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Pathophysiological role of prostanoids in coagulation of the portal venous system in liver cirrhosis

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Abstract

Background

Prostanoids are important regulators of platelet aggregation and thrombotic arterial diseases. Their involvement in the development of portal vein thrombosis, frequent in decompensated liver cirrhosis, is still not investigated.

Methods

Therefore, we used pro-thrombotic venous milieu generation by bare metal stent transjugal intrahepatic portosystemic shunt insertion, to study the role of prostanoids in decompensated liver cirrhosis. Here, 89 patients receiving transjugular intrahepatic portosystemic shunt insertion were included in the study, and baseline levels of thromboxane B₂, prostaglandin D₂ and prostaglandin E₂ were measured in the portal and the hepatic vein.

Results

While the hepatic vein contained higher levels of thromboxane B₂ than the portal vein, levels of prostaglandin E₂ and D₂ were higher in the portal vein (all P<0.0001). Baseline concentrations of thromboxane B₂ in the portal vein were independently associated with an increase of portal hepatic venous pressure gradient during short term follow-up, as an indirect sign of thrombogenic potential (multivariable P = 0.004). Moreover, severity of liver disease was
inversely correlated with portal as well as hepatic vein levels of prostaglandin D₂ and E₂ (all P<0.0001).

Conclusions

Elevated portal venous thromboxane B₂ concentrations are possibly associated with the extent of thrombogenic potential in patients with decompensated liver cirrhosis.

Trial registration

ClinicalTrials.gov identifier: NCT03584204.

Introduction

Cirrhosis has been shown to be a pro- as well as an anticoagulatory condition[1]. Portal vein thrombosis (PVT), in particular, has been described as a sign of progression in disease stage [2]. However, factors related to PVT in cirrhosis have not been elucidated in detail.

Prostanoids are important regulators of the vascular tone, platelet aggregation and vascular remodeling. For biosynthesis of certain prostanoids, arachidonic acid is converted by cyclooxygenases (COXs) to prostaglandin (PG) G₂ and subsequently to PGH₂[3]. In humans, there are two COX isoforms: COX-1, expressed constitutively, and COX-2, which is inducible. PGH₂ is subsequently converted into the prostanoids and thromboxane (TX) depending on specific synthases differentially expressed in each cell type. For example, TXA synthase (TXAS) is mainly expressed in platelets which produce TXA₂, a lipid mediator that promotes proliferation of vascular smooth muscle cells and their constriction, platelet activation and aggregation, and finally thrombosis[4]. Importantly, COX-2 and TXAS are induced by various inflammatory stimuli, resulting in a close relationship between inflammation and regulation of the vascular tone and platelet function[5]. Hence, increased production and activity of TXA₂ is known to contribute to the pathogenesis of diverse thrombotic arterial diseases, such as stroke or coronary heart disease[6,7]. However, little is known about the role of TXA₂ in the portal vein and thrombosis in the portal compartment. One reason for this lack of information is that access to this compartment is difficult. Insertion of transjugular intrahepatic portosystemic stent shunt (TIPS) provides an opportunity to access the portal compartment. According to current guidelines, the major indications for TIPS insertion are treatment and prevention of variceal bleeding and/or intractable or refractory ascites[8,9]. Despite the development of polytetrafluoroethylene (PTFE) covered stents, up to 15% of patients suffer from TIPS dysfunction within the first two years[10]. Especially before the use of PTFE stents, bare metal stent insertion was associated with early TIPS dysfunction, mainly due to pro-thrombotic milieu generation. We hypothesized that prostanoids may contribute to this pro-thrombotic milieu generation. Therefore, blood samples were collected at first puncture of the portal vein during TIPS insertion and certain prostanoids, namely TXB₂, a stable metabolite of TXA₂, PGD₂ and PGE₂ were measured.

Patients and methods

Patients and samples

We included eighty-nine patients with diagnosed liver cirrhosis and severe portal hypertension, undergoing TIPS insertion, in our study. Primary outcome was the increase in portal
hepatic venous pressure gradient (PHPG) after TIPS insertion as a read-out of pro-thrombotic milieu generation. All patients were treated between August 1996 and August 2003 in the University of Bonn, Department of Internal Medicine I, Germany. Patients, older than 18 years with clinical signs of liver cirrhosis and a multidisciplinary defined indication for TIPS insertion, were included in our trial. Exclusion criteria were the presence of systemic infection, hepatic encephalopathy (higher than grade I), bilirubinemia (higher than 5mg/dl) or arterial pulmonary hypertension. We used bare stents for TIPS insertion (8–10 mm Wallstent, Boston Scientific, MA, USA), as previously described[11,12]. Control angiography was performed after a mean of 14 days. Here, position and function of the stent was evaluated in patients with significant decrease in portal venous flow or TIPS flow (N = 64). The process flow was previously specified as routine in the center’s protocol to optimize TIPS function[13]. During the procedures, portal and hepatic venous pressures were invasively measured by the use of a pressure transducer system (Combitrans, Braun, Melsungen, Germany) and a multichannel monitor (Sirecust, Siemens, Germany). Per definition, the difference between the portal and hepatic venous pressure was named portal hepatic pressure gradient (PHPG). Once, the right branch of the portal vein was cannulated, we harvested blood from portal and hepatic vein in EDTA tubes (N = 89) to obtain material for the analyses of TXB$_2$, PGD$_2$ and PGE$_2$. All TIPS insertions were performed without general anesthesia. After collection of the blood, we centrifuged the samples at 3000 revolutions per minute for 15 minutes at 4°C. Afterwards, plasma samples were stored at minus 80°C. Clinical patient data were recorded at their admission to the study. For the analyses of common blood parameters (e.g. creatinine or bilirubin levels), we used standard biochemical tests. The study protocol passed and has been approved by the local ethics committee of the University of Bonn (029/13). All patients signed and agreed to all procedures as declared in the study protocol. The final manuscript was reviewed by all authors. Access to the study data was ensured and all authors approved the final manuscript.

**Liquid chromatography mass spectrometry for prostanoid analysis**

Two hundred µL plasma was porcupined with isotopically labeled internal standards (TXB$_2$-d4, PGD$_2$-d4, PGE$_2$-d4), 100 µL EDTA solution (0.15M) and 600 µL ethyl acetate, to quantify levels of thromboxane B$_2$, prostaglandin D$_2$ and E$_2$. Specimens were vortexed, and subsequently centrifuged at 20.000 g for 15 minutes. We removed the organic phase and rerun the extraction by the addition of 600 µL ethyl acetate. After combination of the organic fractions, a sparing stream of nitrogen was used for evaporation at a temperature of 45°C. Reconstitution of the residues was performed by the addition of 50 µL acetonitrile/water/formic acid (20:80:0.0025, v/v/v) and transferred to glass vials. LC-MS/MS analysis was carried out using an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to the hybrid triple quadrupole linear ion trap mass spectrometer QTRAP 6500+ (Sciex, Darmstadt, Germany) equipped with Turbo V source operating in negative ESI mode. The chromatographic separation was carried out using a Synergi Hydro-RP column (150 × 2 mm, 4 µm particle size and 80 Å pore size; Phenomenex, Aschaffenburg, Germany). A gradient program was employed at a flow rate of 300 µL/min. Mobile phase A was water/formic acid (100:0.0025, v/v) and mobile phase B was acetonitrile/formic acid (100:0.0025, v/v). Separation of the analytes was performed under gradient conditions within sixteen minutes. The injection volume was 10 µL and the gradient program started with 90% A for 1 min. The mobile phase A was decreased to 60% within 1 min. After 1 min biding, the mobile phase was further decreased to 50% within 1 min and another held for 2 min. Within 2 min, mobile phase A was further decreased to 10% and held for 1 min. In the space of one minute, the initial conditions were restored. The column was re-equilibrated for 6 min. For mass spectrometric, parameters were set as follows:
Source temperature 500 °C, ion spray voltage -4500 V, curtain gas 40 psi, nebulizer gas 40 psi and turbo heater gas 60 psi. Both quadrupoles were running at unit resolution. Analyst Software 1.6.3 and MultiQuant Software 3.0.2 (both Sciex, Darmstadt, Germany) were used for analysis and quantification. The following precursor-to-product ion transitions were used for quantification: m/z 369.2 → m/z 195.0 for TXB₂, m/z 351.2 → m/z 233.3 for PGD₂ and m/z 351.2 → m/z 315.0 for PGE₂. Peak area of the corresponding internal standard was used for correction of the peak area of each analyte. Calibration curves were constructed using linear regression with 1/x² weighting. The coefficient of correlation was at least 0.99. Variations in accuracy were less than fifteen percent over the whole range of calibration, except for the lowest limit of quantification, where a variation in accuracy of twenty percent was accepted.

**Measurement of lipopolysaccharide levels**

Using a commercial enzyme-linked immunosorbent assay kit (Cusabio), lipopolysaccharide (LPS) serum levels were measured. Therefore, a specific antibody for LPS was precoated onto a microplate, and 100 μL of sample or standards was plated for 2 hours at room temperature. After incubation, samples were read at 450 nm. Values were expressed as picograms per milliliter; intra- and inter-assay coefficients of variation were 8% and 10%, respectively.

**Statistical analyses**

GraphPad Prism 5 for Windows (GraphPad Software, Inc.) or BIAS® for Windows was used for the performance of statistical analyses. Wilcoxon matched-pairs test was used for paired intra-individual comparisons, namely portal versus hepatic vein prostanoid concentrations. Group differences of unrelated groups were assessed by means of χ² contingency tables or Wilcoxon-Mann-Whitney U tests, as appropriate. Associations of outcomes with continuous variables were assessed in linear regression models. After univariate analyses, multivariate analyses were performed for significant associations using a P value >0.1. Variables with P >0.1 were discharged from the model. Survival analyses were performed using Cox proportional hazards regression analysis. Correlations were assessed by the use of Spearman/Kendall rank correlation. P values < 0.05 were considered to be statistically significant.

**Results**

**Baseline characteristics of patients**

We included eighty-nine patients with a mean age of fifty-nine, undergoing BMS TIPS insertion, in the study. Refractory ascites (N = 41 patients; 46%) was the major indication for TIPS insertion, followed by secondary prophylaxis of variceal bleeding (N = 36 patients; 40%) and treatment of hepatorenal syndrome (N = 6 patients; 7%). In six patients (7%) indication was presence of both, esophageal variceal bleeding and ascites. Development of cirrhosis was induced in most patients by alcohol abuse (51%), followed by viral hepatitis (7%) and primary biliary cirrhosis (3%). Here, patients presented a mean MELD score of twelve points (range 6–33), and most patients were classified as Child-Pugh B at the time of study inclusion. The mean survival time after TIPS insertion was 770 days. Please find the entire patients characteristics in Table 1.

**Prostanoids and coagulation in the portal vein**

Quantification of baseline TXB₂ levels during TIPS insertion revealed significantly lower concentrations in the portal than in the hepatic vein (231.2 pg/mL vs. 726.9 pg/mL; P <0.0001). In contrast, concentrations of PGD₂ (295.7 pg/mL vs. 169.4 pg/mL; P <0.0001) and of PGE₂...
(872.6 pg/mL vs. 693.4 pg/mL; P<0.0001) were significantly higher in the portal than in the hepatic vein (Fig 1, Table 1). Since albumin binding of prostanoids has been shown to reduce their bioavailability and activity in patients with liver cirrhosis[14], we assessed correlations of TXB$_2$, PGD$_2$ and PGE$_2$ with serum albumin concentrations. Here, albumin concentrations correlated inversely with PGD$_2$ and PGE$_2$ concentrations in the portal vein (P = 0.04 each), but neither with portal vein concentrations of TXB$_2$, nor with hepatic vein concentrations of TXB$_2$, PGD$_2$ or PGE$_2$. Patient age did not affect portal or hepatic vein prostanoid levels. Correlation of white blood cell count and portal vein TXB$_2$ levels revealed a significant correlation (P = 0.012). Furthermore, portal and hepatic vein levels of TXB$_2$ correlated with platelet count (PV: P = 0.014; HV: P = 0.002).
The severity of liver disease (MELD score) was inversely correlated with the portal as well as the hepatic vein levels of PGD$_2$ and PGE$_2$ (all $P < 0.0001$). Here, creatinine showed a significant inverse correlation with portal and hepatic vein levels of PGD$_2$ and PGE$_2$ and portal vein levels of TXB$_2$ (Table 2).

### Association between prostanoid concentrations and portal hepatic venous pressure gradient

The mean portal hepatic venous pressure gradient (PHPG) before TIPS insertion was 20.6 mmHg, and it decreased to a mean of 9 mmHg after TIPS insertion. At control angiography, performed in 64 patients due to significant decrease in portal venous flow or TIPS flow, mean PHPG measurement was 14.6 mmHg (Table 3). Out of 64 patients, 38 had shunt dysfunction due to partial thromboses and received angioplasty. Next, uni- and multivariable regression analysis was performed to identify predictors of PHPG increase after TIPS insertion (Table 4). Of note, baseline TXB$_2$ ($P = 0.004$) concentrations in the portal vein showed an independent correlation with PHPG increase at short time follow-up. An additional predictor of PHPG increase after TIPS insertion was baseline PHPG ($P = 0.001$). In contrast, no statistically significant association between portal vein concentrations of PGD$_2$ and PGE$_2$, or hepatic vein concentrations of TXB$_2$, PGD$_2$ and PGE$_2$ was observed.

In a subset of 17 patients, we analyzed portal vein levels of lipopolysaccharide (mean 57.4 pg/ml +/- 9.6pg/ml). Spearmen’s rank correlation did not show significant correlations between portal vein levels of lipopolysaccharide and portal vein levels of PGD$_2$ (R = -0.26; $P = 0.31$), PGE$_2$ (R = -0.23; $P = 0.37$), or TXB$_2$ (R = -0.02; $P = 0.95$).

### Table 2. Correlation of prostanoids and different parameters.

|                  | Albumin | WBC | Platelets | MELD score | Creatinine |
|------------------|---------|-----|-----------|------------|------------|
|                  | Rho P   | Rho P | Rho P | Rho P  | Rho P   |
| **Portal vein**  |         |      |          |           |           |
| TXB$_2$          | -0.3    | 0.8  | 0.27     | 0.01      | 0.26      | 0.014     |
| PGD$_2$          | -0.24   | 0.038| 0.02     | 0.88      | 0.1       | 0.36      |
| PGE$_2$          | -0.23   | 0.039| 0.004    | 0.97      | 0.06      | 0.59      |
| **Hepatic vein** |         |      |          |           |           |
| TXB$_2$          | -0.06   | 0.62 | 0.007    | 0.53      | 0.33      | 0.002     |
| PGD$_2$          | -0.2    | 0.08 | -0.04    | 0.74      | 0.03      | 0.78      |
| PGE$_2$          | -0.16   | 0.15 | -0.06    | 0.57      | 0.02      | 0.89      |

Spearman’s rank correlation test for correlation analysis. MELD: model for end-stage liver disease, WBC: white blood cell count; TXB$_2$: thromboxane B$_2$; PGD$_2$: prostaglandin D$_2$; PGE$_2$: prostaglandin E$_2$. P-values $<0.05$ were considered statistically significant.

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Predictors of survival after TIPS insertion

COX regression analysis of survival after TIPS insertion revealed baseline creatinine (P < 0.0001) concentrations as a negative predictor of survival (S1 Table). In detail, median survival of patients with baseline creatinine concentration ≤1.2 mg/dL was 236 days compared to 820 days in patients with creatinine concentration < 1.2 mg/dL (P = 0.0005)(S1 Fig). However, no significant association between prostanoid concentrations in the portal or hepatic vein and patient survival after TIPS insertion was observed.

Discussion

The major finding of the present study is that concentrations of TXB$_2$ are associated with an increase of PHPG as an indirect sign of thrombogenic potential.

Portal hypertension (PHT) is associated with sequelae, such as ascites, variceal bleeding and hepatorenal syndrome. Besides aggravation of portal hypertension due to cirrhosis, PHT can also be impaired by the development of portal vein thrombosis, which frequently occurs in cirrhosis. These mechanisms are not fully understood. To the best of our knowledge there are no

Table 3. Change of portal hepatic venous pressure gradient through TIPS-insertion over time.

| Character | Before TIPS-insertion$^1$ (n = 89) | P-value$^1$ | After TIPS insertion$^2$ (n = 89) | P-value$^2$ | Follow-up angiography$^3$ (n = 64) | P-value$^3$ |
|-----------|-----------------------------------|------------|-----------------------------------|------------|-----------------------------------|------------|
| PHPG, mean | 20.6 | <0.0001 | 9 | <0.0001 | 14.6 | <0.0001 |
| 1–10 mmHg, n | 0 |  | 60 |  | 16 |  |
| 11–16 mmHg, n | 14 |  | 25 |  | 28 |  |
| 17–20 mmHg, n | 33 |  | 3 |  | 10 |  |
| 21–25 mmHg. n | 32 |  | 1 |  | 9 |  |
| >25 mmHg. n | 10 |  | 0 |  | 1 |  |

Patient characteristics at time point 1 (baseline at insertion of TIPS), time point 2 (directly after TIPS insertion) and time point 3 (Follow-up angiography); TIPS: transjugular intrahepatic portosystemic stent shunt; PHPG: portal hepatic venous pressure gradient. P-values < 0.05 were considered statistically significant.

1 between time point 1 and 2
2 between time point 2 and 3
3 between time point 1 and 3.

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Table 4. Regression analysis of factors associated with increase of PHPG in control angiography compared to baseline after TIPS-insertion.

| | univariable model | multivariable model |
|---|------------------|-------------------|
| | β | P-value | β | P-value |
| TXB$_2$ (PV) | 0.003 | 0.004 | 0.003 | 0.004 |
| PGD$_2$ (PV) | 0.008 | 0.16 |  |
| PGE$_2$ (PV) | -0.005 | 0.08 | -0.014 | 0.15 |
| TXB$_2$ (HV) | 0.0002 | 0.7 |  |
| PGD$_2$ (HV) | 0.02 | 0.2 |  |
| PGE$_2$ (HV) | -0.0003 | 0.9 |  |
| Portal hepatic venous pressure gradient | -0.54 | 0.002 | -0.56 | 0.001 |

Multivariable linear regression analysis. TXB$_2$: thromboxane B$_2$; PGD$_2$: prostaglandin D$_2$; PGE$_2$: prostaglandin E$_2$; PV: portal vein; HV: hepatic vein; P-values < 0.05 were considered statistically significant. N = 64 patients

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correlations of prevalence of thrombosis with portal pressure. Therefore, this study delivers evidence for a potential mechanism leading to portal hypertension, cirrhosis and PVT.

TXA\(_2\) is a potent vasoconstrictor and a major activator of platelets synthesized by TXAS from PGH\(_2\), a downstream metabolite of arachidonic acid which is preferentially catalyzed by constitutively expressed COX-1. In turn, TXA\(_2\) is highly unstable and rapidly converted into its inactive hydrolysis product TXB\(_2\), which is a good surrogate marker of TXAS activity and TXA\(_2\) levels. The observation of higher TXB\(_2\) concentrations in the hepatic vein compared to the portal vein might be explained by the abundance of COX-1 and COX-2 in liver sinusoidal epithelial cells, which was observed in an animal model of liver cirrhosis\[15\]. Moreover, high levels of phospholipase A2, an enzyme needed to catalyze the release of arachidonic acid from membrane phospholipids, were observed in the cirrhotic liver of rats\[16,17\]. However, the experimental evidence about inducible TXB\(_2\) release by Kupffer cells in fibrosis with a consequent increase of portal pressure\[18\] supports our recent finding of elevated TXB\(_2\) levels in the human hepatic vein in decompensated liver cirrhosis. At least in animal models, the distribution of these enzymes results in high levels of hepatic TXB\(_2\), which is in line with our finding of a post-hepatic increase of TXB\(_2\) concentrations\[19\]. Indeed, aggregation of platelets in cirrhosis might lead to obliteration and parenchymal extinction, which is associated with portal hypertension\[20\]. This observation was confirmed by the correlation between platelets and TXB\(_2\). Also, in the portal vein, TXB\(_2\) seems to play a specific role, since it is independently associated with an increase of PHPG and correlated with the white blood count. While in our study, TXB\(_2\) concentrations in the portal vein were lower than in the hepatic vein, they must be considered as high, since significantly lower TXB\(_2\) concentrations were observed in the systemic circulation of healthy individuals (no data on portal venous TXB\(_2\) concentrations have become available to date)\[21,22\]. Another possible explanation of the TXB\(_2\) correlation with increase in PHPG is that TXB\(_2\) induced contraction of hepatic stellate cells\[23\].

Hence, the association observed here between TXB\(_2\) concentrations in the portal vein and increase in PHPG after TIPS insertion appears to be biologically plausible. Furthermore, one may speculate that high levels of TXB\(_2\) in the portal vein possibly predispose to the aggravation of PHT and TIPS dysfunction, particularly since it is clearly linked to inflammation, as suggested by the white blood count correlation.

In contrast to TXB\(_2\), concentrations of PGE\(_2\) and PGD\(_2\) were higher in the portal than in the hepatic vein. While we can only speculate on the functional basis of this observation, it is known that the enzymes generating PGD\(_2\)\[24\] and PGE\(_2\)\[25\] are widely distributed in various tissues and, in particular, in inflammatory cells. Therefore, it appears plausible that the high concentrations of PGD\(_2\) and PGE\(_2\) in the portal vein originate from bacterial translocation, a hallmark of decompensated liver cirrhosis\[26,27\]. Although PGE\(_2\) has rather minor effects on platelets, PGE\(_2\) effects on the vascular tone are the opposite of those of TXA\(_2\), as PGE\(_2\) signaling results in vasodilation\[28\]. The inverse association, even if not statistically significant, between PGE\(_2\) concentration in the portal vein and increase of PHPG after TIPS insertion further supports the potential pathophysiological relevance of our findings.

Interestingly, portal and hepatic vein levels of PGD\(_2\) and PGE\(_2\) were inversely correlated to the severity of liver diseases as determined by the MELD score. While it is known that PGD\(_2\) and PGE\(_2\) are secreted by inflammatory cells, here, we did not find a correlation with the white blood count. This can be explained either by immune paralysis due to advanced liver disease with reduced PGD\(_2\)- and PGE\(_2\) release by inflammatory cells\[29\] or the possibility that other cells are the main producers of PGD\(_2\) and PGE\(_2\) in this pathological setting.

Evaluating inhibitors of prostanoid synthesis, namely of the TXA\(_2\)-pathway, for prevention of portal vein thrombosis will be of interest here. These anti-platelet agents not only have anti-thrombotic functions in liver cirrhosis, but also anti-fibrotic and chemoprevention effects.
inhibiting hepatocellular carcinoma in animal models of liver disease and in clinical association studies[30–33]. On the one hand, non-steroidal anti-inflammatory drugs (NSAID) decrease concentrations of TXA2 by inhibition of COX and may reduce vascular tone in the liver. On the other hand, administration of NSAIDs reduces renal blood flow as well as the glomerular filtration rate in cirrhotic patients with ascites and induces renal failure[34–36]. Moreover, the equilibrium between thrombosis and bleeding is very unstable in liver cirrhosis. Therefore, one should be very careful with systemic drugs in cirrhosis, especially with COX inhibition. Therefore, systemic administration of COX inhibitors is obsolete in cirrhosis and ascites[9]. Since in animal models, COX inhibition seems to be useful in terms of portal hypertension, this liver-specific approach may be considered, as previously shown for Rho-kinase inhibition[37].

Our study has limitations. We were unable to include a control group of healthy individuals, because no medical interventions allowing for withdrawal of portal or hepatic venous blood were performed in these individuals. Unfortunately, the comparison of peripheral prostanoid levels of healthy controls, compensated and decompensated cirrhotic patients was not focus of this study. Furthermore, we quantified total prostanoid concentrations in the knowledge that albumin binding may also play a role in their bioavailability and activity, in particular in patients with decompensated liver cirrhosis[14]. However, similar results were found when PGD2 and PGE2 were normalized to the respective albumin levels in our study. Moreover, we focused only on the stent thrombosis and other territories were not evaluated. Finally, we can’t exclude the role of the bile leakage on the generation of the thrombogenic milieu and a possible interaction with the prostanoids. Future studies may address all these limitations.

Conclusion

In conclusion, elevated portal venous TXB2 concentrations are possibly associated with the extent of thrombogenic potential in patients with decompensated liver cirrhosis and offer suitable targets for future therapies.

Supporting information

S1 Table. Cox regression analysis of factors predicting mortality of patients receiving TIPS.
(DOCX)

S1 Fig. Survival after TIPS insertion in dependency of baseline serum creatinine levels.
(TIF)

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