontamination, prokinetic agents and non selective beta-blockers in this setting.

METHODOLOGICAL STUDIES IN PORTAL HYPERTENSION

ASSESSMENT OF THE AGREEMENT BETWEEN WEDGE HEPATIC VEIN PRESSURE AND PORTAL VEIN PRESSURE IN CIRRHOTIC PATIENTS

Introduction

The measurement of the portal pressure gradient provides prognostic information in the management of patients with cirrhosis (311;312) and we believe it should be more widely used given that it has independent prognostic significance over and above scores of the severity of liver disease (311). For many years, measuring hepatic venous pressures, both the free pressure (FHVP) and the wedged pressure (WHVP), either with a wedge catheter or a balloon catheter (313), has been the standard approach for estimating portal venous pressure (PVP) (311;314-316).

Several studies have compared WHVP with portal pressure in cirrhotic patients but with relatively small cohorts and using different techniques for assessing portal pressure (Table 18). Some of them found good correlation between the two measurements, especially in patients with alcoholic cirrhosis (316-322) and HBV cirrhosis (323); other studies reported less good correlations between the two techniques, especially in patients with non-alcoholic cirrhosis (317;319;324-326). In HCV cirrhotic patients in particular, the data is conflicting (318;324).

Recently, the precision in measuring WHVP per se has been questioned (327). In this study, 61 % of the hepatic venous pressure gradient (HVPG) measurements in two separate hepatic veins differed by as much as 4-34 mm Hg. If confirmed, this finding would raise serious doubts about the validity of WHVP measurement as a reliable way of estimating PVP.

Thus, our aim was to evaluate the agreement between HVPG and portal pressure gradient (PPG) by systematically reassessing all the published data comparing these two measurement techniques. In particular, as the use of correlation for comparing two measurement techniques has been criticised as inappropriate (since it only describes a linear correlation between variables, but not necessarily an agreement), we decided to also apply an alternative approach which has been advocated for this purpose (291).

Materials and methods

We conducted a full Medline search in all languages for studies comparing wedge hepatic venous pressure with portal pressure, using the keywords “cirrhosis”, “wedge hepatic venous pressure”, “WHVP”, “hepatic venous pressure gradient”, “HVPG”, “portal pressure” both alone and in combination between each other. We also searched the bibliographies of all relevant papers. In this way we identified 17 studies, which had individual patient measurements, comparing WHVP with PVP (316-326;328-333). We could only include 11 of these in our study; in 5 of them the measurements were reported as individual numerical values (316;322;325;326;331), in the remaining 6 we were able to extract the values from the graphs (317;318;320;321;323;324). We excluded 6 studies from our evaluation, because in 2 it was not possible to determine which measurements had been taken in patients with liver disease (332;333), in 1 because only pressure changes after vasoactive drug therapy were reported (330), in 1 because it was not possible to extract the data from the graph (319), in 1 because the two different measurements were taken at a long time interval from each other (329) and lastly in 1 because it only considered patients with spontaneous portosystemic encephalopathy who were very likely to have a significant portosystemic shunt (328).

We pooled all the data from the published studies and recalculated the determination coefficient (r^2) with linear regression determined by using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The r^2 gives the fraction of variability in one variable explained by the other variable, in this case the prediction of portal venous pressure by measuring WHVP and PPG by measuring HVPG. Thus the r^2, as compared to the more commonly used r (regression coefficient), which was used in the individual articles correlating portal pressure to WHVP and HVPG, is the most appropriate expression of the correlation we wished to test.

We evaluated agreement between the two measurements using the method described by Bland and Altman (291), i.e. by plotting the difference between the two measurements against their mean. As this method does not allow a quantification of the degree of agreement, we evaluated it by describing the absolute number and percentage of values outside 1.96 standard deviations (i.e. commonly referred to as 2 standard deviations). We considered less then 10 % of the values being outside 1.96 standard deviations as a good agreement.

We also evaluated the difference between the two methods in relation to basal PVP in order to assess if there was an increasing error dependent on the degree of PVP.

Results

We identified a total of 320 comparative measurements of WHVP and PVP and 75 comparative measurements of HVPG and PPG; 102 comparative measurements of WHVP and portal pressure were performed in patients with alcoholic liver disease and 88 in patients

with non-alcoholic liver disease. In the latter group 53 had hepatitis C virus (HCV) related liver disease (an additional 14 had both alcoholic liver disease and HCV related liver disease), other aetiologies included in this group were hepatitis B virus (HBV) related liver disease, primary biliary cirrhosis (PBC) and autoimmune hepatitis, as well as cryptogenic cirrhosis. A total of 194 measurements were performed with a wedge catheter and 113 with a balloon catheter.

All patients

Evaluating the 320 measurements comparing WHVP and PVP, WHVP was greater than PVP in 113 measurements and PVP was greater in 138 measurements. The range of difference between WHVP and PVP was from -22.1 to 10 mm Hg (mean±1.96 S.D. being -0.48±6.33 mm Hg, median 0 mm Hg). We found a coefficient of determination (r^2) of 0.87. From the graph (Figure 9A) it can be seen that 2 cases, both from the same author (325), are outliers. By excluding these two cases as outliers the r^2 is 0.90 and the range of difference was from -10.5 to 10 mm Hg. According to Bland and Altman plot (Figure 9B), there were no absolute systematic errors, no proportional errors and no variation depending on the magnitude of measurement. However, the range of -0.48±6.33 mm Hg is clinically important.

If only the 101 measurements for which individual numerical values were available (predominantly earlier studies) are considered, the r^2 is 0.68 (0.70 excluding the outliers) and according to Bland and Altman plot, there were no absolute systematic errors, no proportional errors and no variation depending on the magnitude of measurement, however, the range of -1.36±7.31 mm Hg is clinically important. Considering only the 219 measurements for which values were extracted from graphs (predominantly later studies), the r^2 was 0.91 and according to Bland and Altman plot, there were no absolute systematic errors, no proportional

errors and no variation depending on the magnitude of measurement, however, the range of -0.08±5.66 mm Hg is clinically important.

Evaluating the 75 measurements comparing HVPG and PPG (316;320;322;331), HVPG was greater than PPG in 21 measurements and PPG was greater in 23 measurements. The range of difference between WHVP and PVP was from -4 to 8.5 mm Hg (mean±1.96 S.D. being -0.05±3.39 mm Hg, median 0 mm Hg). The r^2 was 0.90; according to Bland and Altman plot, there were no absolute systematic errors, no proportional errors and no variation depending on the magnitude of measurement. However, the range of 0.05±3.39 mm Hg is clinically important (Figure 9C and 1D).

Alcoholic liver disease

Evaluating the 102 measurements (317;318;320-322;326) comparing WHVP and PVP, WHVP was greater than PVP in 33 measurements and PVP was greater in 45 measurements. The range of difference between WHVP and PVP was from -8 to 9 mm Hg (mean±1.96 S.D. -0.4±5.23 mm Hg, median 0 mm Hg). There was a r^2 of 0.93; according to Bland and Altman plot, there were no absolute systematic errors, no proportional errors but there was an increasing variation of at least one method depending on the magnitude of measurement. The range of -0.4±5.23 mm Hg is clinically important (Figure 10A and B).

Amongst the 13 measurements (320;322) comparing HVPG and PPG the r^2 is 0.84; considering the Bland and Altman plot, absolute systematic errors, proportional errors and variation depending on the magnitude of measurement were difficult to assess due to the small number of measurements. The range of -0.38±3.63 mm Hg is clinically important (Figure 10C and D).

Non-alcoholic liver disease

Amongst the 88 measurements (317;318;321;323;324) comparing WHVP and PVP, WHVP was greater than PVP in 38 measurements and PVP was greater in 41 measurements. The range of difference between WHVP and PVP was from -10.5 to 10 mm Hg (mean±1.96 S.D. -0.39±6.45 mm Hg, median 0 mm Hg). The r^2 is 0.83; according to Bland and Altman plot, there were no absolute systematic errors, no proportional errors and no variation depending on the magnitude of measurement. However, the range of 0.39±6.45 mm Hg is clinically important (Figure 11A and B).

In particular, in the 53 measurements (318;324) comparing WHVP and PVP in patients with HCV related liver disease, WHVP was greater than PVP in 23 measurements and PVP was greater in 22 measurements. The difference between WHVP and PVP ranged from -10.5 to 10 mm Hg (mean±1.96 S.D. -0.08±7.39 mm Hg, median 0 mm Hg). The r^2 is 0.75; according to Bland and Altman plot, there were no absolute systematic errors, no proportional errors and no variation depending on the magnitude of measurement. However, the range of -0.08±7.39 mm Hg is clinically important (Figure 11C and D).

No measurements comparing HVPG and PPG were available in this subgroup.

Wedge catheter measurement

Evaluating the 194 measurements (316;317;320-322;326;331), WHVP was greater than PVP in 59 measurements and PVP was greater in 82 measurements. The range of difference between WHVP and PVP was from -9 to 8.5 mm Hg (mean±1.96 S.D. -0.55±4.78 mm Hg, median 0 mm Hg). The r^2 is 0.85; according to Bland and Altman plot, there were no absolute systematic errors, no proportional errors and no variation depending on the

magnitude of measurement. However, the range of -0.55±4.78 mm Hg is clinically important (Figure 12A and B).

As all the measurements comparing HVPG and PPG were performed using a wedge cathether, the results are the same as reported for the whole series.

Balloon catheter measurement

Amongst the 113 measurements (318;323;324), WHVP was greater than PVP in 49 measurements and PVP was greater in 41 measurements. The range of difference between WHVP and PVP was from -10.5 to 10 mm Hg (mean±1.96 S.D. -0.19±6.17 mm Hg, median 0 mm Hg). The r^2 is 0.90; according to Bland and Altman plot, there were no absolute systematic errors, no proportional errors but there was an increasing variation of at least one method depending on the magnitude of measurement. However, the range of -0.19±6.17 mm Hg is clinically important (Figure 12C and D).

Assessment of the variability between direct and indirect portal pressure measurement according to basal portal pressure

The assessment of the absolute difference between the two types of measurement, in all measurements and in those performed with a balloon catheter, plotted against the basal PVP showed that the variability was constant for a range of PVP from 1 to 45 mm Hg (Figure 13 A and C). A similar distribution was seen for measurements performed with a wedge catheter and in separate subgroups of patients with and without alcoholic liver disease.

The difference between the two measurements, as a change in percentage from WHVP measurement, plotted against the PVP, showed a tendentially greater change in percentage for

lower PVP pressures (<20 mm Hg) (Figure 13 B), but this was not observed when only the measurements performed with a balloon catheter were considered (Figure 13 D).

Discussion

Our reassessment of the data published to date on comparative measurements of wedged hepatic pressures and portal pressures clearly shows that WHVP correlates well with PVP in a cohort of patients with alcoholic liver disease, HBV and HCV related liver disease, PBC and autoimmune hepatitis, irrespective of the use of a wedge or a balloon catheter. However there was a trend to greater differences between the wedge technique and PVP at lower PVP pressures but this did not reach statistical significance. In particular, it is important to note that this also holds true for the main aetiologies of liver cirrhosis, alcoholic liver disease and HCV related liver disease, despite some claims to the opposite (324). Furthermore, we also confirmed that HVPG correlates significantly with PPG.

These findings are of great importance, as measurement and monitoring of hepatic pressure measurements (HVPG and WHVP) are increasingly recommended in clinical trials and clinical practice regarding the pharmacological prevention of portal hypertension-related bleeding (334-336). Moreover, a higher HVPG measurement is known to be an independent factor associated with mortality in liver cirrhosis (311) and one study has linked pharmacological reduction in HVPG to a decreased long-term risk of complications related to portal hypertension, such as variceal bleeding, ascites, spontaneous bacterial peritonitis, hepatorenal syndrome and hepatic encephalopathy (312;337). A recent report also suggests that changes in HVPG could be considered as an adjunctive end point for the therapeutic evaluation of antiviral therapy in chronic hepatitis C, and HCV related cirrhosis (338).

Our findings do not confirm a recently reported high variability in HVPG measurements (327), as the correlation with PPG measurements was highly significant, across 8 different centres reporting data, given that direct portal pressure measurement has very little variability. The paper by Keiding et al (327) used a wedge catheter technique and did not compare WHVP with PVP. On the whole, WHVP measurement seems to slightly underestimate PVP, but the difference is small (mean -0.48 mm Hg).

We also assessed the agreement between portal pressures and WHVP and HVPG with the method proposed by Bland and Altman (291). This is important since this method has been suggested to be more appropriate for assessing agreement between two methods of clinical measurements, as the use of correlation coefficients in this setting may be misleading. The Bland and Altman method showed a clinically significant range of differences, with 95 % of the differences lying between 3.39 mm Hg above or below the mean and 7.39 mm Hg above or below the mean. This variability is of importance, especially if such small variabilities as 10 % or 20 % reductions from baseline are considered, as have been reported to be important in the setting of prophylaxis of variceal bleeding and complications of portal hypertension in general (339;340).

One possible bias in our evaluation is that the number of measurements might have been too small thus leading to a larger standard deviation. However, even considering the total number of available measurements (i.e. 320) the range of differences is still clinically significant (6.33 mm Hg above or below the mean). Another possible source of heterogeneity is that studies included were performed over a 44 year time span with consequent variation in equipment and technique of measuring pressure. However, the distinction between studies performed using the wedge technique (1955-1985) and those using the balloon technique (1989-1999) takes into account most of these differences.

Lastly, there is an issue of generalisability. All of these studies were performed in specialised centres, and thus these results may not be reproducible in a routine setting. However, recommendations for an accurate technique have been published recently(341) and should make accurate measurements achievable in every institution.

This analysis, although confirmatory of other studies with regard to the correlation between WHVP and PVP and between HVPG and PPG, each with a small number of patients, questions the agreement between these variables and thus the use of WHVP and HVPG measurements as surrogate measurements of PVP and PPG. To our knowledge, this is the first time that an assessment of more than 300 comparative measurements has been performed. The suboptimal accuracy of WHVP and HVPG as indirect indices of portal pressure reported here, should be, we believe, a further stimulus to a more widespread study of these methods. It may well be that performed with a correct and repeatable technique (341), these techniques could result not only in good correlation, but also in acceptable agreement. This is especially important as the use of HVPG holds great promise for prognostic estimation (311;312), for researching the role of target reduction of portal pressure during pharmacological therapy of portal hypertension (334-336) and potentially as a surrogate marker of chronicity or fibrosis (338).

COMMENTS ON THE IMPORTANCE OF DIFFERENT PATHOPHYSIOLOGICAL MECHANISMS IN COMPLICATIONS OF PORTAL HYPERTENSION – THE EXAMPLE OF HAEMODYNAMIC NON RESPONDERS TO PHARMACOLOGICAL TREATMENT

HOW TO PREVENT VARICES FROM BLEEDING: SHADES OF GREY – THE CASE FOR NON-SELECTIVE BETA BLOCKERS

Introduction (Black and white)

The risk of bleeding from oesophagogastric varices is determined by the extent of portal hypertension, liver dysfunction and endoscopic findings (342). Portal hypertension is responsible for many of the complications of cirrhosis and warrants treatment in patients who have already suffered a variceal haemorrhage as well as those who have a risk of bleeding for the first time. This can be pharmacological by lowering portal pressure or local treatment of the varices by ligation at the site of bleeding. Recently some data suggests that reduction in portal pressure might also be beneficial for other outcomes, such as ascites, spontaneous bacterial peritonitis, hepatorenal syndrome, hepatic encephalopathy and overall survival (340;343). Whether a therapeutic benefit can occur without improvement of liver function, providing there is a reduction in portal pressure, has recently been discussed (344;345). However, this has only been assessed in a subgroup of 31 patients (344) so further data is clearly needed. The pharmacological treatment most commonly used is non-selective beta blockers (propranolol, nadolol or timolol) either as monotherapy or in association with nitrates. The presence or absence of a decrease in the hepato-venous pressure gradient (HVPG), a surrogate marker of portal pressure(346;347), either by a certain degree (20 % or more) or below an absolute value (12 mm Hg) have been advocated as a prognostic tool in order to predict the therapeutic effect – the prevention of bleeding and rebleeding (343;348). However the applicability of routine remeasurement of HVPG as well and the generalisability of the published data have been questioned ((339;349-354)).

Patients treated with portal pressure lowering drugs can be divided into “white” (haemodynamic responders) or “black” (haemodynamic non-responders). Thus those patients achieving an HVPG reduction of >20 % from baseline or below 12 mm Hg, are considered to be at far less risk of rebleeding (“white”), whereas the others are believed to be at continued risk of rebleeding which is greater than the responders’ (“black”). These latter patients are then to be considered for alternative therapies such as ligation or more aggressive treatments such as TIPS to evade this fate. However although ligation is often recommended, current data suggests rebleeding still occurs even if patients are switched to endoscopic treatment (355;356). Indeed, the relationship between the absence of a good response with non-selective beta blockers and bleeding is not a close correlation and prediction of bleeding in an individual patient is not sufficiently good for routine clinical practice. This consideration has often been taken as a reason to choose ligation of varices as the choice of first therapy for prevention of rebleeding.

The aim of this paper is to review the evidence for dividing patients into “black” and “white” (a division which we consider to be too simplicistic) and to make a case for propranolol to be used for primary and secondary prevention of bleeding, providing there are no contraindications or intolerance, in all patients.

Haemodynamic response and efficacy of drug treatment – clinical evidence

Non-selective beta blockers, with or without nitrates, are effective for preventing the first variceal bleeding episode in patients with moderate or large varices, and for preventing variceal rebleeding (342;357;358). However, the protective effect of non-selective beta blockers may not only be due to reduction in portal pressure, but may also be due to a

reduction of bacterial translocation (through an effect on intestinal motility, permeability and bacterial overgrowth(359)) and a reduction in bacterial infections(309;359), and through this a reduction in the risk of bleeding.

Several studies have assessed the haemodynamic response with respect to the risk of variceal haemorrhage, mostly in the setting of secondary prophylaxis (360-364), but also in primary prophylaxis (340;365;366) or in cohorts with patients undergoing both primary and secondary prophylaxis (356;367).

For prevention of rebleeding, the association with target reduction of portal pressure is clear cut in those in whom pressure was remeasured between 1 and 3 months after starting therapy. However, the fact that hepatic venous pressure measurements were not measured at baseline or repeated in all patients, especially in those patients who rebled before remeasurement, further complicates the interpretation of the data (350;351).

What can be determined from the current data is that not all haemodynamic responders will stay free of bleeding/rebleeding unless HVPG is below 12 mm Hg (but only a median of 14 % achieve this response) (351) and conversely, not all non-responders will eventually bleed or rebleed.

Considering the 5 studies assessing haemodynamic response for the secondary prophylaxis of variceal bleeding, a total of 138 out of 211 patients on drug treatment who had their haemodynamic response assessed did not rebleed, of which 57 (41.3 %) were non responders (Table 19). Even excluding the study by McCormick and coworkers(361), which has been criticised for assessing haemodynamic response only at 5 months (i.e. very late), 45 out of 110 patients who did not rebleed (40.9 %) were non responders. Moreover, it is often not

emphasized that the percentage of patients not rebleeding was consistently higher than the haemodynamic response rate in all trials (median, 64 % vs 51 %).

With regard to the variability in bleeding rates in relation to pressure response, an interesting parallel can be made by evaluating studies using nitrates. The addition of isosorbide mononitrate (ISMN) has been shown to reduce HVPG, even in patients who have no haemodynamic response to propranolol (368;369). However, this effect does not always translate into a significantly increased clinical efficacy. ISMN as monotherapy does not reduce the incidence of first variceal bleeding (370), and in one (out of two) randomized controlled trials for primary prophylaxis, its addition to propranolol made no improvement to the incidence of variceal haemorrhage (371). The randomized trials evaluating combined treatment versus non-selective beta blockers alone for prevention of rebleeding showed trends, but not clear cut significant benefit for combination treatment (372;373)

In the two randomized controlled studies (from the same centre) of pharmacological versus endoscopic prophylaxis for variceal rebleeding that also assessed haemodynamic response, non-responders to non selective beta-blockers had the same incidence of rebleeding as patients undergoing sclerotherapy (55 % vs 53 %) and a slightly higher incidence compared to those undergoing endoscopic banding ligation (67 % vs 49 %) (363;364).

Overall, 22 to 67 % (median, 47 %) of non-responders rebleed (351), which is lower and at least not worse than historical controls with a 63 % bleeding rate at 2 years without treatment (348). In trials of primary prophylaxis, the bleeding rate in non-responders was 37 % during follow-up for up to 5 years (excluding patients with small varices without red signs) [24], while a more recent study[3] (recruiting all patients with varices) had a bleeding rate of 30 % in the non-responder group during a follow-up period of up to 8 years. This compares favourably to

a historical risk of 26.5 % at one year without treatment (and thus comprising both potential responders and non-responders) [32]. Indeed the positive predictive value of haemodynamic non-response for variceal rebleeding is as low as 52.1 % when pooling the data from the trials (Table 20).

In contrast to the above results, a non randomised study proposing “à la carte” treatment for portal hypertension (356), aimed at the target of achieving a haemodynamic response of lowering HVPG below 12 mm Hg or by at least 20 %. However, those patients not achieving these targets with propranolol alone or in combination to ISMN had their beta blockers discontinued and were switched to endoscopic banding ligation and had a very high rebleeding rate of 87 % - more than twice the median of the other studies. This raises doubts as to whether this cohort is representative of non-responders, or whether indeed those patients had been experiencing a protective effect from the drug treatment before this was suspended, in spite of not achieving a haemodynamic response. It is known that patients under non-selective beta blockers lose all benefits from drug treatment and return to their prior bleeding risk once this is stopped (374). In addition, if the results from this study(356) were representative of non-responders, it would suggest that even banding ligation is not effective for non-responders to beta blockers. A recent uncontrolled study showed a low rebleeding rate in “partial responders” (HVPG reduction of ≥10 % and <20 %) with the combination of pharmacological treatment and banding ligation(375), suggesting that a beneficial effect may also be observed with moderate decreases in HVPG.

Alternative pathopysiological mechanisms for the action of non selective beta blockers (Table 21)

1) Alternative haemodynamic mechanisms

The protective effect of propranolol (376) in preventing variceal bleeding is thought to be mediated in several ways. The major effect is by a reduction of portal inflow (377;378) resulting from a decrease of cardiac output and from splanchnic vasoconstriction (379-381). Another haemodynamic mechanism, often overlooked, is an increase in porto-collateral resistances reducing blood flow to varices. Indeed, propranolol decreases azygos blood flow (382-386) (an index of collateral blood flow) and variceal pressure (367;383;387). Some authors consider the reduction of collateral blood flow and variceal pressure as the principal mechanism by which propranolol reduces bleeding risk in cirrhotic patients (382;383). Finally, propranolol also causes a decrease in total effective vascular compliance (388;389), so that HVPG is further reduced because free hepatic vein pressure (FHVP) is increased.

Propranolol therapy also increases intrahepatic vascular resistance, thereby potentially offsetting, at least partially, its beneficial effect on portal pressure (390;391). For this reason, combination therapy with vasodilators, such as nitrates or prazosin, or the use of carvedilol (a non selective beta-blocker with intrinsic alphalytic activity) have been advocated (392;393). All of these drugs have been shown to increase the haemodynamic response rate as assessed by HVPG. Yet, by looking closely at the other haemodynamic variables in these studies, some differences become evident.

Propranolol reduces azygos venous blood flow by a greater extent (34-38 %) than either cardiac output (23-24 %), HVPG (11-14 %) or hepatic blood flow (13 %) (382;394). As hepatic blood flow is the sum of both portal venous and hepatic arterial blood flow, the fact

that propranolol reduces hepatic blood flow to a lesser extent than azygos blood flow may be due to a relative increase in hepatic artery blood flow after beta-blockade, as seen in an experimental portal hypertension model in rats (395), despite an increase in hepatic resistance (390). Propranolol decreases azygos blood flow (382;384;386;394;396-398) through an effect of beta-blockade (384), and does so even in patients without a haemodynamic response as assessed by HVPG (383;386).

On the other hand, the addition of ISMN to propranolol, whilst further decreasing HVPG, results in a non significant increase (394) of collateral blood flow (as estimated by azygos blood flow). Administration of ISMN alone leads to no change in azygos blood flow (369) or to a slight decrease by 11 % (368). A study comparing propranolol with the combination of propranolol and ISMN found no significantly greater reduction of flow in the combination group as compared to propranolol alone (398). Carvedilol reduces azygos blood flow less than propranolol (by 15-20 % for carvedilol vs 23-27 % for propranolol) despite a greater reduction in HVPG (393;399). As regards prazosin we were not able to find any published data on its effect on azygos blood flow.

Variceal pressure is correlated with azygos blood flow (400). Propranolol decreases variceal pressure (367;383;387;401); again, this is also seen in HVPG non responders (383). It might be considered that reduction of pressure at the site of bleeding is the closest surrogate pressure measurement in relation to bleeding. A decrease in variceal pressure whilst on propranolol treatment was found to be associated with a decreased risk of variceal bleeding or rebleeding (367). Nitrates decrease variceal pressure when given either orally (368) or sublingually (402) or result in no change after intraduodenal infusion despite a marked decrease in arterial pressure (403). No data on long-term effects of nitrates on variceal pressure are published.

This suggests that the effect of propranolol and other non-selective beta blockers on the prevention of variceal bleeding may not correlate solely with the degree of portal pressure reduction. Some of the therapeutic effects may indeed be due to the changes in collateral

blood flow and variceal pressure which also occur in patients who do not achieve a haemodynamic response as assessed by a HVPG decrease of 20 % or more (367;383).

Also, several other factors might be of importance, such as the age of the patients, the dose of beta blockers or nitrates used (which varies quite significantly in the different studies), and even more so the compliance with the treatment (344;350;351). Recently the importance of beta-2 adrenergic receptor gene polymorphism has been investigated(404), but it does not seem to predict hemodynamic response to propranolol in patients with cirrhosis (although it may allow selecting a subgroup of patients likely to benefit from the addition of nitrates)(405). Another possible source of heterogeneity could lie in constitutional factors such as the presence of the metabolic syndrome, but little data has been published so far on this issue. The importance of technical differences in HVPG measurement and the need for standardization have also been pointed out recently (406;407).

2) Non-haemodynamic mechanisms

The role of bacterial infection in variceal bleeding in cirrhotic patients is an ever more attractive hypothesis (59;359). Bacterial infections in cirrhotic patients are common (3). There is a predisposition to intestinal bacterial overgrowth, intestinal dysmotility and increased intestinal permeability, all leading to an increase in bacterial translocation (359). Bacterial translocation is the probable mechanism for some of the most common infections in cirrhosis, such as spontaneous bacterial peritonitis, but is also the source of bacterial by-products such as endotoxin which can cause an increase in portal pressure (culminating in the increased portal pressure in the presence of spontaneous bacterial peritonitis(118;408)), an impairment of liver function and a worsening of haemostasis (359).

The effects of bacterial infection and bacterial products on the portal and systemic haemodynamics in cirrhosis and clinical data on infection, from both retrospective and prospective studies**,** of variceal bleeding and other settings, demonstrate the importance of infection in pathophysiological mechanisms in cirrhosis (359). This has been followed by recent clinical evidence that antibiotic therapy reverses systemic vasodilation (25;108), and prevents early variceal rebleeding in two randomised studies (22;299).

Propranolol has been found to result in faster intestinal transit and lower rates of bacterial overgrowth and translocation in cirrhotic rats (36). Interestingly, in a randomised study (albeit in abstract form) propranolol reduced the risk of post surgical infections from 42 % to 15 % in a cohort of 73 cirrhotic patients (57). Moreover, two recent retrospective studies of 134 and 139 patients suggested that propranolol may prevent spontaneous bacterial peritonitis (58;409), a finding also supported by data from trials for the prevention of variceal bleeding (340) or rebleeding (343;410) Indeed, this beneficial effect seems to be obtainable with a reduction in portal pressure of as little as 11 %(340), which again points towards the efficacy of propranolol even in those who do not have a 20 % reduction of HVPG (309). This effect of propranolol is probably due to increasing bowel motility by its sympatholytic action, indirectly decreasing microbial translocation as shown in animal models(36). Given this data propranolol may have another mode of action in reducing variceal bleeding by preventing or reducing bacterial translocation, or infection, which may trigger bleeding (59;359).

The argument against a pure mechanical understanding of variceal haemorrhage

Rupture of oesophageal varices is frequently seen as an event explainable merely by the terms of Laplace's law, as a direct function of variceal pressure and radius and thickness of the variceal wall. However, the known risk factors for variceal bleeding (HVPG, liver function,

size of varices and presence of red signs) do not readily explain why bleeding and early rebleeding occur unpredictably in patients with cirrhosis. Portal pressure rises significantly with daily meals as well as exercise (199;200), and oesophageal peristaltic contractions have been reported to increase variceal pressure to more than 80 mm Hg (411). Yet despite these marked daily changes bleeding episodes are relatively infrequent.

In the setting of non cirrhotic portal hypertension, where often there are large oesophageal varices with red signs, the incidence of bleeding is much lower than in cirrhotics with similar varices (196). The bleeding rate in portal vein thrombosis has been documented as 12,5 episodes per 100 patient years (197;198), whereas the risk of first bleeding with Child grade A cirrhotics with large varices and moderate red signs is 24 % in 1 year and in Child grade C with small varices and no red signs it is 20 % (195). The difference probably lies in the presence of liver disease (although the thrombophilic conditions often associated with portal vein thrombosis cannot be discounted). Cirrhosis predisposes to risk of infection, which is not reported in non-cirrhotic portal hypertension. In particular bacterial translocation is much less frequent in rats with portal vein ligation as compared to cirrhotic rats (64;65). Also, in patients with cirrhosis, circulating bacterial DNA fragments (a marker of bacterial translocation) are only observed in the presence of ascites suggesting that ascites and advanced liver failure contribute to the risk of infection. In the setting of extra-hepatic portal vein obstruction, especially in children, haemorrhage may be initiated by a minor, intercurrent infection (412). Lastly variceal columns can be composed of several dilated vessels rather than a single vein, thus making it more difficult to directly apply Laplace's law (413).

Thus a merely mechanical understanding of variceal bleeding as a consequence of portal pressure and tension on the variceal wall might not entirely explain the pattern of variceal bleeding. Endotoxaemia secondary to bacterial infection may indeed be the critical trigger for variceal haemorrhage (59), since it produces a wide series of effects that may predispose the

cirrhotic patient to bleeding: impairment of primary and secondary haemostasis, increase of portal pressure and worsening of the hyperkinetic syndrome and of liver function (359).

Conclusion (Shades of grey)

Portal hypertension causes the development of oesophageal varices and ultimately leads to variceal haemorrhage in many patients with cirrhosis. The degree of portal hypertension as assessed by HVPG measurement is an independent prognostic factor for survival in cirrhotic patients (414). There is little doubt that a low HVPG (<12 mm Hg), either at baseline (415) or achieved after a therapeutic intervention such as abstinence from alcohol (416) or pharmacological treatment (363-366) is associated with a very low risk of subsequent variceal bleeding. An HVPG reduction of 20 % from baseline seems to be of value in terms of improving survival and rebleeding(339), even though the effect on the former may be related at least in part to changes in liver function(344).

Interestingly different thresholds of portal pressure reduction (≥10 % but < 20 %) have been published with regard to protection from complications of portal hypertension other than variceal bleeding (309;417), such as SBP (340) and development of oesophageal varices (417). In particular Turnes and coworkers (340)showed that an 11 % reduction in HVPG was the best cut off in predicting no risk for SBP, thus (since bacterial infection can be the trigger for variceal bleeding (359)) protection against haemorrhage might already take place at a lower level of HVPG reduction. Furthermore, a haemodynamic response also seems to be correlated with an overall better prognosis (343). Thus haemodynamic responders do have a better outcome and thus HVPG measurement retains its important prognostic role in determining survival (414). A final consideration is that the more commonly used cut-off

value (i.e. a 20% reduction in HVPG or to <12 mmHg) was selected to maximize the specificity, but is not very sensitive. It could be that the use of a less stringent cut-off (i.e., a 10% decrease in HVPG) would be more sensitive and markedly reduce the "grey zone", while still being of prognostic value (as an example, the proportion of non-responders using the 10% cut-off is of about 25%, which is much lower than for the conventional cut-off values) (*JB, unpublished observations*).

It is thus difficult to consider all haemodynamic non-responders as treatment failures i.e. "black". The effect of propranolol on other haemodynamic parameters such as azygos blood flow and on bacterial translocation, together with the fact that more patients do not rebleed than achieve the target "protective" HVPG thresholds suggest a more complex picture. While some of the haemodynamic non-responders might indeed be "black" and thus doomed to a worse prognosis, a substantial part of non-responders are only "grey": i.e. they do not share the appealing pure status of haemodynamic responders, yet are protected from adverse outcomes (in particular variceal bleeding) by treatment with non-selective beta blockers (Table 22). This is either due to a decrease of collateral and thus variceal blood flow, even without a marked decrease in HVPG, and/or due to a reduction of bacterial translocation and bacterial infections that may trigger the bleeding itself. Further studies should clarify if responses of portal pressure achieved by a proportion of patients (for instance, a decrease of 10%, 12% or 15%) can uncover part of this "grey" zone. Another way to define this "grey" area would be through a randomised controlled trial assigning haemodynamic non-responders either to continuation of treatment with non-selective beta blockers, to placebo or to endoscopic treatment – but this might well become obsolete in view of the fact that the combination of endoscopic banding ligation with non-selective beta blockers seems to be superior to endoscopic treatment alone(418;419). Interestingly, a recent study suggested that the addition of banding ligation to non-selective beta blockers compared to non-selective beta blockade alone was of little added value(420), again suggesting that beta blockers may be

acting via other mechanisms to protect against bleeding. Alternatively patients could be randomised to long-term antibiotic therapy to test the hypothesis of infection being the trigger for variceal bleeding(59;359). Thus, propranolol (or other non-selective beta blockers) may well turn out to be the Hepatologist's "aspirin" – cheap, few contraindications, relatively little intolerance and of universal application in cirrhotics irrespective of the presence of varices or history of bleeding (344), as they affect outcomes other than bleeding. This therapeutic possibility needs urgent exploration, not only because of its potential clinical importance, but also because it may define the need for HVPG measurement in the future.

FIGURES

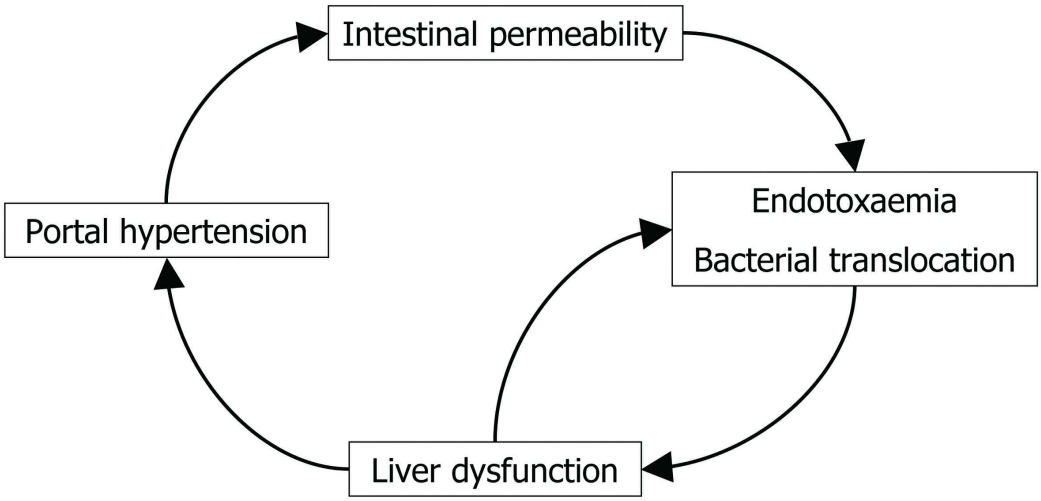


Figure 1: Damage to the intestinal barrier leads to bacterial translocation and endotoxaemia and thus to impairment of liver function and increase in portal pressure, possibly causing further damage to the gut: a vicious circle

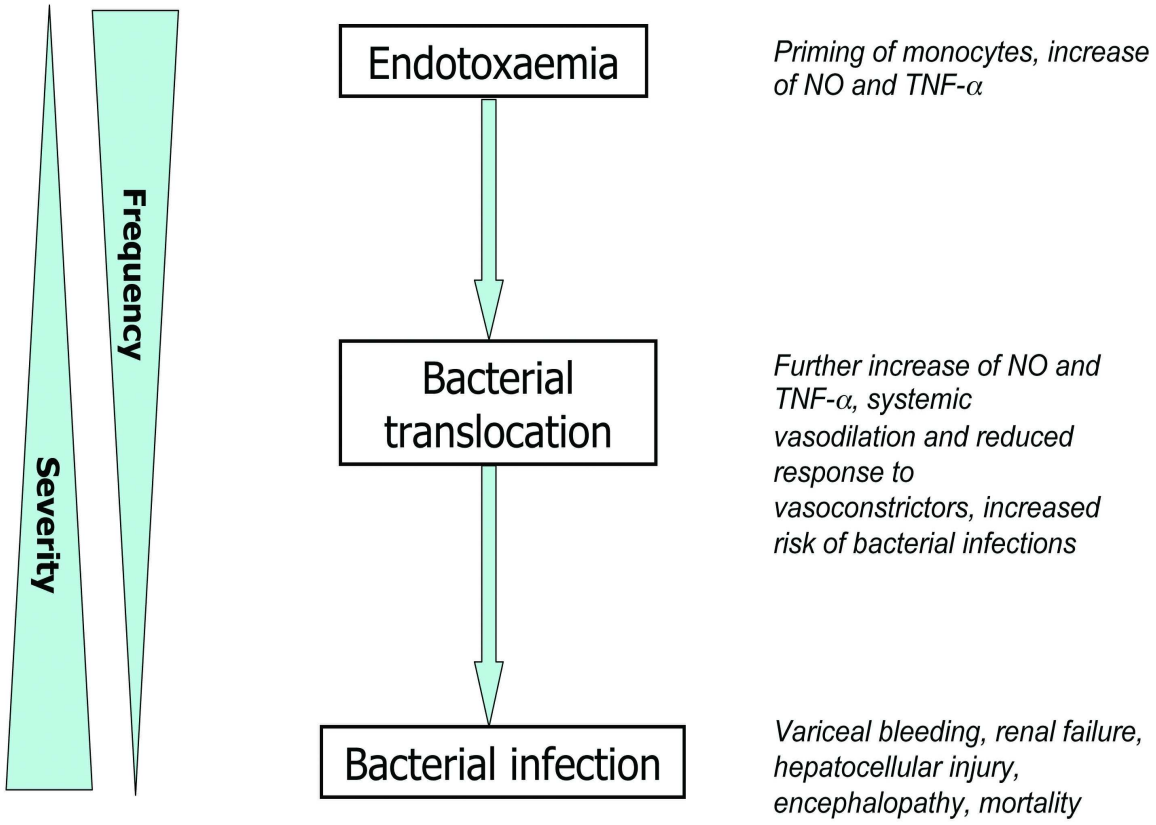


Figure 2: Endotoxaemia, bacterial translocation and bacterial infection may be different expressions of the same process at different degress of severity, and are associated with increasingly severe complications

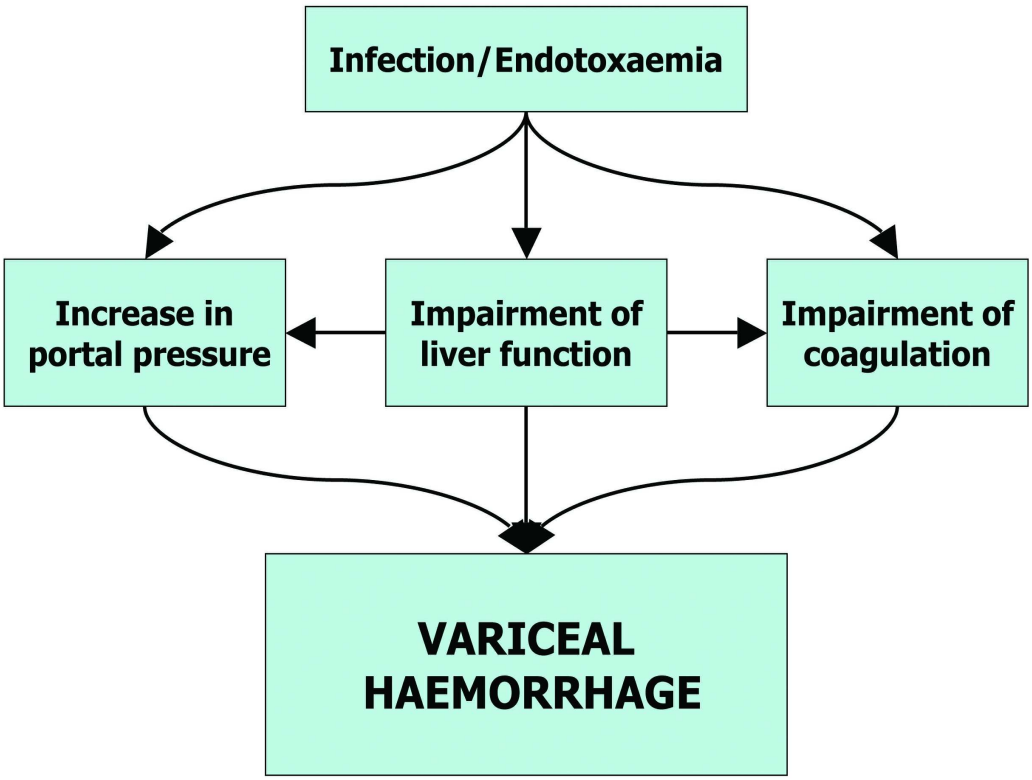


Figure 3: Possible pathophyisiological pathways through which bacterial infection can trigger variceal haemorrhage

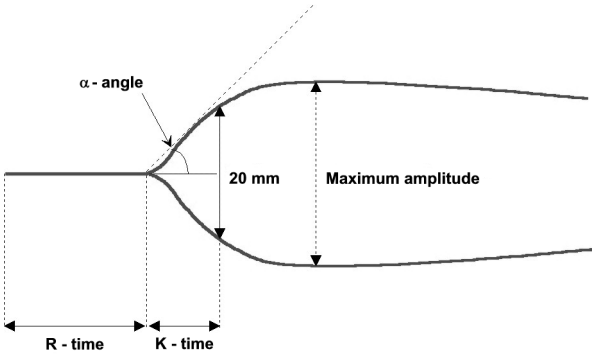
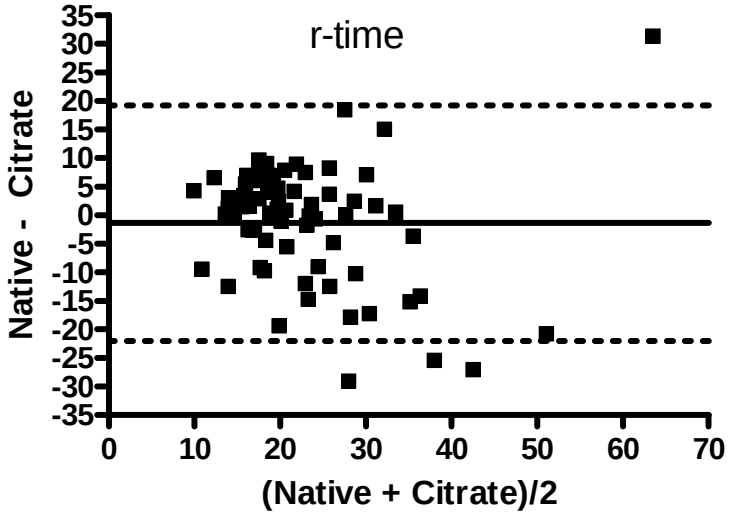
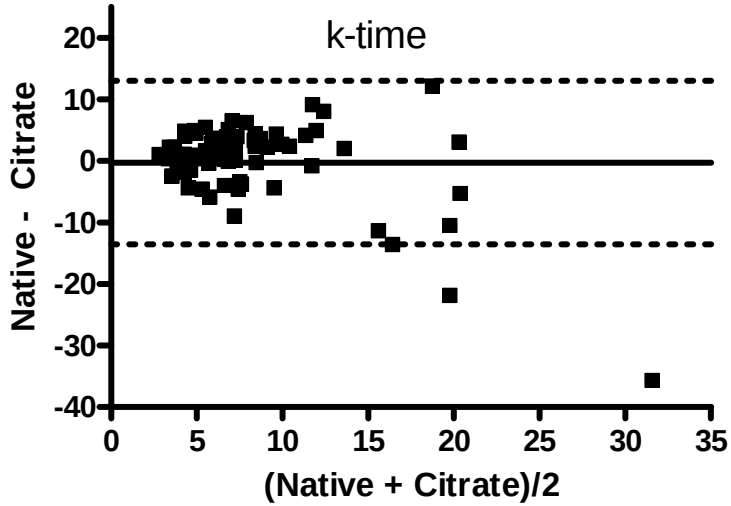
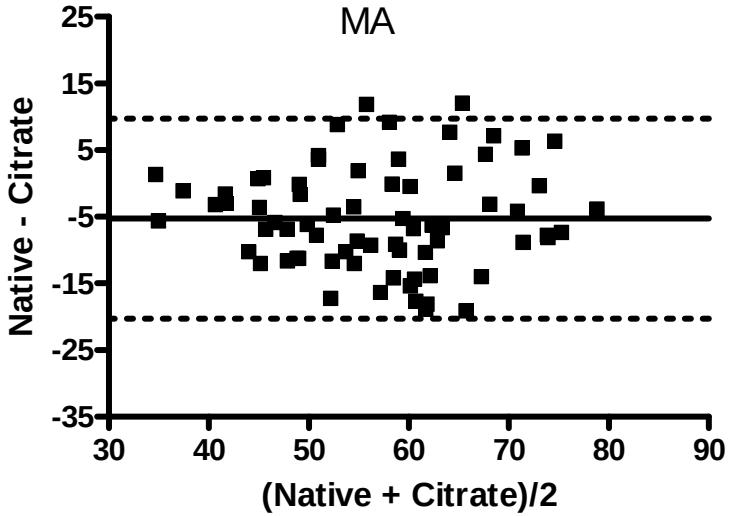


Figure 4: TEG variables. The R time is the time between activation of the sample with calcium, and the point where the amplitude of the tracing reaches 2mm, representing the formation of the first fibrin strands. The K time is measured from this point until the amplitude reaches 20mm, representing the time taken for a fixed degree of viscoelasticity to develop, as a result of fibrin build-up and crosslinking. The α-angle is that formed by the slope of the TEG tracing between the R time and the K time, and denotes the speed at which solid clot forms. The maximum amplitude is the greatest amplitude on the TEG trace and is a reflection of the absolute strength of the fibrin clot. The TEG parameters can be affected by qualitative and quantitative differences in platelets and the proteins of the clotting cascade, and hyperfibrinolytic states.


r-time
Native - Citrate
(Native + Citrate)/2
35
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
0
10
20
30
40
50
60
70


k-time
Native - Citrate
(Native + Citrate)/2
20
10
0
-10
-20
-30
-40
0
5
10
15
20
25
30
35


MA
Native - Citrate
(Native + Citrate)/2
25
15
5
-5
-15
-25
-35
30
40
50
60
70
80
90

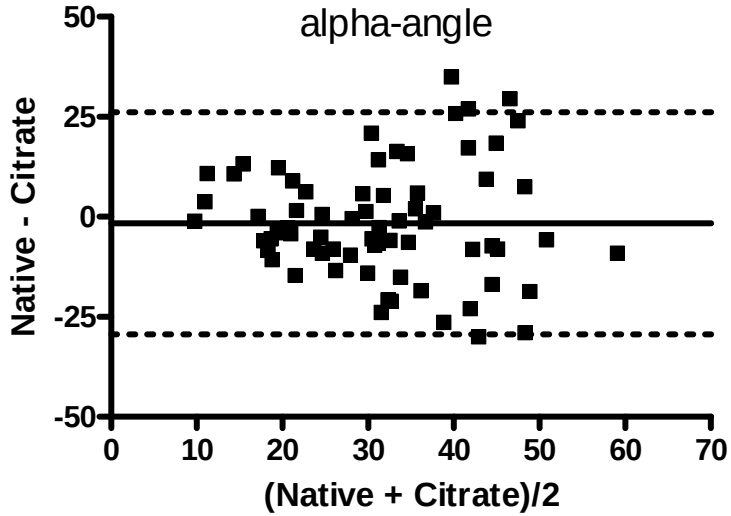

alpha-angle
Native - Citrate
(Native + Citrate)/2
50
25
0
-25
-50
0
10
20
30
40
50
60
70

Figure 5: Bland-Altman plots of the degree of agreement for each TEG parameter in both patients (n=50) and healthy volunteers (n=21) between non-kaolin activated native blood and non-kaolin activated citrated blood. Dotted lines indicate 1.96 standard deviations from the mean.

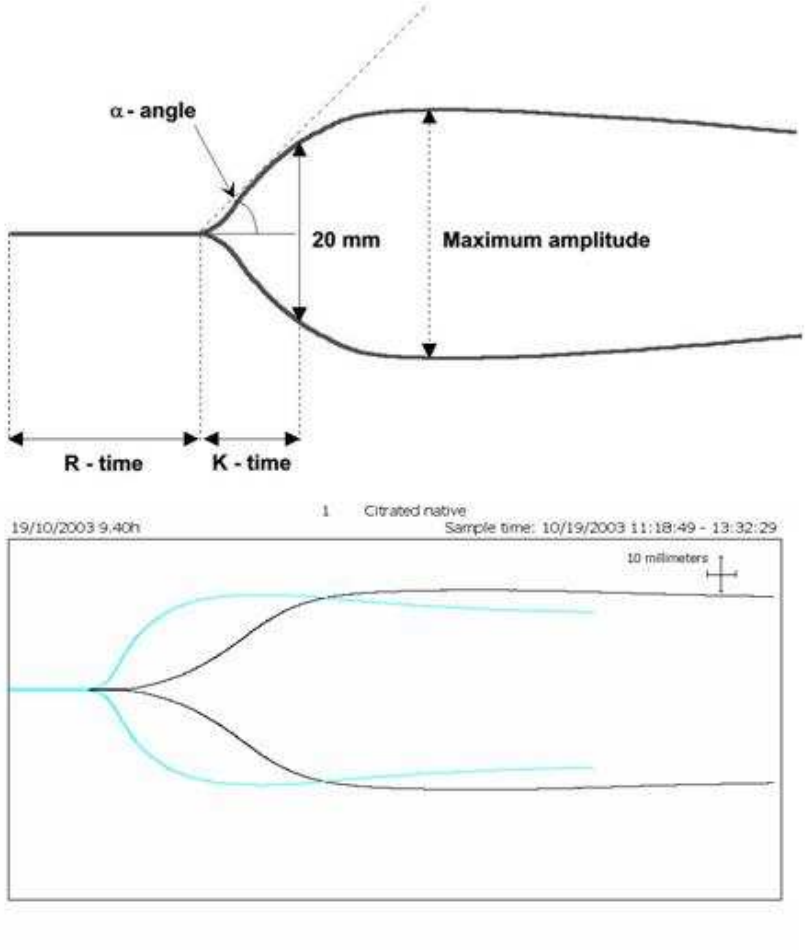


Figure 6: Panel A) Diagramatic thromboelastographic trace showing standard TEG parameters. Panel B) Thromboelastographic trace showing a typical heparinase effect as found in patient 2, 42 hours after the first haematemesis (Black line standard TEG, Grey line heparinase I-modified TEG)

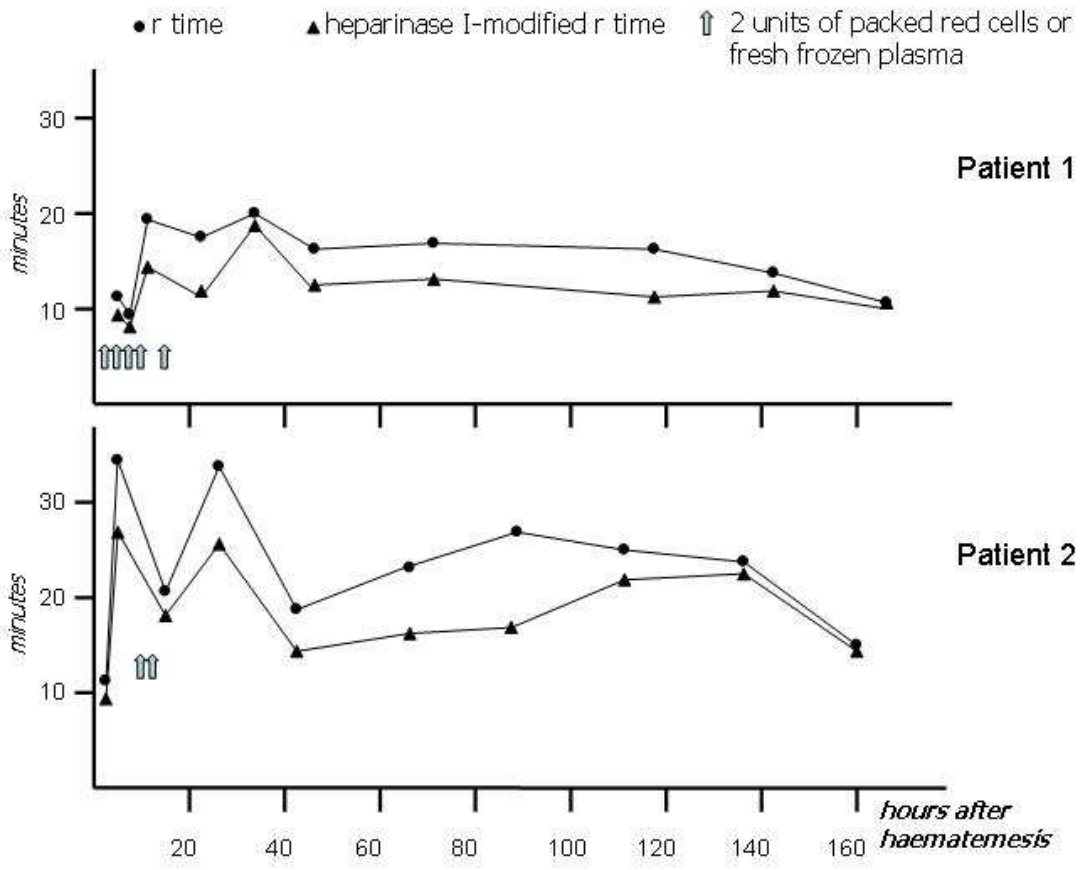


Figure 7: Comparison of standard and heparinase I-modified thromboelastography with respect to r time in two patients with variceal bleeding.

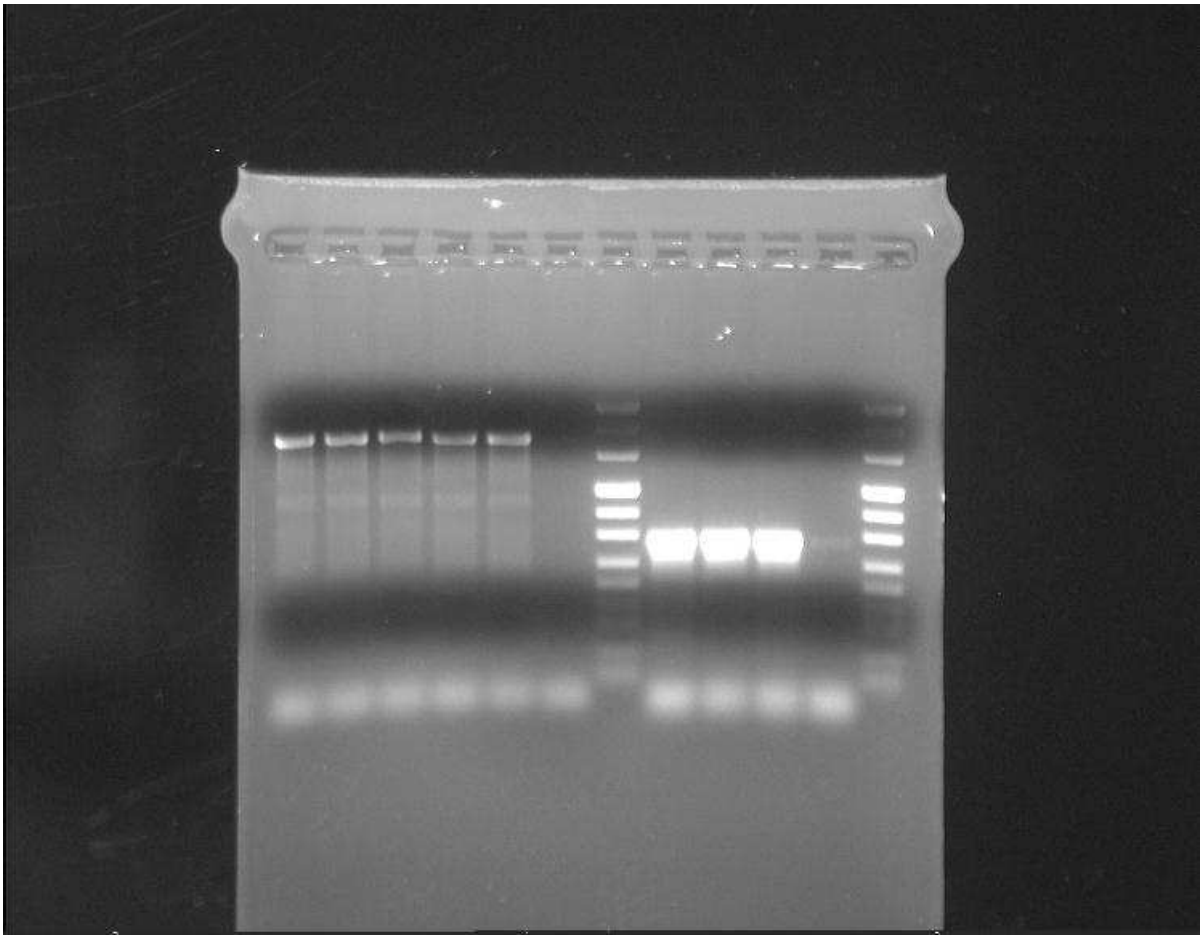
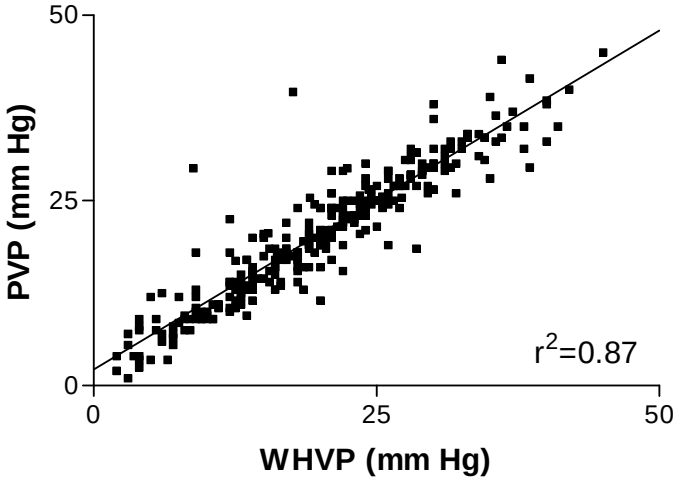
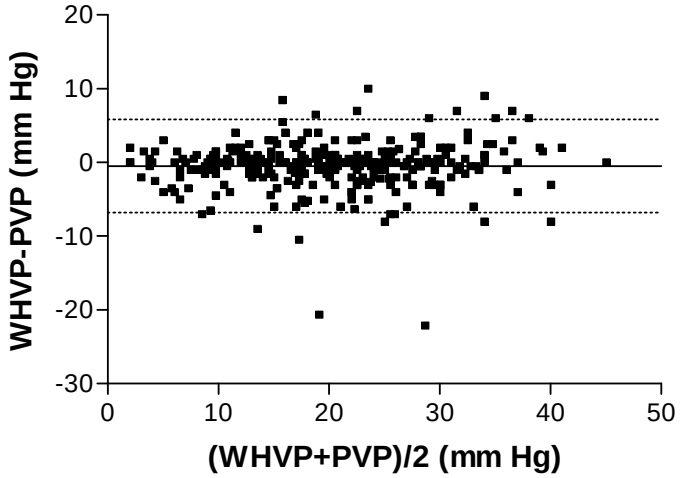


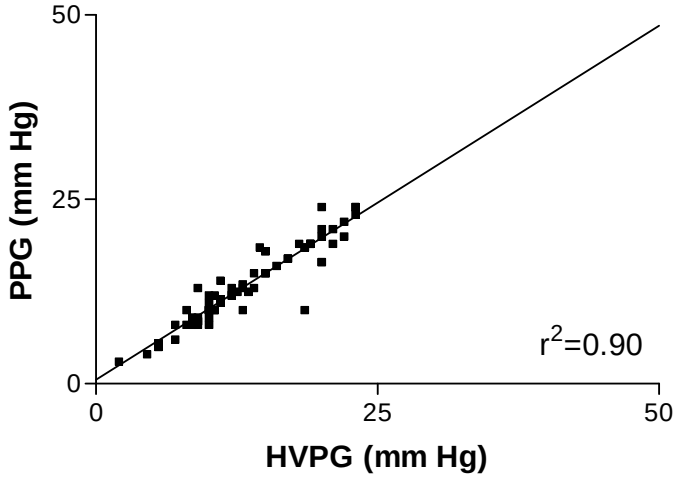
Figure 8: PCR detection of bacterial DNA in ascitic fluid and serum samples from patient 3. From left to right: Lane 1, positive control (*E.coli* DNA); Lane 2, ascitic fluid; Lanes 3-5 serum from days 1, 2 and 3; Lane 6, negative control (water instead of DNA); Lane 8, positive control (*E.coli* DNA); Lane 9: ascitic fluid; Lane 10, serum from day 1; Lane 11, negative control (water).
Lanes 1 to 6, PCR performed with BR1 universal primers; Lanes 8 to 11, PCR performed with EntB primers. Lanes 7 and 12: molecular weight markers.


50
25
0
0
25
50
PVP (mm Hg)
WHVP (mm Hg)
r2=0.87

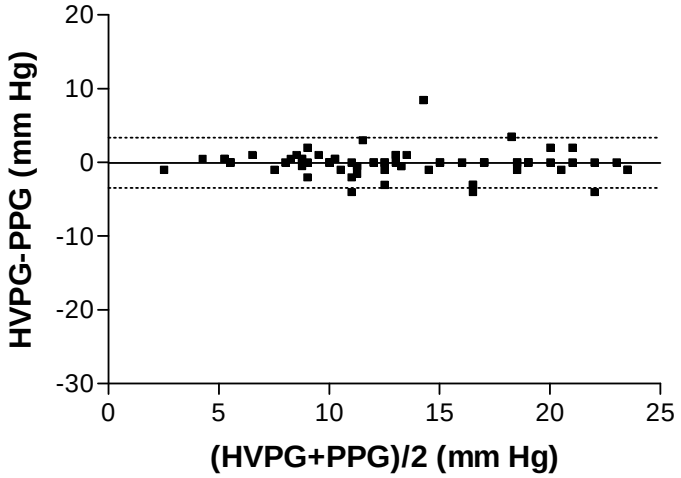
A


20
10
0
-10
-20
-30
0
10
20
30
40
50
WHVP-PVP (mm Hg)
(WHVP+PVP)/2 (mm Hg)

B

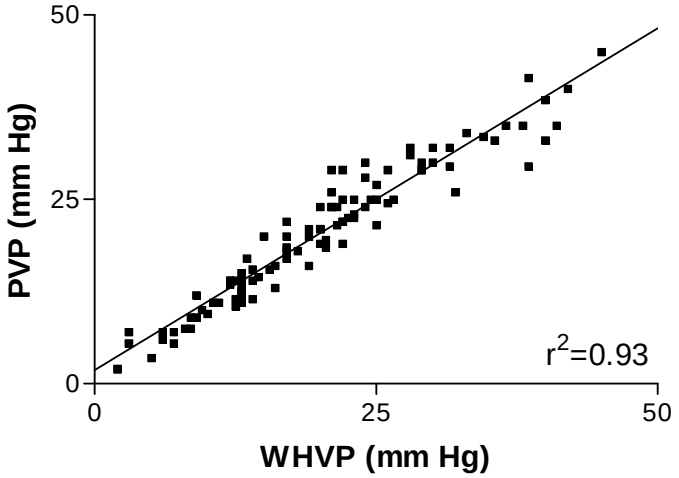

50
25
0
0
25
50
PPG (mm Hg)
HVPG (mm Hg)
r2=0.90

C

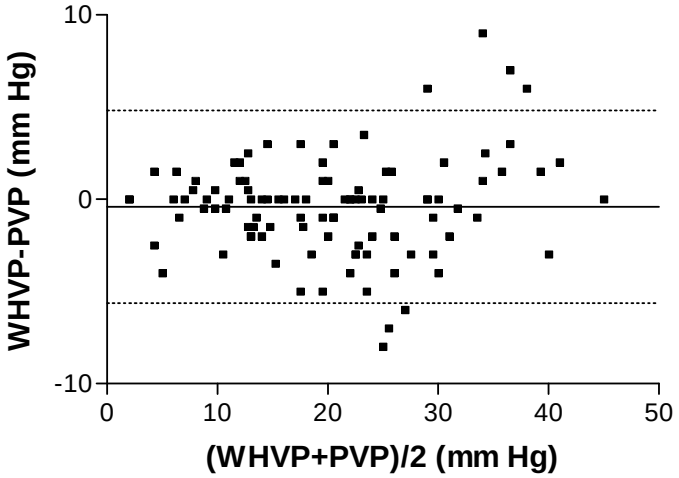

20
10
0
-10
-20
-30
0
5
10
15
20
25
HVPG-PPG (mm Hg)
(HVPG+PPG)/2 (mm Hg)

D

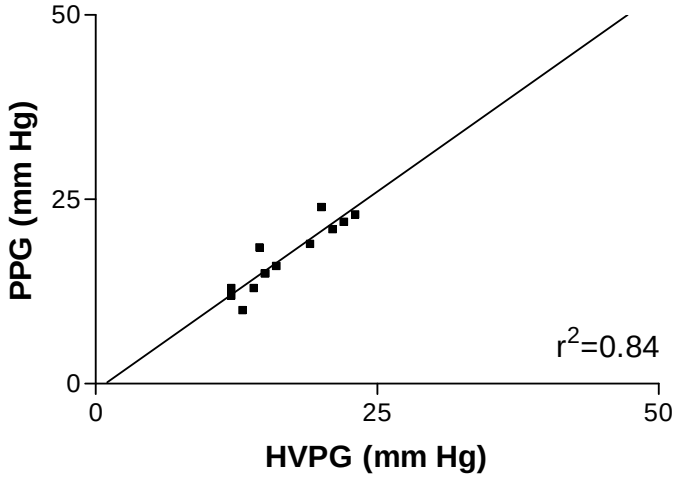
Figure 9: Data of measurements in all patients. A) Correlation between WHVP and PVP (n=320); B) corresponding agreement between WHVP and PVP according to Altman and Bland; C) correlation between HVPG and PPG (n=75); D) corresponding agreement between HVPG and PPG according to Altman and Bland. Dotted lines: 1.96 standard deviations.


PVP (mm Hg)
WHVP (mm Hg)
r2=0.93
0
25
50

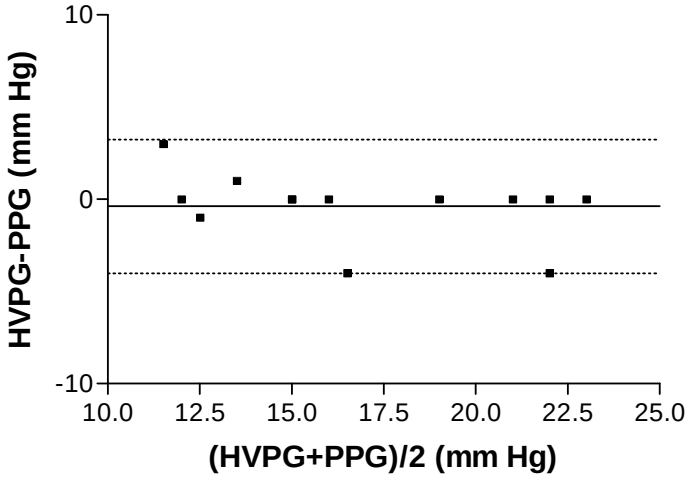
A


WHVP-PVP (mm Hg)
(WHVP+PVP)/2 (mm Hg)
10
0
-10
0
10
20
30
40
50

B

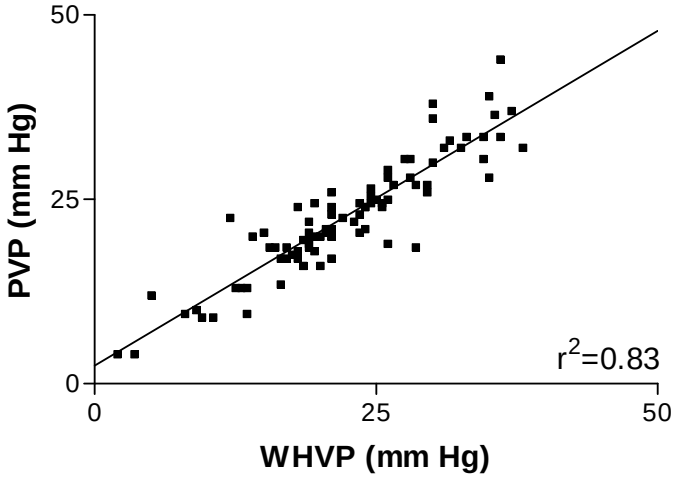

PPG (mm Hg)
HVPG (mm Hg)
r2=0.84
0
25
50

C

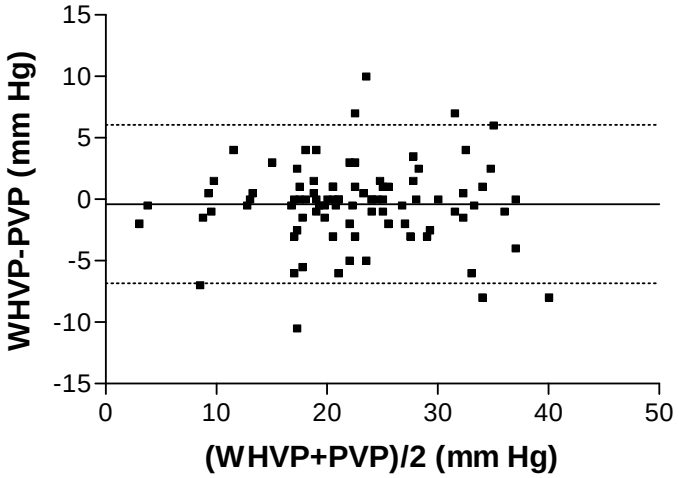

HVPG-PPG (mm Hg)
(HVPG+PPG)/2 (mm Hg)
10
0
-10
10.0
12.5
15.0
17.5
20.0
22.5
25.0

D

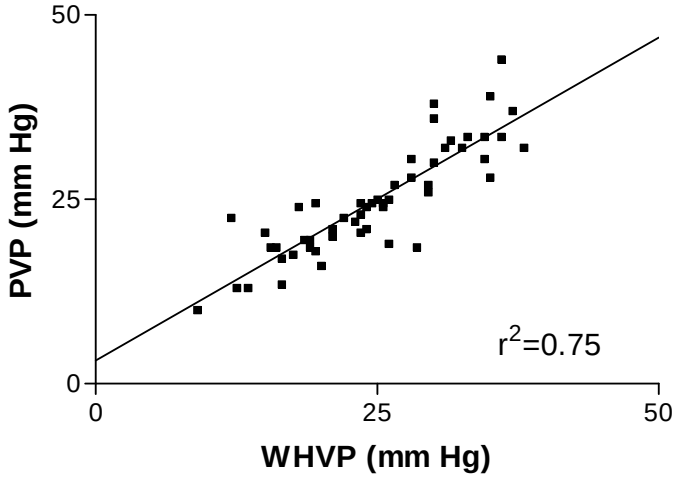
Figure 10: Data of measurements in patients with alcoholic liver disease. A) Correlation between WHVP and PVP (n=102); B) corresponding agreement between WHVP and PVP according to Altman and Bland; C) correlation between HVPG and PPG (n=13); D) corresponding agreement between HVPG and PPG according to Altman and Bland. Dotted lines: 1.96 standard deviations.


PVP (mm Hg)
WHVP (mm Hg)
r2=0.83
0
25
50

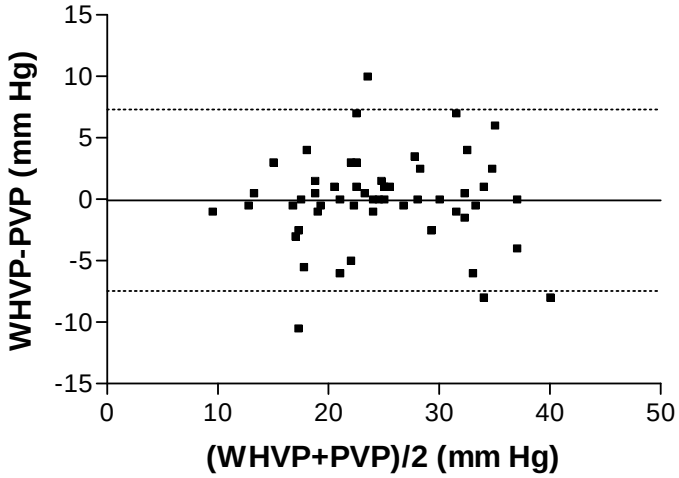
A


WHVP-PVP (mm Hg)
(WHVP+PVP)/2 (mm Hg)
15
10
5
0
-5
-10
-15
0
10
20
30
40
50

B

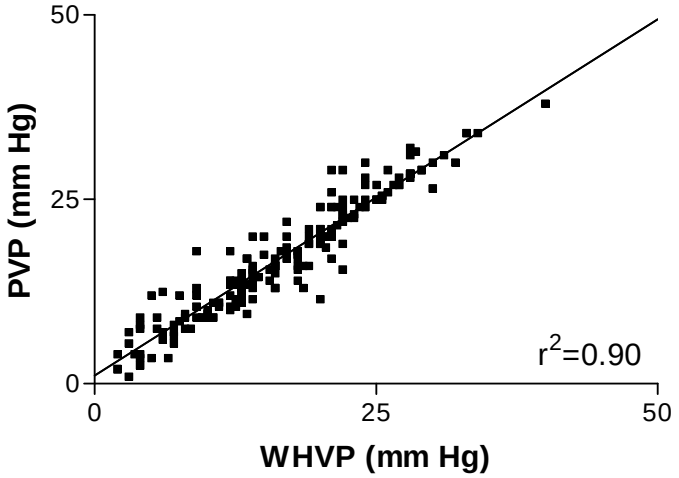

PVP (mm Hg)
WHVP (mm Hg)
r2=0.75
0
25
50

C

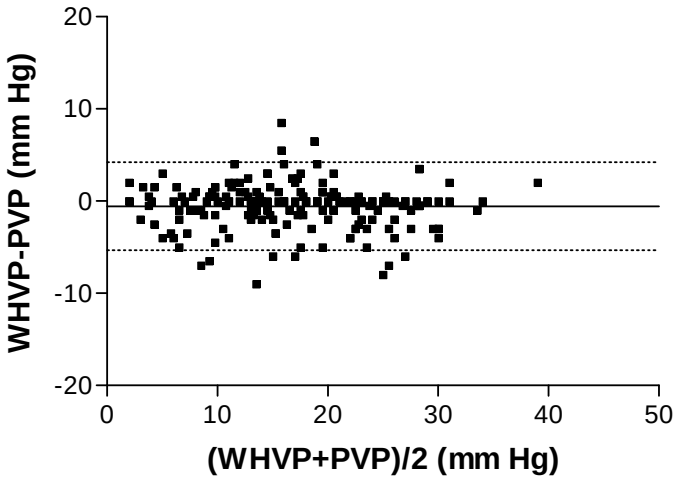

WHVP-PVP (mm Hg)
(WHVP+PVP)/2 (mm Hg)
15
10
5
0
-5
-10
-15
0
10
20
30
40
50

D

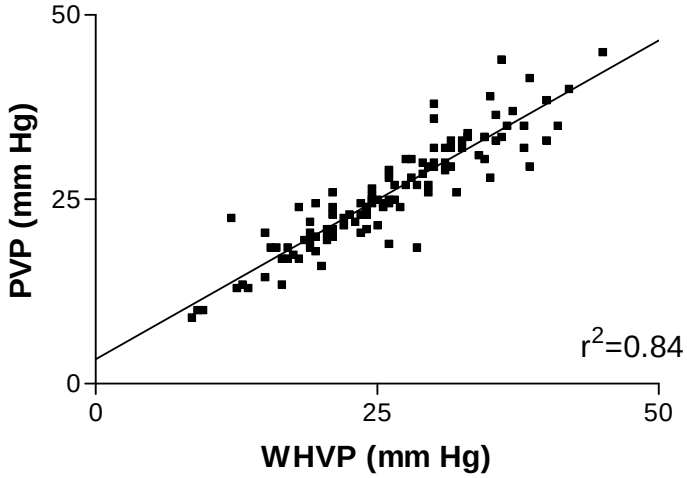
Figure 11: A) Correlation between WHVP and PVP in patients with non-alcoholic liver disease (n=88); B) corresponding agreement between WHVP and PVP according to Altman and Bland in patients with non-alcoholic liver disease; C) correlation between WHVP and PVP in patients with HCV related liver disease (n=53); D) corresponding agreement between WHVP and PVP according to Altman and Bland in patients with HCV related liver disease. Dotted lines: 1.96 standard deviations.


PVP (mm Hg)
WHVP (mm Hg)
r2=0.90
0
25
50

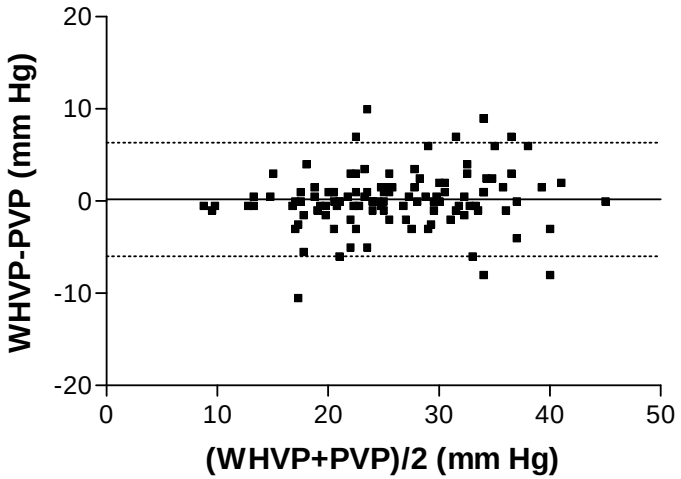
A


WHVP-PVP (mm Hg)
(WHVP+PVP)/2 (mm Hg)
20
10
0
-10
-20
0
10
20
30
40
50

B

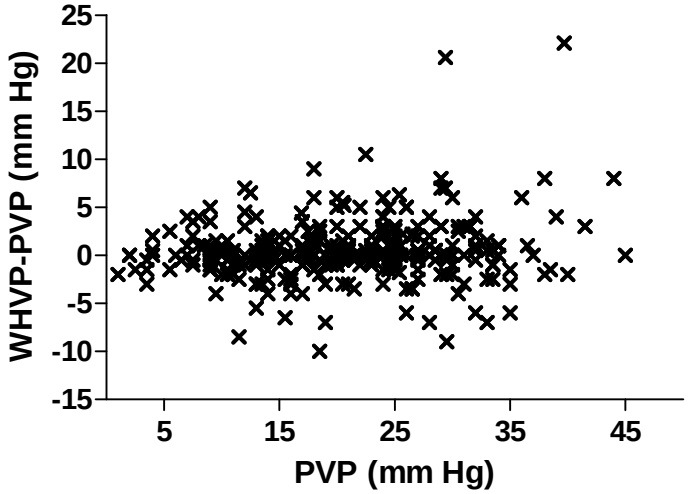

PVP (mm Hg)
WHVP (mm Hg)
r2=0.84
0
25
50

C

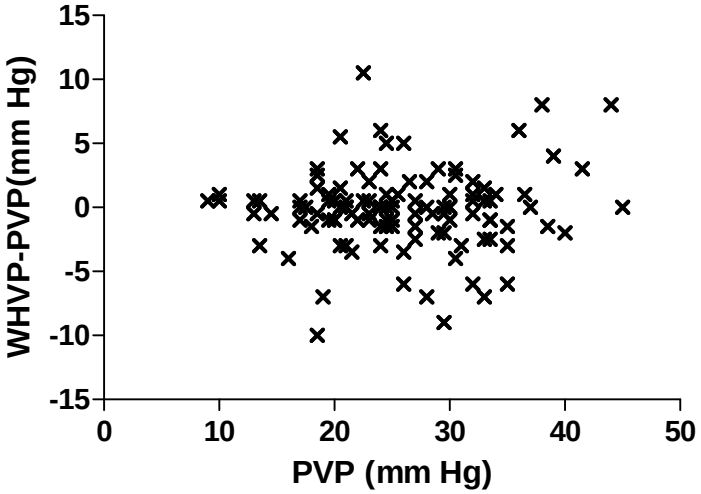

WHVP-PVP (mm Hg)
(WHVP+PVP)/2 (mm Hg)
20
10
0
-10
-20
0
10
20
30
40
50

D

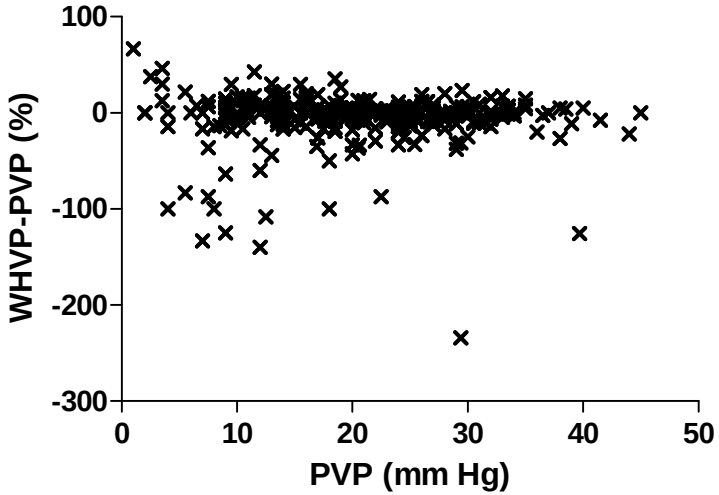
Figure 12: A) Correlation between WHVP and PVP measurements performed with a wedge catheter (n=194); B) corresponding agreement between WHVP and PVP according to Altman and Bland measurements performed with a wedge catheter; C) correlation between WHVP and PVP measurements performed with a balloon catheter (n=113); D) corresponding agreement between WHVP and PVP according to Altman and Bland measurements performed with a balloon catheter. Dotted lines: 1.96 standard deviations.


WHVP-PVP (mm Hg)
25
20
15
10
5
0
-5
-10
-15
5
15
25
35
45
PVP (mm Hg)

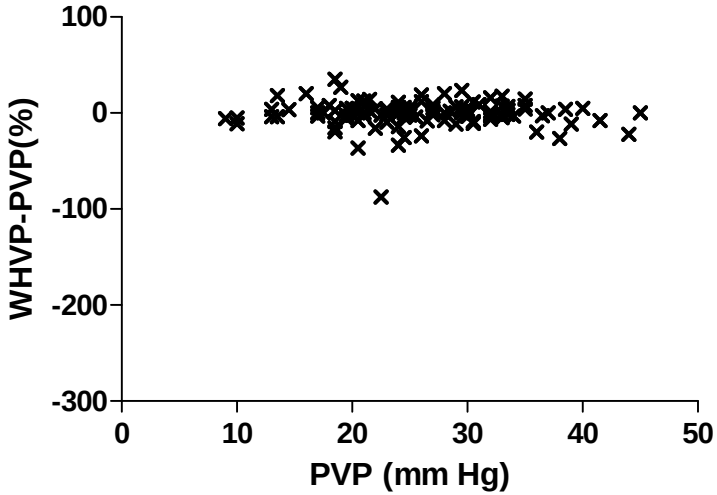
A


WHVP-PVP(mm Hg)
15
10
5
0
-5
-10
-15
0
10
20
30
40
50
PVP (mm Hg)

C


WHVP-PVP (%)
100
0
-100
-200
-300
0
10
20
30
40
50
PVP (mm Hg)

B


WHVP-PVP(%)
100
0
-100
-200
-300
0
10
20
30
40
50
PVP (mm Hg)

D

Figure 13: Assessment of the difference between the two types of measurement plotted against portal vein pressure for measurements in all patients (absolute difference, **A** and percentage change from WHVP, **B**) and measurements with balloon catheter (absolute difference, **C** and percentage change from WHVP, **D**).

TABLES

Reaction (R) time (9.4 - 27.4 minutes)

U / ml	Saline		UFH		LMWH		DPD	
0.025	14.2	± 1.3	17.0	± 1.4	17.8	± 1.1	16.7	± 2.0
0.050	13.8	± 1.0	19.0	± 1.9	17.2	± 1.7	20.1	± 4.1
0.100	12.7	± 0.8	26.4	± 1.8	15.9	± 1.2	19.6	± 2.1
0.250	15.0	± 1.6	46.0	± 5.0	26.5	± 3.4	27.0	± 3.7
0.500	13.6	± 1.5	55.6	± 4.4	50.6	± 5.5	43.3	± 4.4
1.000	14.2	± 1.5	60.0	± 0.0	59.3	± 0.7	56.7	± 3.3

K time (1.9-8.9 minutes)

U / ml	Saline		UFH		LMWH		DPD	
0.025	5.5	± 0.4	7.1	± 0.8	9.8	± 1.3	8.3	± 1.5
0.050	5.2	± 0.5	8.8	± 1.0	9.1	± 1.8	8.9	± 1.2
0.100	5.0	± 0.5	15.2	± 1.6	9.3	± 1.3	9.6	± 1.9
0.250	5.2	± 0.4	41.7	± 7.5	19.1	± 5.0	17.2	± 2.6
0.500	5.2	± 0.5	55.0	± 5.0	50.2	± 6.6	34.7	± 6.5
1.000	4.5	± 0.6	60.0	± 0.0	60.0	± 0.0	56.4	± 3.6

α-angle (22-58 degrees)

U / ml	Saline		UFH		LMWH		DPD	
0.025	39.9	± 3.1	28.9	± 3.1	24.1	± 2.6	29.3	± 4.7
0.050	40.1	± 2.2	26.0	± 3.1	25.7	± 2.4	29.2	± 3.7
0.100	41.2	± 3.3	16.6	± 2.1	30.0	± 2.4	27.5	± 4.1
0.250	39.1	± 2.9	8.0	± 3.5	17.7	± 2.2	19.8	± 3.2
0.500	39.6	± 2.5	2.0	± 2.0	6.0	± 3.7	9.8	± 3.1
1.000	45.5	± 2.8	0.0	± 0.0	0.0	± 0.0	1.1	± 1.1

Maximum amplitude (MA) (44.4-63.6 mm)

U / ml	Saline		UFH		LMWH		DPD	
0.025	48.3	± 1.6	43.6	± 1.3	41.8	± 1.6	43.9	± 2.5
0.050	46.3	± 1.6	41.2	± 1.6	42.8	± 2.1	38.2	± 3.7
0.100	50.1	± 1.4	40.3	± 1.8	42.7	± 1.4	43.1	± 1.8
0.250	48.6	± 1.2	15.5	± 5.3	35.1	± 4.5	39.1	± 2.3
0.500	47.8	± 0.9	3.7	± 3.7	9.2	± 5.8	24.0	± 6.7
1.000	53.2	± 1.2	0.0	± 0.0	1.4	± 1.4	5.4	± 5.4

Table 1A

Prothrombin time (PT) (12 - 16 s)

U / ml	Saline	UFH	LMWH	DPD
0.025	14.0 ± 0.2	14.2 ± 0.3	14.0 ± 0.3	14.22 ± 0.3
0.050	13.8 ± 0.2	14.2 ± 0.2	14.2 ± 0.3	14.34 ± 0.3
0.100	14.0 ± 0.2	14.8 ± 0.2	14.8 ± 0.6	14.44 ± 0.3
0.250	14.3 ± 0.3	15.9 ± 0.7	15.8 ± 0.7	14.79 ± 0.2
0.500	14.3 ± 0.3	18.0 ± 0.7	16.6 ± 0.4	16.04 ± 0.3
1.000	14.7 ± 0.3	26.9 ± 2.4	19.0 ± 0.4	17.51 ± 0.3

Activated partial thromboplastin time (aPTT) (28 - 38 s)

U / ml	Saline	UFH	LMWH	DPD
0.025	32.4 ± 0.8	33.2 ± 1.2	32.4 ± 2.0	33.5 ± 1.1
0.050	32.3 ± 0.7	34.3 ± 1.5	33.4 ± 2.2	35.8 ± 1.2
0.100	32.6 ± 1.0	41.1 ± 3.2	39.5 ± 3.6	37.5 ± 1.2
0.250	33.1 ± 0.7	78.3 ± 13.4	43.9 ± 2.1	46.1 ± 1.7
0.500	33.6 ± 0.6	130.4 ± 12.7	66.5 ± 5.9	59.2 ± 2.6
1.000	34.8 ± 0.8	146.1 ± 14.2	125.0 ± 10.0	90.1 ± 5.6

Thrombin time (TT) (13 - 17 s)

U / ml	Saline	UFH	LMWH	DPD
0.025	14.7 ± 0.4	15.6 ± 0.5	16.3 ± 1.3	14.7 ± 0.4
0.050	15.3 ± 0.5	16.7 ± 1.2	15.1 ± 0.4	15.1 ± 0.4
0.100	15.1 ± 0.3	49.2 ± 5.6	23.6 ± 4.3	15.0 ± 0.3
0.250	15.4 ± 0.6	55.4 ± 4.6	60.0 ± 0.0	27.6 ± 5.6
0.500	15.2 ± 0.4	60.0 ± 0.0	60.0 ± 0.0	30.5 ± 5.1
1.000	15.7 ± 0.4	60.0 ± 0.0	60.0 ± 0.0	50.1 ± 5.1

Anti-Xa activity (U/ml)

U / ml	Saline	UFH	LMWH	DPD
0.025	0.000 ± 0.000	0.019 ± 0.007	0.026 ± 0.006	0.031 ± 0.010
0.050	0.000 ± 0.000	0.049 ± 0.012	0.053 ± 0.007	0.081 ± 0.031
0.100	0.000 ± 0.000	0.133 ± 0.024	0.099 ± 0.008	0.101 ± 0.013
0.250	0.000 ± 0.000	0.321 ± 0.033	0.211 ± 0.021	0.302 ± 0.033
0.500	0.000 ± 0.000	0.703 ± 0.043	0.599 ± 0.043	0.611 ± 0.036
1.000	0.000 ± 0.000	0.894 ± 0.043	0.924 ± 0.060	0.925 ± 0.049

Table 1B

Tables 1A and 1B: Comparison of TEG parameters (table 1a) with conventional coagulation tests (table 1b) for detecting the presence of heparins and Danaparoid in blood. The mean results ± SEM from 10 subjects with each drug are presented. Saline control values shown are the mean of 9 samples (3 subjects, 3 drugs) ± SEM. The reference range for each parameter is shown in brackets. Values outwith the reference range are underlined. UFH = unfractionated heparin; LMWH = low molecular weight heparin (Fragmin); DPD = Danaparoid (Orgaran). Straight-line traces were arbitrarily assigned R and K values of 60 mins, α-angles of 0° and MAs of 0mm

Reaction R time (9.4 - 27.4 minutes)

U / ml	UFH		+Hep'ase		P<	LMWH		+Hep'ase		P<	DPD		+Hep'ase		P<
0.005	16.4	±1.6	14.4	±0.9	.2	16.0	±0.9	12.6	±1.1	.001	15.4	±1.4	12.3	±1.3	.01
0.010	16.5	±1.3	15.3	±1.3	.2	17.8	±1.6	13.1	±0.8	.01	16.8	±1.5	13.6	±1.1	.05
0.025	18.5	±1.2	13.7	±1.1	.001	18.7	±1.1	12.9	±0.9	.001	17.2	±1.9	12.4	±1.2	.01
0.050	21.7	±1.8	14.4	±1.6	.001	18.7	±2.0	13.1	±0.6	.02	21.8	±3.9	13.2	±1.1	.05

K time (1.9-8.9 minutes)

U / ml	UFH		+Hep'ase		P<	LMWH		+Hep'ase		P<	DPD		+Hep'ase		P<
0.005	7.1	±0.9	5.1	±0.6	.05	9.2	±1.1	4.1	±0.6	.01	8.2	±1.2	3.7	±0.5	.01
0.010	6.7	±0.5	5.4	±0.5	.01	10.0	±1.4	4.4	±0.5	.01	9.5	±1.5	4.2	±0.4	.01
0.025	8.8	±0.8	4.5	±0.6	.01	11.2	±1.1	4.1	±0.5	.001	9.0	±1.3	4.0	±0.7	.01
0.050	12.2	±1.4	4.6	±0.6	.001	11.5	±2.1	4.4	±0.4	.01	10.6	±1.0	4.4	±0.7	.001

α-angle (22-58 degrees)

U / ml	UFH		+Hep'ase		P<	LMWH		+Hep'ase		P<	DPD		+Hep'ase		P<
0.005	29.4	±2.5	38.3	±2.9	.01	24.0	±2.2	45.6	±4.6	.01	27.2	±3.1	48.3	±3.8	.01
0.010	29.9	±2.1	37.1	±2.5	.001	22.2	±2.4	44.4	±3.4	.01	24.3	±2.9	44.3	±2.8	.001
0.025	23.7	±1.9	40.9	±3.9	.01	20.0	±1.9	46.4	±3.4	.001	25.8	±4.1	46.3	±4.4	.01
0.050	18.6	±1.8	41.6	±3.5	.001	21.8	±2.9	42.2	±2.6	.001	24.2	±3.4	43.8	±4.2	.01

Maximum amplitude (MA; 44.4-63.6 mm)

U / ml	UFH		+Hep'ase		P<	LMWH		+Hep'ase		P<	DPD		+Hep'ase		P<
0.005	44.8	±2.0	47.7	±2.3	.2	40.0	±1.9	51.1	±2.9	.01	42.6	±2.0	51.5	±2.8	.01
0.010	45.8	±1.2	50.1	±2.3	.001	40.6	±2.5	51.5	±2.3	.02	42.6	±1.7	51.5	±1.8	.01
0.025	43.1	±1.4	51.8	±2.6	.01	40.0	±1.2	53.5	±2.8	.001	42.5	±2.4	50.9	±2.5	.01
0.050	41.0	±1.4	50.7	±3.1	.01	39.5	±2.0	51.3	±2.2	.01	36.5	±3.6	51.1	±3.0	.01

Prothrombin time (PT) (12 - 16s)

U / ml	UFH		LMWH		Danaparoid	
0.005	14.4	± 0.3	14.3	± 0.3	14.4	± 0.4
0.010	14.2	± 0.3	14.4	± 0.4	14.4	± 0.3
0.025	14.3	± 0.3	14.3	± 0.3	14.4	± 0.3
0.050	14.4	± 0.3	14.5	± 0.3	14.6	± 0.4

aPTT (28 - 38s)

UFH		LMWH		Danaparoid	
33.8	± 1.4	34.3	± 1.4	33.5	± 1.1
33.6	± 1.3	34.2	± 1.3	33.3	± 1.1
34.4	± 1.4	34.4	± 1.4	35.0	± 1.2
35.4	± 1.6	35.8	± 1.6	37.4	± 1.3

Thrombin time (TT) (13 - 17s)

U / ml	UFH		LMWH		Danaparoid	
0.005	15.1	± 0.5	14.5	± 0.2	15.5	± 0.7
0.010	14.1	± 0.2	14.2	± 0.3	14.3	± 0.3
0.025	15.6	± 0.5	16.3	± 1.2	14.9	± 0.4
0.050	17.0	± 1.1	15.4	± 0.4	15.5	± 0.4

Anti-Xa activity (U/ml)

UFH		LMWH		Danaparoid	
0.034	± .013	0.007	± .004	0.004	± .002
0.034	± .014	0.013	± .005	0.009	± .003
0.044	± .029	0.018	± .006	0.029	± .010
0.052	± .022	0.038	± .010	0.083	± .031

Table 2: Comparison of standard and heparinase modified TEG parameters with conventional coagulation tests for detecting the presence of low concentrations of heparins and Danaparoid in blood. The mean results ± SEM from 10 subjects with each drug are presented. Paired t-test p-values demonstrate statistically significant differences between TEG parameters with and without heparinase modification at all but the lowest concentrations of UFH used in this study

Summary Slopes	UFH			LMWH			Danaparoid		
	Mean	**±SEM**	**P <**	**Mean**	**±SEM**	**P <**	**Mean**	**±SEM**	**P <**
R time	44.4	1.3	**0.0001**	48.0	1.1	**0.0001**	41.8	3.3	**0.0001**
K time	56.2	1.3	**0.0001**	57.9	1.4	**0.0001**	51.1	4.0	**0.0001**
α angle	-27.4	2.5	**0.0001**	-29.5	1.6	**0.0001**	-30.4	3.2	**0.0001**
MA	-47.2	1.6	**0.0001**	-47.1	2.6	**0.0001**	-39.0	6.5	**0.0001**

Table 3A: Mean (n=10) of summary slopes representing the change in each TEG parameter vs. anticoagulant concentration (0.025 to 1.0 U/ml)

Summary Slopes	UFH			LMWH			Danaparoid		
	Mean	**±SEM**	**P <**	**Mean**	**±SEM**	**P <**	**Mean**	**±SEM**	**P <**
R time	121.7	37.5	**0.02**	47.9	29.7	**0.2**	133.4	86.9	**0.2**
K time	123.4	38.3	**0.02**	47.4	30.6	**0.2**	40.7	35.6	**0.3**
α angle	-259.0	72.9	**0.01**	-40.3	55.5	**0.5**	-40.5	112.2	**0.8**
MA	-119.32	40.9	**0.02**	-11.9	36.9	**0.8**	-136.2	83.2	**0.2**

Table 3B: Mean (n=10) of summary slopes representing the change in each TEG parameter vs. anticoagulant concentration (0.005 to 0.5 U/ml)

Slope Pairs (Standard vs heaprinase)	UFH			LMWH			Danaparoid		
	Mean	**± SEM**	**P <**	**Mean**	**± SEM**	**P <**	**Mean**	**± SEM**	**P**
R time	133.5	37.5	0.01	42.0	28.3	0.2	126.5	81.9	0.2
K time	137.8	40.3	0.01	44.0	31.2	0.2	29.6	43.1	NS (0.51)
α angle	-352.0	104.0	0.01	22.9	80.5	NS (0.78)	23.6	182.9	NS (0.9)
MA	-179.8	65.1	0.05	-13.3	56.4	NS (0.82)	-126.5	100.4	NS (0.24)
Anti-Xa	0.3	0.4	NS (0.43)	0.7	0.2	0.02	1.8	0.7	0.05

Table 4: Mean difference (n=10) between standard and heparinase modified TEG summary slopes representing the change in each parameter vs. anticoagulant concentration (0.005 to 0.5 U/ml), compared with mean (n=10) of summary slopes representing the change in anti-Xa activity vs. anticoagulant concentration (0.005 to 0.5 U/ml)

	Age (±S.D.)	Male/Female	Child score(±S.D.)	INR (±S.D.)	Enoxaparin 20/40 mg
Group 1	31.8±4.7	13/8			
Group 2	52.3±8.2	10/10	9±1.8		
Group 3	49.4±11.4	4/6			
Group 4	55.2±17.5	6/4		3.0±1.0	
Group 5	55.8±15.9	8/2			4/6

Table 5: Demographics of healthy volounteers (Group1) and patients (Group 2-5)

	r time	k time	α angle	MA
Total	0.70	0.46	0.52	0.69
Group 1	0.18	0.53	0.58	0.69
Group 2	0.68	0.67	0.67	0.77
Group 3	0.60	- 0.56	- 0.68	0.22
Group 4	0.77	0.31	0.57	- 0.02
Group 5	0.90	0.71	0.78	0.75

Group 1 – 21 healthy volunteers
Group 2 – 20 patients with non-biliary cirrhosis
Group 3 – 10 patients with PBC/PSC
Group 4 – 10 patients on warfarin
Group 5 – 10 patients on enoxaparin prophylaxis

Table 6: Correlation (Spearman r) between native blood and kaolin-activated native blood

	r time	k time	α angle	MA
Total	0.37	0.07	0.19	0.80
Group 1	0.46	0.44	0.56	0.68
Group 2	0.19	0.14	0.31	0.80
Group 3	0.47	- 0.13	- 0.07	0.7
Group 4	0.53	- 0.28	- 0.20	0.49
Group 5	0.73	0.33	0.32	0.49

Group 1 – 21 healthy volunteers
Group 2 – 20 patients with non-biliary cirrhosis
Group 3 – 10 patients with PBC/PSC
Group 4 – 10 patients on warfarin
Group 5 – 10 patients on enoxaparin prophylaxis

Table 7: Correlation (Spearman r) between citrated blood and kaolin-activated citrated blood

	r time	k time	α angle	MA
Total	0.46	0.46	0.47	0.71
Group 1	0.47	0.56	0.62	0.71
Group 2	0.35	0.34	0.36	0.71
Group 3	0.17	0.08	- 0.31	0.19
Group 4	0.36	0.66	0.82	0.19
Group 5	0.84	0.90	0.82	0.75

Group 1 – 21 healthy volunteers
Group 2 – 20 patients with non-biliary cirrhosis
Group 3 – 10 patients with PBC/PSC
Group 4 – 10 patients on warfarin
Group 5 – 10 patients on enoxaparin prophylaxis

Table 8: Correlation (Spearman r) between native blood and citrated blood (non kaolin-activated)

	Total	Group1	Group 2	Group 3	Group 4	Group 5
r-time native vs citrated	0.7980	0.0021*	0.2820	0.3750	0.0098*	0.0645
k-time native vs citrated	0.2145	0.0002*	0.7724	0.9219	0.0195*	0.1934
α angle native vs citrated	0.2042	0.0001*	0.9777	1.0000	0.1055	0.3750
ma native vs citrated	<0.0001*	0.0004*	0.0067*	0.6250	0.0645	0.0840

* $p<0.05$

Group 1 – 21 healthy volunteers
Group 2 – 20 patients with non-biliary cirrhosis
Group 3 – 10 patients with PBC/PSC
Group 4 – 10 patients on warfarin
Group 5 – 10 patients on enoxaparin prophylaxis

Table 9: Wilcoxon's matched pair test p values for native and citrated blood samples (kaolin activated and non-kaolin activated)

	Group1	Group2	Group3	Group4	Group5	Kruskal-Wallis p
Native r time	23.314±5.243	20.290±7.169	17.170±4.480	23.890±8.619	27.770±19.988	0.0437
Native k time	9.238±2.830*	7.620±4.939	5.490±2.015*	9.080±4.642	9.460±6.754	0.0320
Native α angle	24.305±6.533*	32.670±13.598	40.160±10.488*	29.800±16.234	33.080±14.292	0.0166
Native MA	48.524±6.082*$	48.570±8.716&%	63.350±8.027*&	57.280±9.732	65.940±11.300$%	0.0000
Citrated r time	19.290±3.933§	22.670±6.867	21.340±10.282#	37.380±12.211§#!	23.590±17.842!	0.0038
Citrated k time	6.095±2.108§	7.415±3.586	5.540±2.641#	18.980±13.849§#	8.340±7.021	0.0211
Citrated α angle	34.648±10.646	31.155±9.950	39.010±14.289#	20.600±12.000#	36.130±16.911	0.0281
Citrated MA	55.414±8.625$	53.215±9.862&"%	65.410±8.686&	65.000±8.043"%	69.840±9.807$	0.0001
Native r time (Kaolin)	5.914±1.182*	4.890±1.130	4.400±0.924*	5.630±2.213	4.650±1.439	0.0109
Native k time (Kaolin)	1.448±0.218*$	1.905±1.528&%	1.060±0.222*&	1.340±0.420	1.060±0.222$%	0.0004
Native α angle (Kaolin)	69.157±3.145*$	66.290±10.448&%	74.760±2.950*&	71.300±5.283	75.980±2.693$%	0.0000
Native MA (Kaolin)	64.762±4.393$	55.415±9.465&"%	69.350±5.817&	66.930±10.243"	74.330±4.996$%	0.0000

* Significant difference between groups 1-3
$ Significant difference between groups 1-5
& Significant difference between groups 2-3
% Significant difference between groups 2-5
§ Significant difference between groups 1-4
# Significant difference between groups 3-4
! Significant difference between groups 4-5
"Significant difference between groups 2-4

Table 10: Differences between group

Age (mean±2*S.D.)	48.8±28.6
Gender (M/F)	6/4
Aetiology	
Alcohol	6
Budd Chiari	2
Hepatitis C Virus	1
Cryptogenic	1
Indication for TIPS	
Recurrent bleeding	4
Refractory ascites/hydrothorax	3/1
Budd Chiari	2
Child class (A/B/C)	1/5/4
Child score (mean±2*S.D.)	8.8±3.6

Table 11: Demographics of the patients recruited for the study.

Patient	**Aetiology**	**Indication**	**Child score**	**TEG variable**	**Baseline**	**Day 0**	**Day 1**	**Day 2**
Patient 1	Hepatitis C	Hydrothorax	8					
				r time	-	-	-	
				k time	-	-	-	
				alpha angle	-	-	-	
				ma	-	-	-	
Patient 2	Cryptogenetic	Ascites	9					
				r time	-	-	+	-
				k time	-	+	+	-
				alpha angle	-	-	+	-
				ma	-	-	-	-
Patient 3	Alcohol	Bleeding	7					
				r time	-	-	-	
				k time	-	-	+	
				alpha angle	-	-	-	
				ma	-	-	-	
Patient 4	Alcohol	Bleeding	10					
				r time	-	-	-	-
				k time	+	-	-	-
				alpha angle	+	-	-	-
				ma	-	-	-	-
Patient 5	Alcohol	Ascites	10					
				r time	-	+	+	-
				k time	-	+	-	-
				alpha angle	-	+	-	-
				ma	-	-	-	-
Patient 6	Alcohol	Bleeding	7					
				r time	-	+	-	-
				k time	-	+	-	-

				alpha angle	-	+	-	-
				ma	-	-	-	-
Patient 7	Alcohol	Bleeding	9					
				r time	-	+	-	
				k time	-	-	-	
				alpha angle	-	+	-	
				ma	-	-	-	
Patient 8	Alcohol	Ascites	7					
				r time	+	-	-	-
				k time	-	+	-	-
				alpha angle	-	+	-	-
				ma	-	-	-	-
Patient 9	Budd Chiari	Budd Chiari	6					
				r time	-	+	-	
				k time	-	+	-	
				alpha angle	-	+	-	
				ma	-	-	-	
Patient 10	Budd Chiari	Budd Chiari	10					
				r time	-	+	-	
				k time	-	+	-	
				alpha angle	-	+	-	
				ma	-	-	-	

Table 12: Summary of findings

Primer	Sequence	Amplified gene	Gene product	Amplicon size (in bp)	Detected bacteria
BR1	GGACTACCAGGGTATCTAAT AGAGTTTGATCCTGGCT (See Ref. 9)	16S rDNA gene	Ribosomic DNA 16S	804	All
EntB	TGAATCACAAAGTGGTAAGCG TGGGGATGACGTCAAGTCAT	*hly*	Haemolysin	300	Enterobacteriaceae
Eco	ATCATGGAAGTAAGACTGC TTGCTGTGCCAGGCAGTTT	*uidA*	β glucuronidase	356	*E. coli*
pbp5	CATGAGCAATTAATCGG CATAGCCTGTCGCAAAAC	*pbp5*	Penicillin binding protein 5	444	*E. faecalis*

Table 13: Characteristics of the primers used in this study

Age (mean±2*S.D.)	57.7±25.1
Sex (M/F)	6/1
Aetiology	
Alcohol	3
HCV	2
HBV	1
HCV+Alcohol	1
Ascites (present/absent)	4/3
Child class (A/B/C)	4/3/0
Child score (mean±2*S.D.)	6.7±3.8
Diuretics (yes/no)	4/3
Varices (yes/no)	6/1

Table 14: Demographics of the patients studied

Albumin (mean±2*S.D.)	34.3±14.6 g/L
Bilirubin (mean±2*S.D.)	1.9±2.8 mg/dL
INR (mean±2*S.D.)	1.2±0.3
Haemoglobin (mean±2*S.D.)	13.6±3.4 g/dL
White cell count (mean±2*S.D.)	6050±3958 per mm^3
Platelets (mean±2*S.D.)	124000±43000 per mm^3
Creatinine (mean±2*S.D.)	0.86±0.37 mg/dL
Sodium (mean±2*S.D.)	139±4 mmol/L
AST (mean±2*S.D.)	122±115 IU/L
ALT (mean±2*S.D.)	101±171 IU/L
Urinary sodium (mean±2*S.D.)	92±72 mmol/24 hours
Mean arterial blood pressure (mean±2*S.D.)	92±10 mm Hg

Table 15: Baseline values. INR: International normalised ratio, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase

Patient	Aetiology	Ascites	DNA in serum or ascites	Transit time	Permeability	Bacterial overgrowth
1	HCV	Yes	No	Decreased	Increased	No
2	HCV + Alcohol	No	No	Normal	Increased	No
3	HCV	Yes	Yes	Decreased	Increased	No
4	Alcohol	Yes	No	Decreased	Increased	No
5	HBV	No	No	Decreased	Increased	Yes
6	Alcohol	No	No	Decreased	Increased	No
7	Alcohol	Yes	No	Decreased	Increased	No

Table 16: Summary of findings

Patient	Urine volume (mL)	Urinary mannitol (mmol/L)	Urinary lactulose (mmol/L)	%M	%L	%L/%M
1	590	4.9	0.25	10.5	0.5	0.047
2	530	4.8	0.31	9.42	0.56	0.059
3	150	11.1	0.866	6.1	0.44	0.072
4	230	21.01	1.2	17.7	0.94	0.053
5	320	6.5	0.24	7.6	0.26	0.036
6	140	16.1	0.72	8.2	0.35	0.042
7	200	12.1	0.36	8.8	0.24	0.027

Table 17: Excretion of mannitol and lactulose in patients with cirrhosis. %M: percentage of mannitol excreted; %L: percentage of lactulose excreted; %L/%M: excrection ratio.

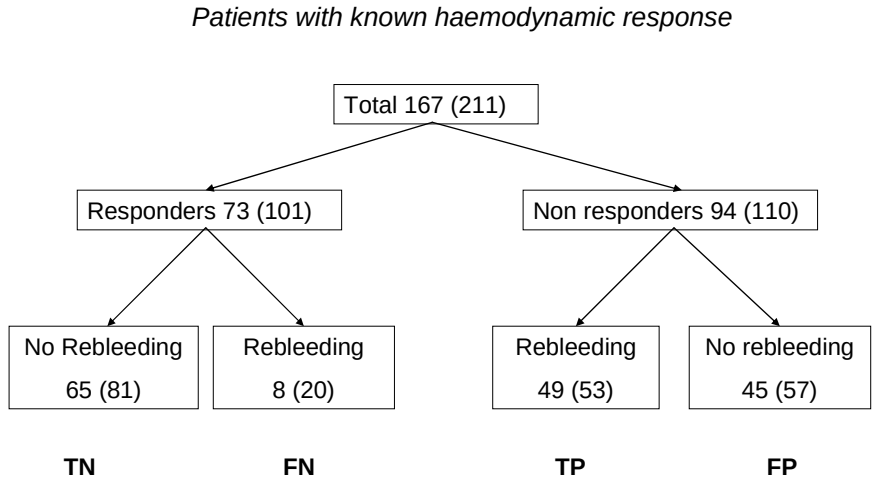
Author	*Year*	*Patients*	*Aetiology*	*ALD*	*Cirrhosis*	*Method of*	**Method of PVP**
		n		*%*	*%*	*WHVP*	
Reynolds (16)	1955	19	ALD	100	100	wedge	Direct portal vein catheterization
Joly (22)	1968	19	n/a	n/a	100	wedge	Umbilical vein catheterization
Reynolds (11)	1970	22	ALD, Non ALD	73	<73	wedge	Umbilical vein catherization
Viallet (6)	1970	43	ALD, Non ALD	93	100	wedge	Umbilical vein catherization
Boyer (7)	1977	78	ALD, Non ALD	58	n/a	wedge	Percutaneous transhepatic puncture
Okuda (15)	1977	13	n/a	n/a	100	n/a	Percutaneous transhepatic puncture
Valla (12)	1984	7	ALD	100	100	wedge	Percutaneous transhepatic puncture
Rector (10)	1985	6	ALD	100	100	wedge	Percutaneous transhepatic puncture
Lin (13)	1989	21	HBV	0	100	balloon	Percutaneous transhepatic puncture
Deplano (14)	1999	21	HCV	0	100	balloon	Percutaneous transhepatic puncture
Perello (8)	1999	71	ALD, HCV	55	89	balloon	Percutaneous transhepatic and transjugular puncture

ALD – Alcoholic liver disease; Non ALD – causes other than alcoholic liver disease; HBV – hepatitis B virus related liver disease; HCV – hepatitis C virus related liver disease; n/a – not available

Table 18: Studies comparing portal and wedge pressures included in the systemical evaluation

Total n of patients with known haemodynamic response	167 (211)
Haemodynamic responders	73 (101)
% Rebleeding	10.9% (19.8%)
Haemodynamic non responders	94 (110)
% Rebleeding	52.1% (48.2%)
Patients rebleeding	57 (73)
% Non responders	85.9% (72.6%)
Patients not rebleeding	110 (138)
% Non responders	40.9% (41.3%)

Table 19: Breakdown according to haemodynamic response and rebleeding of patients with known haemodynamic response in 5 trials for prevention of variceal rebleeding (360-364). In parentheses the same data including the outlier study (361)



Positive predictive value (PPV): 52.1 % (48.1 %)

Negative predictive value (NPV): 89 % (80.2 %)

Table 20: Positive predictive value (PPV) and negative predictive value (NPV) of haemodynamic non-response for variceal rebleeding. In parentheses the same data including the outlier study (361). TN: True negatives, FN: False negatives, FP: False positives, TP: True positives

Effects of non-selective beta blockers

Mechanism	Comment
Reduction of HVPG by ≥20% or to ≤12 mm Hg	By definition only in responders
Reduction of HVPG by <20%	Possible in non responders
Reduction of azygos blood flow/collateral blood flow	Both in responders and non responders
Reduction of variceal pressure	Both in responders and non responders, but greater in responders
Reduction of bacterial translocation	Both in responders and non responders

Table 21: Different protective effects of non-selective beta blockers in haemodynamic responders and non responders

BLACK, WHITE & GREY

BLACK: Haemodynamic non responders who bleed or rebleed from varices (? and haemodynamic responders who bleed or rebleed from varices)

WHITE: Haemodynamic responders who do not bleed or rebleed

GREY: Haemodynamic non responders who do not bleed or rebleed

Table 22: Three different patient populations as identified by response to non-selective beta blockers and risk of variceal haemorrhage

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