# Biomarkers of liver dysfunction correlate with a prothrombotic and not with a prohaemorrhagic profile in patients with cirrhosis


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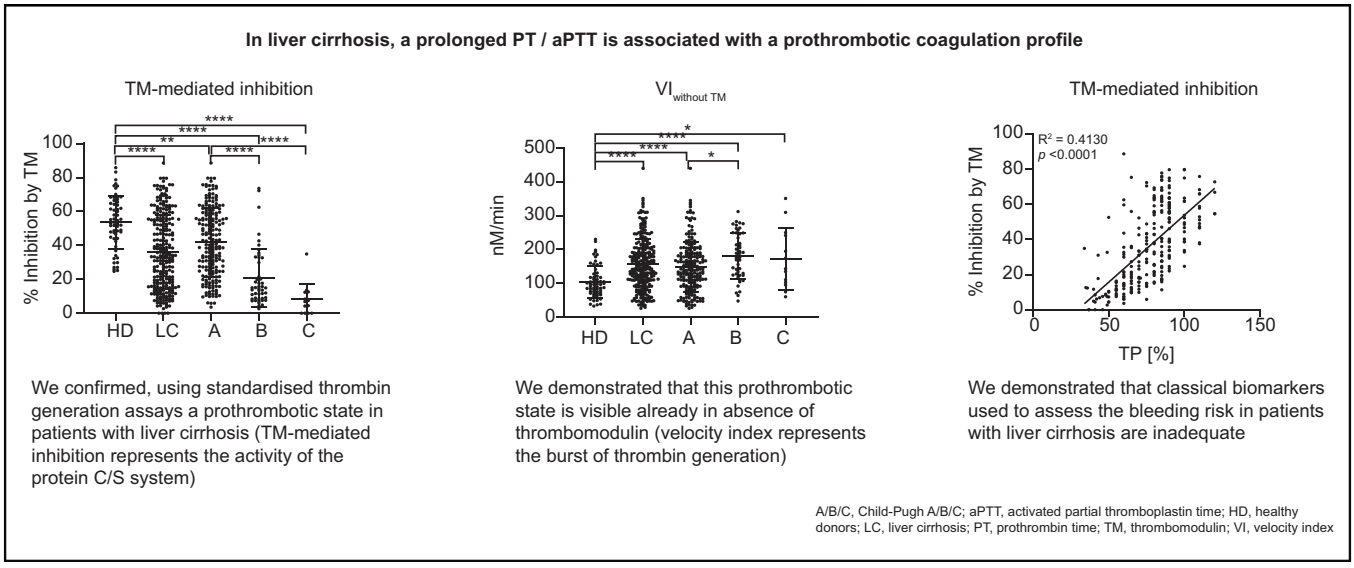
## Graphical abstract




## Highlights

- Patients with cirrhosis display a prothrombotic coagulation profile.
- This is due to a relative decrease of natural anti-coagulants compared with procoagulants.
- In cirrhosis, PT and aPTT correlate with a pro-thrombotic state, and are inadequate as bleeding risk biomarkers.

## Lay summary

We demonstrate that the laboratory parameters used to assess bleeding risk of patients with liver disease, *e.g.* prothrombin time/international normalised ratio (PT/INR) and activated partial thromboplastin time (aPTT), are inadequate for this purpose because they are correlated with a prothrombotic coagulation profile. In this article, we highlight the need for alternative parameters to assess bleeding risk in patients with liver disease.

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Research article

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# Biomarkers of liver dysfunction correlate with a prothrombotic and not with a prohaemorrhagic profile in patients with cirrhosis

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**Background & Aims:** Different liver dysfunction biomarkers are used to assess the bleeding risk of patients with cirrhosis, either as such or included in bleeding risk assessment scores. Since the current model of coagulation in patients with cirrhosis describes a procoagulant tendency with increasing severity according to Child-Pugh stage, we decided to investigate the relation between liver dysfunction biomarkers and thrombin generation. Our aim was to verify their adequacy for bleeding risk assessment.

**Methods:** We performed a prospective single-centre study including 260 patients with liver cirrhosis. Thrombin generation was measured using ST Genesia® Thrombin Generation System without and with thrombomodulin in order to assess the role of proteins C and S. Relations between thrombin generation and Child-Pugh/model for end-stage liver disease (MELD) scores, prothrombin time (PT)/international normalised ratio (INR), activated partial thromboplastin time (aPTT), factor V activity, albumin, and total bilirubin were assessed.

**Results:** Thrombomodulin-mediated inhibition of thrombin generation was significantly decreased in patients with liver cirrhosis compared with healthy donors ($p$ <0.0001) and in Child-Pugh B and C compared with A ($p$ <0.0001 [A–B], 0.4515 [B–C], <0.0001 [A–C]). Thrombomodulin-mediated inhibition significantly decreased with increasing PT/INR, aPTT, and total bilirubin levels and with decreasing factor V activity and albumin levels.

**Conclusions:** Worsening liver dysfunction biomarkers reflect an increasing prothrombotic profile in patients with liver cirrhosis. In particular, prolonged PT/INR and aPTT as well as decreasing factor V activity are related to an increasing thrombotic risk and not to an increasing bleeding risk. These parameters should not be used to assess bleeding risk due to haemostatic anomalies in patients with liver cirrhosis. Alternative biomarkers for bleeding risk assessment in patients with liver cirrhosis need to be developed.



## Introduction

For a long time, patients with liver cirrhosis were considered to have a bleeding diathesis. This was due to prolonged routine coagulation assays, *i.e.* prothrombin time (PT)/international normalised ratio (INR) and activated partial thromboplastin time (aPTT). However, in the last 2 decades, *in vitro* evidence has accumulated in favour of a procoagulant shift in patients with liver cirrhosis.[1–7] Indeed, the coagulopathy of liver cirrhosis is complex and involves prohaemorrhagic and prothrombotic changes. The main prohaemorrhagic changes include decreased coagulation factors (with the exception of factor VIII) and possible dysfibrinogenemia. The main prothrombotic changes include increased factor VIII and decreased natural anticoagulants, *i.e.* proteins C/S and antithrombin.[8] Routine coagulation assays, such as PT or aPTT, are not sensitive to the changes occurring in natural anticoagulants and they only assess the reduced levels of coagulation factors. In contrast, thrombin generation assays without and with thrombomodulin (TM) or Protac®, as protein C activators, can evaluate both prohaemorrhagic and prothrombotic changes. These assays showed an increased endogenous thrombin potential (ETP) when using TM,[4,5,7] an increased ratio of ETP with and without TM or Protac,[2,3,5–7] and an increased velocity index[7] in patients with liver cirrhosis compared with normal subjects. These characteristics have been shown to be markers of thrombotic risk in patients

Keywords: Liver cirrhosis; Thrombosis; Blood coagulation tests; Serum albumin; Bilirubin; Coagulation.

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with cirrhosis[9] as well as in other populations.[10–12] Therefore, *in vitro* analyses showed that patients with liver cirrhosis generally display a prothrombotic coagulation state, an observation that is in line with recent clinical studies.[13]

This prothrombotic state seems to increase with the severity of liver dysfunction assessed by Child-Pugh[2,5] or MELD scores[4,6] (which include PT/INR), but also with increasing INR.[7] However, in clinical settings, patients with liver cirrhosis are still broadly considered as having a bleeding diathesis, and increasing PT/INR and aPTT continue to be widely used as bleeding risk assessment parameters in patients with liver cirrhosis.[14,15] Moreover, several bleeding risk assessment scores include an abnormal liver function, which is differently defined by the various scores: increasing PT/INR,[16] biochemical evidence of liver dysfunction (HAS-BLED score; liver cirrhosis or increased bilirubin and transaminases)[17] or not specified (HEMORR2AGES score).[18] This contradicts the accumulating evidence of a procoagulant profile in patients with liver cirrhosis, which worsens in parallel to cirrhosis severity. However, to date, there are no published data regarding the alteration of the haemostatic potential of patients with liver cirrhosis depending on liver dysfunction biomarkers, including PT/INR, aPTT, factor V, albumin, and total bilirubin.

In this study, we aimed to assess the relation between liver cirrhosis stage, liver dysfunction biomarkers, and thrombin generation. This should determine the adequacy of these biomarkers for bleeding risk assessment.

## Patients and methods

### Study design

We performed a prospective single-centre study at the Division of Haematology and Central Haematology Laboratory as well as at the Division of Gastroenterology and Hepatology, Lausanne University Hospital (CHUV), Lausanne, Switzerland.

### Patients and study procedure

We prospectively included 260 patients older than 18 years with a diagnosis of liver cirrhosis Child-Pugh A–C of any aetiology as assessed by experienced gastroenterologists and hepatologists, between 22 January 2019 and 29 February 2020. Pregnant women, patients under anticoagulation, and patients unable to provide informed consent were excluded. Of note, patients with a history of splanchnic vein thrombosis and other venous thromboembolic events or cancer were not excluded. Additional blood was drawn during routine blood collection. Blood for thrombin generation assay was drawn into S-Monovettes® (Sarstedt AG & Co. KG, Nümbrecht, Germany) containing 0.106 mmol/L trisodium citrate as anticoagulant. Platelet-free plasma (PFP) was obtained by double centrifugation at 1,800× *g* for 10 min and immediately stored at −80°C. The following liver dysfunction biomarkers were analysed: PT, INR, aPTT, factor V activity, total bilirubin, and albumin.

We included 59 healthy donors, who had been recruited for the internal validation of ST Genesia® Thrombin Generation System (Stago, Asnières-sur-Seine, France). The project was presented to the patients during a scheduled routine control by the treating hepatologists. Clinical data of interest were filled in a standardised questionnaire by the physician in charge.

Patients who were included in this study gave their written informed consent. The study was approved by the local Ethical Committee (protocol CER-VD 2018-02157) and conducted according to the Declaration of Helsinki.[19]

### Laboratory assays

PT/INR, aPTT, and factor V activity were analysed on a Sysmex CS5100 coagulation analyser (Siemens Healthcare, Erlangen, Germany) with the following reagents: Dade®-Innovin®, Pathromtin SL, human standard plasma, factor V deficient plasma, and calcium chloride (Siemens Healthcare).

Albumin and total bilirubin levels were analysed on a Cobas 8000 (Roche Diagnostics International Ltd, Rotkreuz, Switzerland) using Albumin Gen. 2 (Roche Diagnostics International Ltd) and Bilirubin Total Gen. 3 (Roche Diagnostics International Ltd), respectively.

### Automated thrombin generation assay

Thrombin generation was analysed using the ST Genesia® Thrombin Generation System according to the manufacturer instructions. Analyses were performed on thawed citrated PFP. PFP was thawed for 10 min in a water bath at 37°C. Calibration was performed every day to correct for the inner filter effect, the effect of α2-macroglobulin, and the substrate consumption.[20] FluoSet (Stago) was used for plasma-specific calibration for colour. The initiator (ThromboScreen [Stago; intermediate tissue factor concentration] with and without TM) was automatically added to plasma and incubated for 10 min. After this incubation, the coagulation was triggered by the automatic addition of FluoStart (Stago) and the measurement was started. Analyses were performed without and with TM in duplicate. Corn trypsin inhibitor was not used as inhibitor of contact activation because the tissue factor concentration was >1 pM.[21–23]

The following parameters without and with TM were measured: time until thrombin generation (lag time, min), maximal concentration of thrombin (peak height$_{\text{without TM}}$, peak height$_{\text{with TM}}$, nM), thrombin generation velocity (velocity index$_{\text{without TM}}$, velocity index$_{\text{with TM}}$, nM/min), and area under the curve for total thrombin generation (ETP$_{\text{without TM}}$, ETP$_{\text{with TM}}$, nM min). The software calculated the TM-mediated inhibition as follows:

$$\frac{\text{ETP}_{\text{without TM}} - \text{ETP}_{\text{with TM}}}{\text{ETP}_{\text{without TM}}}$$

For the other parameters, we calculated ratios of values with and without TM (TM ratios).

Normalisation of the results using a reference plasma (RefPlasma) was performed by the ST Genesia software.

TM-mediated inhibition is the most frequently used parameter in the literature for patients with liver cirrhosis and the most interesting one in case of protein C/S-system disturbances. The velocity index$_{\text{without TM}}$ showed a prothrombotic haemostatic profile in patients with liver cirrhosis already without TM[7] and therefore reflects prothrombotic changes that are independent of the protein C/S system.

### Statistics

Comparisons of thrombin generation parameters (ETP$_{\text{without TM}}$, ETP$_{\text{with TM}}$, TM-mediated inhibition, peak height$_{\text{without TM}}$, peak height$_{\text{with TM}}$, velocity index$_{\text{without TM}}$, velocity index$_{\text{with TM}}$, and TM ratios for the peak height and the velocity index) between healthy donors and patients with liver cirrhosis were assessed graphically and compared using *t* tests for parameters with

**Table 1. Clinical and laboratory characteristics of healthy donors and patients.**

| | Number (%) or mean (range) | |
|---|---|---|
| | Healthy donors (n = 59) | Patients with cirrhosis (n = 260) |
| Age, years | 34 (19–63) | 58 (18–81) |
| Sex | | |
| Male | 33 (55.9) | 209 (80.7) |
| Female | 26 (44.1) | 51 (19.6) |
| BMI, kg/m$^2$ | n.a. | 27.7 (14.5–46.7) |
| Cancer | n.a. | 50 (19.2) |
| Hepatocellular carcinoma | n.a. | 41 |
| Child-Pugh A | n.a. | 33 (16) |
| Child-Pugh B | n.a. | 5 (10) |
| Child-Pugh C | n.a. | 3 (23) |
| Other gastrointestinal cancers | n.a. | 1 |
| Lung cancer | n.a. | 3 |
| Others | n.a. | 5 |
| History of thromboembolic events | n.a. | 24 (9.2%) |
| Splanchnic vein thrombosis | n.a. | 16 (6.2%) |
| Other VTE events | n.a. | 8 (3.1%) |
| Aetiology | | |
| Alcohol | n.a. | 89 (34.2) |
| Viral hepatitis | n.a. | 53 (20.4) |
| HCV | n.a. | 37 (14.2) |
| HBV | n.a. | 14 (5.4) |
| Combined (HBV + HCV) | n.a. | 2 (0.8) |
| Alcohol + NASH | n.a. | 41 (15.8) |
| Alcohol + viral hepatitis | n.a. | 22 (8.5) |
| NASH | n.a. | 22 (8.5) |
| Autoimmune liver disease/others | n.a. | 22 (8.5) |
| Alcohol + NASH + viral hepatitis | n.a. | 8 (3.1) |
| NASH + viral hepatitis | n.a. | 3 (1.2) |
| Child–Pugh | n.a. | 5.9 (5–12) |
| A | n.a. | 200 (77.0) |
| B | n.a. | 47 (18.1) |
| C | n.a. | 13 (5) |
| MELD score | n.a. | 9.7 (6–29) |
| MELD-Na score | n.a. | 10.7 (6–30) |
| Albumin substitution in the previous month | n.a. | 13 (5%) |
| Routine assays | | |
| Prothrombin time, % | n.a. | 77.2 (34.0–120.0) |
| INR | n.a. | 1.2 (1.0–2.0) |
| aPTT, seconds | n.a. | 35.0 (25.0–62.0) |
| Factor V activity, % | n.a. | 81.3 (23–185) |
| Albumin, g/L | n.a. | 40.4 (18.0–57.0) |
| Total bilirubin, μmol/L | n.a. | 23.6 (3.0–382) |
| Fibrinogen, g/L | n.a. | 2.7 (0.9–9.1) |
| Thrombin generation | | |
| Lag time$_{\text{without TM}}$, min | 2.4 (1.7–3.7) | 2.3 (1.0–5.8) |
| Lag time$_{\text{without TM}}$ norm., ratio | 1.4 (0.7–1.9) | 1.2 (0.6–3.0) |
| Lag time$_{\text{with TM}}$, min | 2.7 (1.8–4.2) | 2.6 (1.1–7.2) |
| Peak height$_{\text{without TM}}$, nM | 200.0 (85.2–339.2) | 219.6 (73.1–395.1) |
| Peak height$_{\text{without TM}}$ norm., % | 72.4 (31.5–120.5) | 74.3 (22.5–127.1) |
| Peak height$_{\text{with TM}}$, nM | 136.5 (31.1–291.0) | 164.8 (21.0–325.2) |
| Time to peak$_{\text{without TM}}$, min | 5.2 (3.8–7.7) | 4.5 (2.6–10.0) |
| Time to peak$_{\text{without TM}}$ norm., ratio | 1.2 (0.9–1.8) | 1.2 (0.6–2.4) |
| Time to peak$_{\text{with TM}}$, min | 4.5 (3.4–6.8) | 4.4 (2.8–10.3) |
| Velocity index$_{\text{without TM}}$, nM/min | 105.0 (32.7–230.1) | 156.4 (26.0–440.0) |
| Velocity index$_{\text{without TM}}$, norm., % | 66.3 (21.3–149.7) | 84.6 (13.2–266.4) |
| Velocity index$_{\text{with TM}}$, nM/min | 107.8 (13.7–261.7) | 137.9 (8.3–351.9) |
| ETP$_{\text{without TM}}$, nM min | 1,231 (655–2029) | 1,168 (450–1892) |
| ETP$_{\text{without TM}}$ norm., % | 81.2 (43.4–125.4) | 76.5 (28.0–127.5) |
| ETP$_{\text{with TM}}$, nM min | 586 (165–1528) | 742 (123–1483) |
| TM–mediated inhibition, % | 53.7 (24.7–85.9) | 36.4 (0.0–88.6) |

(*continued on next page*)

**Table 1** (*continued*)

aPTT, activated partial thromboplastin time; ETP, endogenous thrombin potential; INR, international normalised ratio; MELD, model for end-stage liver disease; n.a., not applicable or not available; NASH, non-alcoholic steatohepatitis; norm., normalised; TM, thrombomodulin; VTE, venous thromboembolism.

normal distribution (ETP$_{\text{without TM}}$ normalised, peak height$_{\text{without TM}}$ normalised). For parameters with non-normal distribution, the Mann-Whitney test was used (ETP$_{\text{without TM}}$, ETP$_{\text{with TM}}$, TM-mediated inhibition, peak height$_{\text{without TM}}$, peak height$_{\text{with TM}}$, peak height ratio, velocity index$_{\text{without TM}}$, velocity index$_{\text{without TM normalised}}$, velocity index$_{\text{with TM}}$, velocity index ratio). Comparisons between healthy donors and Child-Pugh categories were done using Kruskall-Wallis tests with multiple comparisons. For the MELD score and liver dysfunction biomarkers, we chose to analyse absolute and normalised ETP$_{\text{without TM}}$, absolute and normalised velocity index$_{\text{without TM}}$, as well as TM-mediated inhibition (see above). Relations between MELD score and thrombin generation parameters were assessed graphically and fitted using linear regressions. Relations between PT, aPTT, factor V activity, albumin and total bilirubin and thrombin generation parameters were assessed graphically and fitted using linear regression or quadratic function as required. All statistical analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA).

## Results

Patient characteristics including age, sex, liver cirrhosis aetiology, and severity of liver dysfunction as well as results of routine laboratory tests and thrombin generation parameters are given in Table 1, which also includes the characteristics of the healthy donors.

### Thrombin generation according to Child-Pugh score

Results of thrombin generation assays are presented in Table 1 and Fig. 1. The ETP$_{\text{without TM}}$ was slightly but significantly reduced in patients with liver cirrhosis compared with healthy donors (1172 ± 240 [median ± standard deviation] nM min *vs.* 1227 ± 233 nM min; $p$ = 0.0487) (Fig. 1A). A trend towards a decrease of ETP$_{\text{without TM}}$ with increasing Child-Pugh score was observed but reached significance only when comparing Child-Pugh A with C (1198 ± 230 nM min [A] *vs.* 1065 ± 208 nM min [B] *vs.* 972 ± 333 nM min [C]; $p$ = 0.0509, >0.9999 and 0.0233 [A *vs.* B, B *vs.* C, and A *vs.* C, respectively]). When normalised, ETP$_{\text{without TM}}$ significantly decreased from Child-Pugh A to B and from B to C (78.7 ± 15.0 % [A] *vs.* 68.5 ± 12.3 % [B] *vs.* 63.8 ± 20.2 % [C]; $p$ = 0.0232, 0.5928, and 0.0034 [A *vs.* B, B *vs.* C and A *vs.* C, respectively] [Fig. 1B]). With TM, ETP was significantly increased in patients with liver cirrhosis compared with healthy donors (752 ± 287 nM min *vs.* 583 ± 268 nM min; $p$ <0.0001) and significantly increased in Child-Pugh B compared with Child-Pugh A

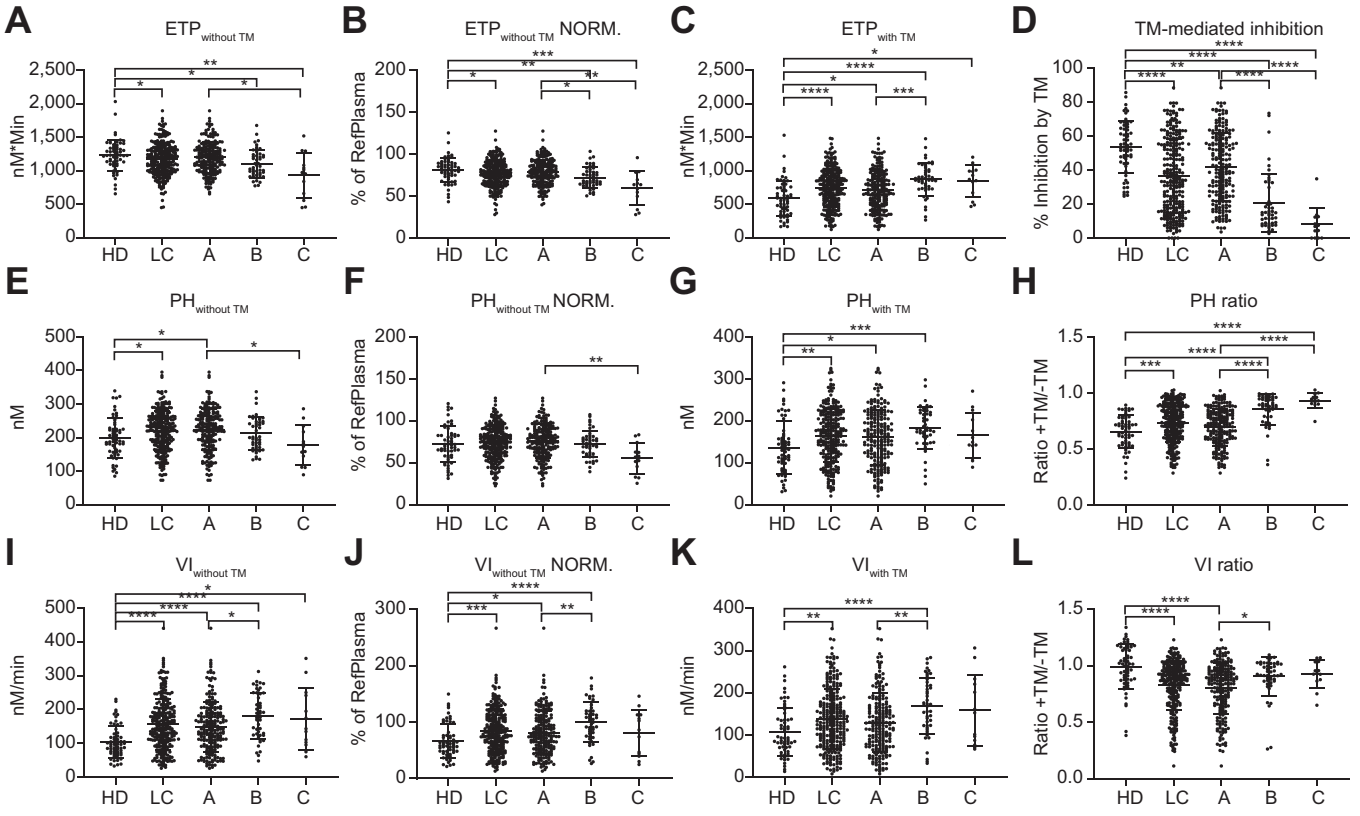


**Fig. 1. Thrombin generation in healthy individuals and patients with liver cirrhosis.** Thrombin generation parameters in healthy donors, patients with liver cirrhosis, and according to Child-Pugh stage (A/B/C). Results of *t* test, Mann-Whitney test, and ANOVA with multiple comparisons, or Kruskall-Wallis tests with multiple comparisons. Error bars represent mean and standard error. **p* = 0.01-0.05, ***p* = 0.001–0.0099, ****p* = 0.0001–0.00099, *****p* <0.0001. ETP, endogenous thrombin potential; HD, healthy donors; NORM., normalised; PH, peak height; TM, thrombomodulin; VI, velocity index.

patients (865 ± 245 nM min *vs.* 691 ± 289 nM min; $p = 0.0010$) (Fig. 1C). This resulted in a significant decrease of TM-mediated inhibition (Fig. 1D) in patients with liver cirrhosis compared with healthy donors (33.8 ± 21.8 % *vs.* 53.7 ± 15.6 %; $p < 0.0001$) and a significant decrease of TM-mediated inhibition in Child-Pugh B and C compared with A (40.5 ± 20.2 % [A] *vs.* 14.9 ± 17.1 % [B] *vs.* 6.9 ± 9.5 % [C]; $p$ <0.0001, 0.4515 and <0.0001 [A *vs.* B, B *vs.* C, and A *vs.* C, respectively]).

The peak height$_{\text{without TM}}$ was significantly increased in patients with liver cirrhosis compared with healthy donors (224.9 ± 59.7 nM *vs.* 193.6 ± 59.3 nM; $p = 0.0127$). However, it significantly decreased from Child-Pugh A to C (229.3 ± 61.1 nM [A] *vs.* 201.3 ± 49.0 nM [B] *vs.* 173.9 ± 58.9 nM [C]; $p = 0.9706$, 0.4936 and 0.0422 [A *vs.* B, B *vs.* C, and A *vs.* C, respectively]) (Fig. 1E). When normalised, no differences were observed between healthy donors and patients with liver cirrhosis (71.1 ± 21.3% *vs.* 75.0 ± 19.6, $p = 0.5147$ [Fig. 1F]). However, the decrease observed between Child-Pugh A and C was confirmed (77.4 ± 19.9 % (A) *vs.* 71.8 ± 15.9 % (B) *vs.* 56.9 ± 18.3 % (C); $p$ >0.9999, 0.0919, 0.0038 (A *vs.* B, B *vs.* C, and A *vs.* C, respectively)]. With TM, the peak height was significantly increased in patients with liver cirrhosis compared with healthy donors (168.7 ± 62.9 nM *vs.* 131.7 ± 62.7 nM; $p = 0.0011$), but no differences were noted between Child-Pugh categories (162.7 ± 65.5 nM [A] *vs.* 177.4 ± 50.5 nM [B] *vs.* 149.2 ± 54.2 nM [C]; $p = 0.1030$, >0.9999, >0.9999 [A *vs.* B, B *vs.* C and A *vs.* C]) (Fig. 1G). This resulted in a significantly increased peak height ratio in patients with liver cirrhosis compared with healthy donors (0.76 ± 0.17 *vs.* 0.69 ± 0.15; $p = 0.0004$) and in Child-Pugh B and C compared with A (0.71 ± 0.17 [A] *vs.* 0.89 ± 0.14 [B] *vs.* 0.94 ± 0.07 [C]; $p$ <0.0001, 0.7419, <0.0001 [A *vs.* B, B *vs.* C and A *vs.* C, respectively]) (Fig. 1H).

The velocity index was already significantly increased without TM in patients with liver cirrhosis compared with healthy donors (146.0 ± 74.9 nM/min *vs.* 97.1 ± 47.4 nM/min; $p$ <0.0001) (Fig. 1I). It increased significantly from Child-Pugh A to B (141.3 ± 74.1 nM/min [A] *vs.* 182.2 ± 68.2 nM/min [B] *vs.* 143.2 ± 91.9 nM/min [C]; $p = 0.0222$, >0.999, 0.999 [A *vs.* B, B *vs.* C and A *vs.* C, respectively]) as well. Using normalised data, similar results were obtained (59.9 ± 30.0 [healthy donors] *vs.* 81.6 ± 38.2 % [cirrhosis]; 75.9 ± 37.9% [A] *vs.* 102.4 ± 35.5 % [B] *vs.* 86.2 ± 40.5 % [C]; $p = 0.0003$, 0.0056, 0.5426, >0.9999 [healthy donors *vs.* patients with cirrhosis, A *vs.* B, B *vs.* C, and A *vs.* C, respectively] [Fig. 1J]). Addition of TM seems to have a greater impact on velocity index in patients with liver cirrhosis compared with healthy donors (velocity index ratio: 1.02 ± 0.20 [healthy donors] *vs.* 0.89 ± 0.22 % [cirrhosis]; 0.86 ± 0.23 [A] *vs.* 0.92 ± 0.17 % [B] *vs.* 0.93 ± 0.12 % [C]; $p$ <0.0001, 0.0179, >0.9999, 0.3180 [healthy donors *vs.* patients with liver cirrhosis, A *vs.* B, B *vs.* C, and A *vs.* C, respectively] [Fig. 1L]).

### Thrombomodulin-mediated inhibition and velocity index without thrombomodulin according to MELD scores

The results of absolute and normalised ETP$_{\text{without TM}}$, TM-mediated inhibition, and velocity index$_{\text{without TM}}$ according to MELD score are presented in Fig. 2. The absolute and normalised ETP$_{\text{without TM}}$ had an inverse linear relation to the MELD

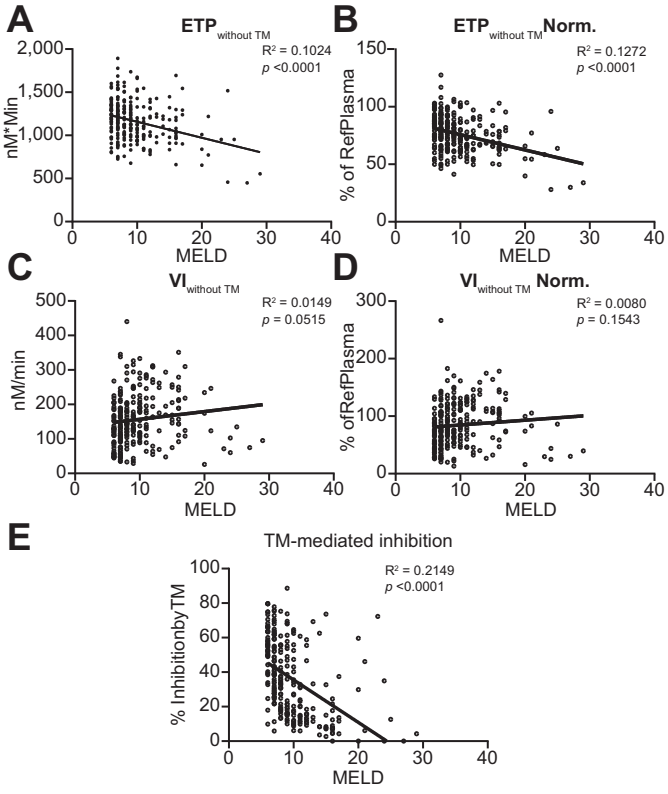


**Fig. 2. Thrombin generation according to MELD score.** TM-mediated inhibition of ETP, velocity index$_{\text{without TM}}$, and velocity index$_{\text{without TM}}$ normalised according to MELD score. Results of linear regression are presented here ($p$ values for differences of the slope than 0, $R^2$; lines represent the linear regression line). ETP, endogenous thrombin potential; MELD, model for end-stage liver disease; NORM., normalized; TM, thrombomodulin; VI, velocity index.

score that was much lower than expected (slope significantly different than 0 [$p$ <0.0001]; $R^2$ = 0.1024 and 0.1272, respectively) (Fig. 2A, B). The absolute and normalised velocity index$_{\text{without TM}}$ was not correlated with MELD score (slope not significantly different than 0 [$p$ = 0.0515 and 0.1543], $R^2$ = 0.0149 and 0.0080) (Fig. 2C and 2D). The TM-mediated inhibition (Fig. 2E) had an inverse linear relation to MELD score, which was of much better quality than for ETP$_{\text{without TM}}$ (slope very highly significantly different than 0 ($p$ <0.0001) and $R^2$ = 0.2149). This indicates an increasing prothrombotic state with increasing cirrhosis stage.

### ETP$_{\text{without TM}}$, Thrombomodulin-mediated inhibition, and velocity index according to liver dysfunction biomarkers

The results of absolute and normalised ETP$_{\text{without TM}}$, the absolute and relative velocity index$_{\text{without TM}}$, and TM-mediated inhibition according to liver dysfunction biomarkers are presented in Fig. 3. The absolute ETP$_{\text{without TM}}$ had a direct relation to TP (Fig. 3A) and factor V activity (Fig. 3D), indirect to INR (Fig. 3B), aPTT (Fig. 3C), and a quadratic relation to total bilirubin (Fig. 3F) (slopes significantly different than 0 [$p$ <0.0001, 0.0070, <0.0001, <0.0001, respectively; no $p$ value for the total bilirubin because the relation was quadratic], $R^2$ = 0.0553, 0.0321, 0.0614, 0.0660, 0.0681). No correlation was observed with albumin ($p$ = 0.1945, $R^2$ = 0.0066; Fig. 3E). Similar results were observed using normalised values (Fig. 3G–L). However, the quality of the relation between these biomarkers and ETP was much lower than expected, considering the use of these biomarkers as bleeding risk biomarkers. Moreover, the velocity index$_{\text{without TM}}$ had an inverse relation to PT (Fig. 4A), factor V activity (Fig. 4D), albumin (Fig. 4E), a direct relation to INR (Fig. 4B), aPTT (Fig. 4C), and a quadratic relation to total bilirubin (Fig. 4F) (all slopes significantly different than 0: $p$ <0.0001 [PT, factor V activity, albumin], 0.0103 [INR], 0.0002 [aPTT]; no $p$ value for total bilirubin, which was assessed by quadratic functions due to their graphical appearance, $R^2$ = 0.0767 [PT], 0.2611 [factor V activity], 0.1020 [albumin], 0.0253 [INR], 0.0577 [aPTT], 0.0989 [total bilirubin]). The results obtained with normalised values of velocity index$_{\text{without TM}}$ were very slightly better than those of absolute values (Fig. 4G–L). Therefore, the velocity index$_{\text{without TM}}$ increases with decreasing PT (expressed in percent), factor V activity as well as albumin and with increasing INR, aPTT, and total bilirubin, indicating an increasing prothrombotic state with increasing disturbances of these parameters, contradicting the results observed for ETP$_{\text{without TM}}$.

The TM-mediated inhibition had a good direct linear relation to PT (Fig. 4M), factor V activity (Fig. 4P), and albumin (Fig. 4Q) and a good inverse linear relation to INR (Fig. 4N) (all slopes very highly significantly different than 0 [$p$ <0.0001], $R^2$ = 0.4130 (PT), 0.4722 (factor V activity), 0.2915 (albumin), 0.3431 (INR)). This indicates an increasing prothrombotic state with decreasing PT, factor V activity, and albumin and with increasing INR. Due to their graphical appearances, the relations between TM-mediated inhibition and aPTT (Fig. 4O) and total bilirubin (Fig. 4R) were analysed using quadratic functions. The TM-mediated inhibition had a good quadratic relation to aPTT and total bilirubin ($R^2$ = 0.3205 and 0.2709, respectively). This indicates an increasing prothrombotic state with increasing aPTT and total bilirubin.

## Discussion

The aim of our work was to evaluate the relationship between liver dysfunction biomarkers and thrombin generation. First, using a fully automated, standardised, and normalised novel assay, we confirmed previous observations obtained with the calibrated automated thrombogram system on thrombin generation in patients with liver cirrhosis (Fig. 1). Indeed, we observed a relatively preserved thrombin generation without TM. Of note, even though ETP$_{\text{without TM}}$ was only slightly decreased and the peak height similar in patients with liver cirrhosis compared with healthy donors, these parameters tended to decrease with increasing cirrhosis severity. This could indicate a slight decrease of the procoagulant potential by itself which, however, appears to be compensated by an increased thrombin generation velocity. In presence of TM, however, we observed increased thrombin generation in patients with liver cirrhosis compared with healthy donors. This indicates the presence of an acquired protein C resistance and a prothrombotic state in these patients.[1,2,5,7] Moreover, we confirm that such a prothrombotic state increases with cirrhosis severity, as assessed by Child-Pugh[2,5] or MELD[4,6] scores (Figs. 1 and 2). For example, ETP$_{\text{with TM}}$ (Fig. 1C), the peak height ratio (Fig. 1H), and the velocity index$_{\text{without TM}}$ (Fig. 1I) increased and the TM-mediated inhibition (Fig. 1D) decreased significantly from Child-Pugh A to B. Additionally, the MELD score showed an inverse relation with TM-mediated inhibition (Fig. 2E). In sum, the novel assay employed in this study confirms a prothrombotic state in patients with liver cirrhosis, which worsens with increasing liver dysfunction.

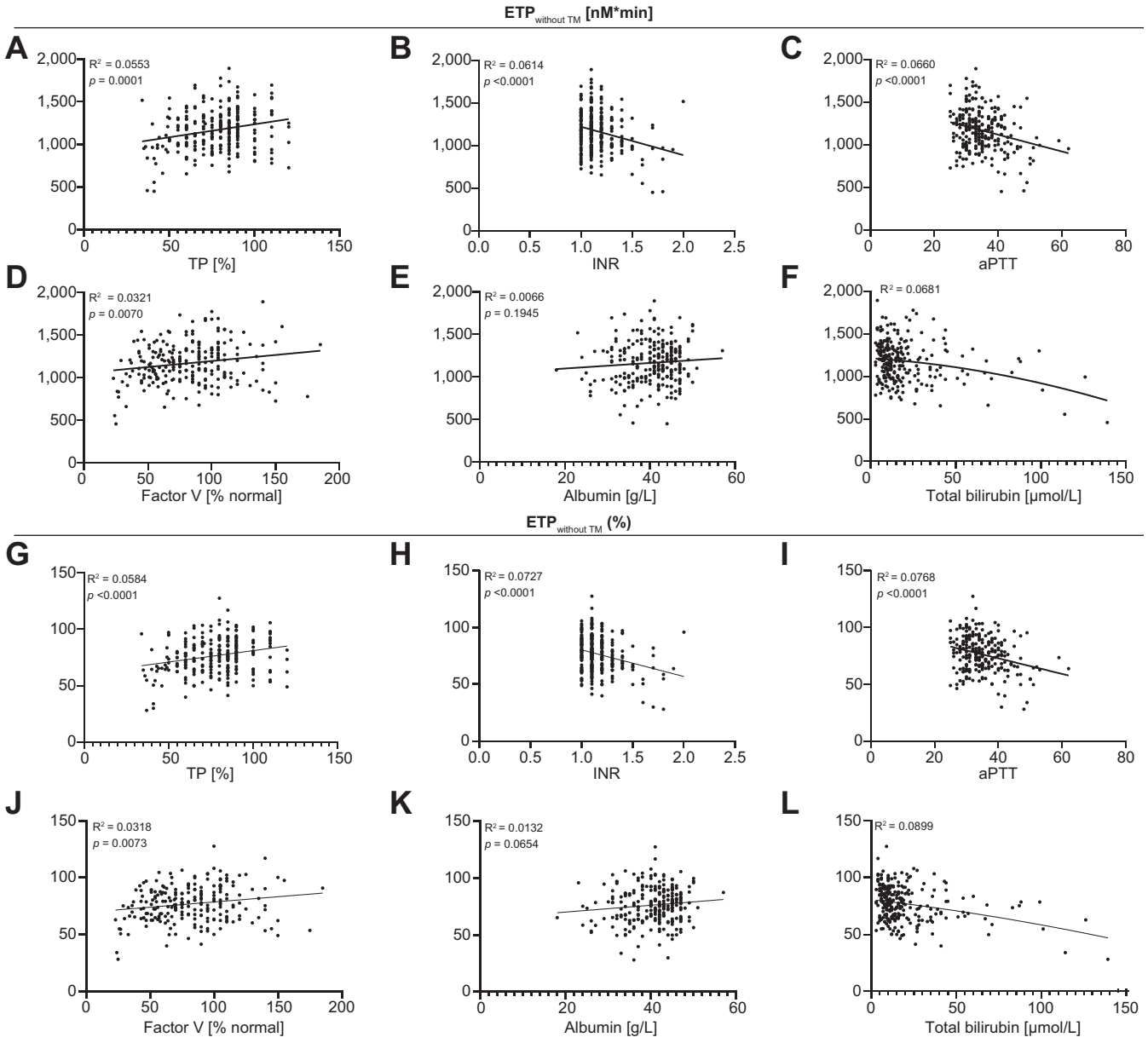


**Fig. 3. $ETP_{without\ TM}$ and normalised $ETP_{without\ TM}$ according to various biomarkers of cirrhosis severity.** $ETP_{without\ TM}$ and normalised $ETP_{without\ TM}$ according to PT, INR, aPTT, factor V activity, albumin, and total bilirubin. Results of linear regressions ($p$ values for differences of the slope than 0, $R^2$; lines represent the linear regression lines), or nonlinear regressions ($R^2$; lines represent the nonlinear regression lines). aPTT, activated partial thromboplastin time; ETP, endogenous thrombin potential; INR, international normalised ratio; NORM., normalized; PT, prothrombin time.

Although a prothrombotic shift of the haemostatic balance has been demonstrated in patients with liver cirrhosis, little is known about the relation between liver dysfunction biomarkers and the haemostatic profile, except for a single study of Gatt *et al.*[7] that reported an increasing prothrombotic profile with increasing INR. In fact, liver dysfunction biomarkers continue to be commonly considered as markers of increased bleeding risk in patients with liver cirrhosis in clinical practice.[14–18] In this study, we evaluated thrombin generation using a fully automated thrombin generation assay in 260 patients with liver cirrhosis and compared it with liver dysfunction biomarkers and cirrhosis stage.

We demonstrated that prolonged PT/INR and aPTT and decreasing factor V activity are linked to an increasing prothrombotic shift in patients with liver cirrhosis (Figs. 3 and 4). Indeed, the relation between $ETP_{without\ TM}$ and the coagulation assays was much lower than expected. Moreover, the relation between the $velocity\ index_{without\ TM}$ and the TM-mediated inhibition and these coagulation assays indicates an increasing prothrombotic state in these patients. These coagulation assays have been reported to be only poorly or not at all correlated with the occurrence of bleeding in patients with cirrhosis.[24–26] Our results explain the absence of correlation between these biomarkers and the bleeding risk. Therefore, and counterintuitively, a patient with liver cirrhosis with a prolonged PT and/or aPTT appears to be at increased risk of venous thromboembolism. This contradicts the rather widespread clinical practice, in which physicians continue to estimate the bleeding risk of

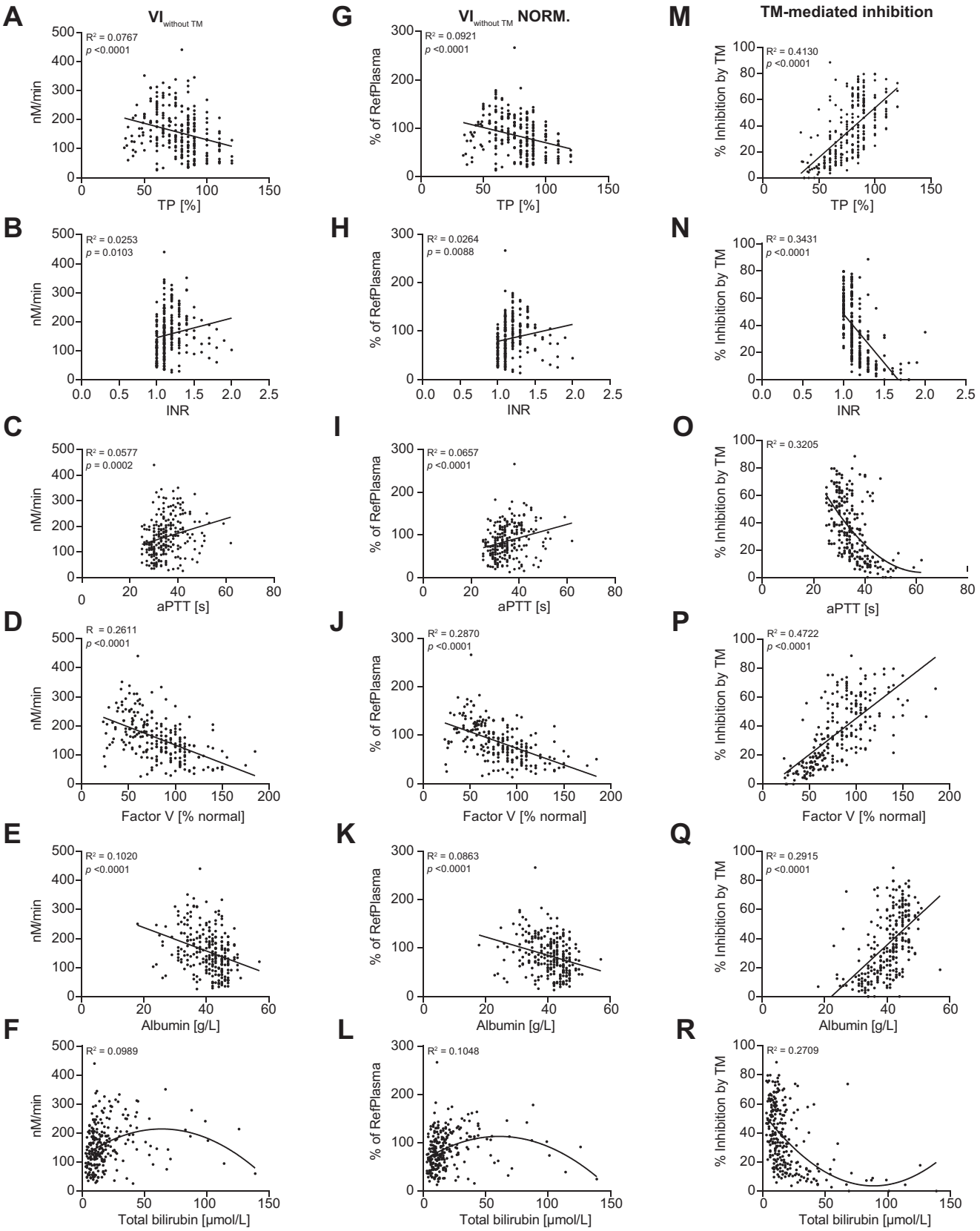


**Fig. 4. TM-mediated inhibition of ETP, velocity index$_{\text{without TM}}$ and normalised velocity index$_{\text{without TM}}$ according to various biomarkers of cirrhosis severity.** TM-mediated inhibition of ETP, velocity index$_{\text{without TM}}$, and normalised velocity index$_{\text{without TM}}$ according to PT, INR, aPTT, factor V activity, albumin, and total bilirubin. Results of linear regressions ($p$ values for differences of the slope than 0, $R^2$; lines represent the linear regression lines), or nonlinear regressions ($R^2$; lines represent the nonlinear regression lines). aPTT, activated partial thromboplastin time; ETP, endogenous thrombin potential; INR, international normalised ratio; PT, prothrombin time; VI, velocity index.

patients with liver cirrhosis using PT/INR and aPTT. This leads to a mismanagement of patients with liver cirrhosis, with withdrawal of prophylactic anticoagulation[14,15,27] or administration of unnecessary blood product transfusion,[28–30] potentially increasing their thrombotic risk and complication rate. Based on our results, we should advise physicians to stop measuring these parameters in patients with liver cirrhosis for evaluating haemorrhagic risk and refrain from correcting these laboratory parameters with prothrombotic drugs.

In this study, we also demonstrate that increasing total bilirubin is associated with an increasing prothrombotic profile (Figs. 3 and 4). Moreover, we confirm previous observations reporting an increasing prothrombotic profile with increasing cirrhosis severity[2,4–6] (see above). Similar to PT/INR and aPTT, total bilirubin and cirrhosis severity are included in several bleeding risk assessment scores.[16–18] Based on our results, the inclusion of liver dysfunction biomarkers within bleeding risk assessment scores appears inadequate and should be re-evaluated.

The presence of liver dysfunction parameters in bleeding risk assessment scores is very likely due to their indirect relation to gastrointestinal haemodynamic changes (*e.g.* portal hypertension) associated with advanced liver cirrhosis, which leads to an increased gastrointestinal bleeding risk (*e.g.* due to rupture of oesophageal varices). Indeed, the prevalence of bleeding from oesophageal varices is increased in decompensated liver disease. However, as demonstrated here, liver dysfunction parameters are in fact markers of a thrombotic risk and, therefore, are inadequate for the purpose of identifying a bleeding risk linked to haemostatic causes. Alternative biomarkers, either specific for gastrointestinal haemodynamic changes or identifying subgroups of patients with liver cirrhosis with a prohaemorrhagic profile, should be evaluated and should substitute 'classical' liver dysfunction biomarkers. This would prevent falsely highlighting the above-mentioned biomarkers as indicators of bleeding risk, eventually improving the quality of the bleeding risk assessment scores.

The observed relation between total bilirubin and a prothrombotic profile is likely an epiphenomenon related to the severity of the liver dysfunction. However, some published data support a direct association between bilirubin and hypercoagulability.[31,32] For instance, thrombin generation has been reported to increase with bilirubin levels up to a concentration of 211 μmol/L in spiking experiments.[33] All bilirubin values observed in our study were lower than this threshold. Therefore, bilirubin could play a direct role in the increasing thrombin generation detected in our population. In contrast, a protective role of bilirubin has also been described,[34] which could be due to its antioxidant effect.[35] Of note, further increase of bilirubin concentrations over 211 μmol/L in spiking experiments led to a decline of thrombin generation.[33] Based on our results and the published evidence, we postulate that the directly proportional relation between total bilirubin and prothrombotic profile observed in this study is most likely due to the correlation of total bilirubin with the degree of liver dysfunction severity in cirrhosis. However, bilirubin could also play a direct procoagulant role in the range of total bilirubin levels observed in our population.[33]

We describe here an indirect relation between albumin and a prothrombotic haemostatic profile in patients with liver cirrhosis (Figs. 3E, K and 4E, K, Q). Different studies showed that elevated INR,[36] low albumin,[36,37] and elevated bilirubin[36] are risk factors for the development of portal vein thrombosis. Our results demonstrate that this is most likely explained by the fact that alterations of PT/INR, aPTT, and albumin[36,37] reflect an intrinsic prothrombotic profile more than just altered haemodynamic factors. Thus, biomarkers reflecting liver synthetic dysfunction should be recognised as indicators of a procoagulant state in liver cirrhosis. Of note, a direct antiplatelet effect of albumin *via* the inhibition of NADPH oxidase 2 and anti-oxidative effect has been postulated.[37] An anti-oxidative effect could also influence coagulation and the prothrombotic haemostatic profile.[8] Therefore, the relation between albumin levels and procoagulant state in patients with liver cirrhosis could be based not only on its relation to cirrhosis severity but also to a direct pathophysiologic role of albumin in haemostasis.

Our study has limitations. First, we investigated only coagulation and not primary haemostasis or fibrinolysis. However, coagulation is the most important determinant of the haemostatic balance in patients with liver cirrhosis, and thrombin generation without and with TM is a well-accepted approach for assessing the coagulation potential in patients with liver cirrhosis. Of note, the platelet count seems not to be predictive for either spontaneous or post-procedural bleeding complications in patients with liver cirrhosis.[38–40] This is likely due to the 'rebalanced' primary haemostasis by hyperactive platelets[8,40] and increased von Willebrand factor levels.[8] Moreover, there is no generally accepted gold standard for the analysis of primary haemostasis and fibrinolysis in patients with liver cirrhosis. For these reasons, in the present study we focused on thrombin generation. Second, although this study on thrombin generation in patients with liver cirrhosis is, to the best of our knowledge, the largest published yet, the number of included patients was insufficient to perform reliable subgroup analyses between aetiologies. Indeed, haemostatic differences were described across the different cirrhosis aetiologies.[8] The relation between liver dysfunction biomarkers and the prothrombotic state could be different according to cirrhosis aetiology. Therefore, larger studies are required in order to identify potential differences in the relation between liver dysfunction biomarkers and thrombin generation for diverse cirrhosis aetiologies. Third, the healthy and the cirrhotic cohorts are not balanced for age and sex, with younger and more female subjects in the healthy cohort. However, the focus of this study is not the comparison between healthy donors and patients with cirrhosis, but the comparison between groups with increasing degrees of liver dysfunction. While some differences can be seen in the thrombin generation parameters between males and females of the healthy population (Fig. S1), previous studies observed no differences between sexes.[41] Moreover, a higher percentage of males in the healthy population would actually increase the differences observed between healthy subjects and patients with liver cirrhosis. No clear tendency for clinically significant changes of thrombin generation parameters with increasing age was observed in the healthy population (Fig. S2) and previous studies concluded that the changes of thrombin generation parameters with age were too weak to be of importance.[41,42] Therefore, the imbalance observed between the 2 populations studied is not expected to influence the presented results.

In conclusion, we demonstrate an increasing prothrombotic profile correlating with worsening alterations of liver dysfunction biomarkers in patients with liver cirrhosis. In particular, prolonged PT/INR and aPTT and decreased factor V activity are related to an increasing thrombotic and not bleeding risk. These

parameters should not be used to assess bleeding risk of patients with liver cirrhosis. Alternative biomarkers for bleeding risk assessment in patients with liver cirrhosis need to be identified and investigated.

## Abbreviations

aPTT, activated partial thromboplastin time; ETP, endogenous thrombin potential; INR, international normalised ratio; MELD, model for end-stage liver disease; PFP, platelet-free plasma; PT, prothrombin time; NASH, non-alcoholic steatohepatitis; TM, thrombomodulin; VTE, venous thromboembolism.

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## Conflicts of interest

The authors disclose no other relevant conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

## Authors' contributions

MGZ designed research, performed laboratory and statistical analysis, interpreted data, and wrote the paper. MF and DM helped to design research, supervised the patient recruitment, and helped to write the paper. DBC and AA helped to design research, analysed data, and helped to write the paper. LA designed research, analysed data, and wrote the paper. All authors read and approved the final paper.

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## Supplementary data

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*Author names in bold designate shared co-first authorship*

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