Hemostasis and anticoagulant therapy in liver diseases
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Preserved clot formation detected by the Thrombodynamics analyzer in patients with cirrhosis

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Abstract

Introduction: Patients with cirrhosis have substantial alterations in their hemostatic system, which are paradoxically associated with the risk of both bleeding and thrombotic complications. However, it still remains difficult to predict those risks, because results from conventional coagulation tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), do not reflect the complex hemostatic changes in these patients. More sophisticated global hemostasis tests, such as thrombin generation assays, are not standardized for routine use yet. Here we examined the spatial clot growth in plasma from patients with cirrhosis using the novel Thrombodynamics assay, which uses a fundamentally new approach to test plasma hemostatic capacity.

Materials and Methods: Thrombodynamics assays were performed in plasma from thirty-one patients with cirrhosis and twenty-five healthy controls. Results were compared to results with thrombin generation testing and PT/APTT test results.

Results: Rates of clot growth, clot size, and clot density from the Thrombodynamics assay were comparable between patients and controls. Thrombin generation in the presence of thrombomodulin was increased in the patients, despite prolonged PT and APTT test results. There was little correlation between parameters derived from the Thrombodynamics assay and the PT, APTT, or thrombin generation data.

Conclusions: The Thrombodynamics assay showed preserved clot formation in plasma from patients with cirrhosis, which is in line with the results of the thrombin generation assay in this study and previously reported by others.
**Introduction**

Conventional coagulation tests, such as the prothrombin time (PT) and activated partial thromboplastin time (APTT), are frequently prolonged in patients with cirrhosis suggesting a hypocoagulable state [1]. Traditionally, these findings have been considered as pointing towards a bleeding tendency in patients with a chronic liver disease. However, these coagulation tests are only sensitive for selected procoagulant factors and do not take the reduction in anticoagulant factors, which also occurs in these patients, into account. In fact, the hemostatic system in patients with a chronic liver disease is nowadays considered to be rebalanced [2]. However, this balance is more unstable compared to that in healthy individuals and there is thus a frequent clinical need for predicting the risk of bleeding or thrombosis in patients with a liver disease [2,3].

In contrast to the conventional coagulation tests, the thrombin generation test in the presence of thrombomodulin, the main activator of protein C, is sensitive to all anticoagulant systems in plasma and thus measures the true balance between the pro- and anticoagulant proteins. This test has shown normal to even increased thrombin generation in patients with a chronic liver disease [4–8]. However, the thrombin generation assay is not widely available yet. The test is currently too complicated for routine use in diagnostics laboratories, and the addition of thrombomodulin is not yet standardized.

Recently a new plasma-based global hemostasis assay, Thrombodynamics, has been developed that allows a continuous monitoring of clot growth in non-stirred plasma initiated by a thin layer of immobilized tissue factor (TF) [9,10]. This assay was designed to better mimic the in vivo conditions of clot formation by taking into account both the biochemical reactions of the coagulation cascade and the spatial aspects of clot formation. Indeed, while in other coagulation tests (e.g. PT and thrombin generation) clotting is activated by TF that is homogenously distributed over the plasma sample, in this test only a thin layer of plasma is exposed to TF and clot formation starts on this surface and propagates into the bulk of plasma. This spatial clot growth assay previously showed defective clot formation (with lower rate of clot growth and thinner clots) in both patients with hemophilia A and B [11]. In addition, another study showed that spatial clot growth in plasma can be used to predict an increase in D-dimer levels in sepsis patients [9]. Furthermore, the test was useful for detecting procoagulant changes caused by an aptamer antagonist of tissue factor pathway inhibitor [10], recombinant factor VIIa [12], or platelet microparticles [13].

Here we aimed to examine the spatial clot growth in plasma from patients with a chronic liver disease using the novel Thrombodynamics assay and compared results with thrombin generation testing and PT/APTT test results.

**Materials and Methods**

**Patients**

Thirty-one adult patients with cirrhosis, who were seen on an outpatient basis or were admitted to the department of Hepatology of the University Medical Center Groningen, were included in the study. Patients were classified according to the Child-Pugh classification [14]. Eleven patients were classified as Child A, ten patients as Child B, and ten patients as Child C cirrhosis. Exclusion criteria were documented history of congenital coagulation disorders, presence of active infection (<2 weeks), presence of acute liver failure, use of anticoagulant
drugs in the past 10 days, pregnancy, human immunodeficiency virus (HIV) positivity, and recent (<7 days) transfusion with blood products.

Twenty-five healthy volunteers working at our institute were included as controls. Exclusion criteria for the control group were a documented history of congenital coagulation disorders, documented history of hepatic disease, recent viral infection (<2 weeks), use of anticoagulant drugs in the past 10 days, pregnancy, and HIV positivity.

The study protocol was approved by the medical ethical committee of the University Medical Center Groningen, Groningen, The Netherlands and written informed consent was obtained from each subject before inclusion in the study.

**Plasma Samples**
Blood samples from each patient and control were drawn by venipuncture and collected into vacuum tubes containing 3.8% trisodium citrate as an anticoagulant, at a blood to anticoagulant ratio of 9:1. Platelet poor plasma was prepared by double centrifugation at 2000 g and 10.000 g respectively for 10 min. Plasma was snap-frozen in liquid nitrogen and stored at -80 °C until use.

**Thrombodynamics Assay**
The general concept of the Thrombodynamics test was previously described by others [9,10,15,16]. Briefly, in a thin layer of plasma coagulation is activated when it is brought in contact with tissue factor (TF) immobilized on a plastic surface. The clot formation starts on the activator and propagates into the bulk of plasma in which no TF is present. Light scattering by fibrin allows observation of spatial clot formation in real time by using time lapse imaging [17].

In this study, the Thrombodynamics assay was performed using an experimental device provided by HemaCore LLC (Moscow, Russia). Reagents (Thrombodynamics kit, Hemacore LLC, Moscow, Russia) and protocols from the manufacturer were used. According to these instructions, plasma was pre-treated with Corn Trypsin Inhibitor for 10 minutes at 37 degrees Celcius prior to initiation of the assay. The following parameters were analyzed: lag time, initial and stationary rates of clot growth, clot density, and clot size at 30 minutes. The lag time is defined as the time between clotting initiation and actual appearance of the fibrin clot. The initial rate of clot growth (Vi) is the slope of the curve on a clot vs. time graph during the first 2-6 minutes of clot growth. Stationary rate of clot growth (Vst) is measured as a slope of the curve on a clot size vs. time graph within the interval 15-25 minutes after clot growth begins.

**Coagulation Tests**
Thrombin generation testing was performed using platelet-poor plasma (PPP) with the fluorimetric method described by Hemker, Calibrated Automated Thrombography® (CAT) [18]. Reagents and protocols were purchased from Thrombinoscope BV, Maastricht, The Netherlands. Coagulation was activated using commercially available reagents containing recombinant TF (final concentration 5 pM), phospholipids (final concentration 4 mM), in absence or presence of soluble thrombomodulin. To calibrate the thrombin generation curves, Thrombin Calibrator (Thrombinoscope BV) was added, and a fluorogenic substrate
with CaCl2 (FluCa-kit, Thrombinoscope BV, Maastricht, The Netherlands) was used to allow a continuous registration of thrombin generation. Fluorescence was read in time by a fluorometer, Fluoroskan Ascent® (ThermoFisher Scientific, Helsinki, Finland).

The PT, APTT, and fibrinogen levels were assessed on an automated coagulation analyzer (ACL 500 TOP) with reagents (Recombiplastin 2G for PT, SynthaSil for APTT, and QFA thrombin (Hemosil) for fibrinogen) and protocols from the manufacturer (Instrumentation Laboratory, Breda, the Netherlands).

**Statistical Analysis**

Data are expressed as means (with standard deviations (SDs)), medians (with interquartile ranges), or numbers (with percentages) as appropriate. Means of two groups were compared by Student’s t-test or distributions in the two groups by Mann-Whitney U test as appropriate. Multiple groups were compared using one-way ANOVA (with the Bonferroni post test) or Kruskal-Wallis H test (with Dunn’s post test) as appropriate. Spearman’s correlation coefficient was used to assess correlation between continuous variables. P values of 0.05 or less were considered statistically significant. GraphPad Prism (San Diego, USA) and IBM SPSS Statistics 20 (New York, USA) were used for analyses.

**Results**

**Patient Characteristics**

The main characteristics of the study population are presented in Table 1. Thirty-one patients with cirrhosis (20 males and 11 females) were included, and they were categorized according to the severity of liver disease as expressed by the Child Pugh classes (11 Child A, 10 Child B and 10 Child C patients). Twenty-five healthy subjects (10 males and 15 females) with a mean age of 33.9 ± 11.1 (mean ± SD) were included as controls. The most common etiology of liver disease was alcoholic, especially in the Child class C patients. Three healthy subjects and none of the patients used oral contraceptives (P=0.08). None of the healthy subjects and three patients used antiplatelet agents or nonsteroidal anti-inflammatory drugs (two used carbasalate calcium and one used naproxen) (P= 0.25).

**Thrombodynamics Assay**

The results of the Thrombodynamics test in the plasma from patients and controls are presented in Table 2 and Figure 1. The lag time was slightly, but significantly prolonged in the patients compared to the controls (P = 0.025). Vi was significantly increased in the Child class C patients (63.7 μm/min (59.4-68.6) (median with range)) compared to controls (57.0 μm/min (50.0-63.4); P < 0.01). However, the Vst was comparable between patients and controls. Clot size was also comparable between patients and controls. Finally, clot density was decreased in the Child class C patients (8093 arbitrary units (a.u.) (4712-10923)) compared to controls (11228 a.u. (6181-20868); P < 0.01).

**Correlations Between Thrombodynamics Results and Other Coagulation Tests**

There was no significant correlation between the Thrombodynamics data and the PT, APTT, or thrombin generation data in the controls. The PT was significantly prolonged in the patients (12.8 s (9.8-23.8)) compared to the controls (10.8 s (9.9-12.1); P < 0.0001), especially in the Child class B and C patients (Table 2). There was a significant negative correlation between
the PT and the clot density of the Thrombodynamics test in the patients ($r = -0.72$; $P < 0.001$). Furthermore, the Vi of the Thrombodynamics test showed a positive correlation with the PT ($r = 0.53$; $P = 0.002$). The APTT was also significantly prolonged in the patients (38.7 s (33.3-48.7)) compared to the controls (33.4 s (27.6-39.1), $P < 0.0001$). There was a significant negative correlation between the APTT and the clot density of the Thrombodynamics test in the patients ($r = -0.51$; $P = 0.004$).

The endogenous thrombin potential (ETP) was decreased in the patients compared to the controls (Table 2). However, in the presence of thrombomodulin, the ETP was increased in the patients (616.4 nM*min (144.6-1046.7)) compared to the controls (355.4 nM*min (168.3-1251.4); $P = 0.003$), especially in the Child class C patients (Table 2). Peak thrombin generation was also increased in the patients compared to the controls in the presence of thrombomodulin (Table 2). In the patients, both ETP and peak thrombin generation showed a significant negative correlation with clot density ($r = -0.61$; $P = 0.0002$ for ETP and $r = -0.46$; $P = 0.01$ for peak) and a positive correlation with Vi of the Thrombodynamics test ($r = 0.48$; $P = 0.006$ for ETP and $r = 0.43$; $P = 0.02$ for peak).

| Table 1. Clinical and demographic characteristics of the study population. |
| Data are expressed as number (%), mean [SD], or median [range]. NSAIDs: Nonsteroidal anti-inflammatory drugs, HCV: Hepatitis C virus, NASH: Non-alcoholic steatohepatitis, PSC: Primary sclerosing cholangitis. |
In addition, the lag time of the thrombin generation assay showed a significant positive correlation with the clot density of the Thrombodynamics assay \( (r = 0.47; P = 0.007) \), and the velocity index of the thrombin generation correlated with the clot density \( (r = -0.45; P = 0.01) \) as well as the Vi of the Thrombodynamics test \( (r = 0.45; P = 0.01) \). Finally, fibrinogen levels strongly correlated with the clot density of the Thrombodynamics test in the patients \( (r = 0.97; P < 0.0001) \).

Discussion
This study shows preserved clot formation in plasma from patients with cirrhosis using the novel Thrombodynamics assay. These results are in line with the results of previous studies using the thrombin generation in the presence of thrombomodulin, which demonstrated normal to increased thrombin generation in patients with a chronic liver disease \([4,6,7]\). Also in this study we observed increased thrombin generation in patients with cirrhosis when thrombomodulin was added to the plasma.

Although overall the Thrombodynamics assay showed comparable test results between patients and controls, in patients with Child C cirrhosis both hyper- and hypocoagulable features were present, with an increase in initial rate of clot growth and decrease in clot density, respectively. The decrease in clot density might be partially explained by a decrease in fibrinogen in patients with severe cirrhosis \([19,20]\). Patients with, especially severe, cirrhosis may thus have a precarious rebalanced hemostasis, explaining the risk of both bleeding and thrombotic complications \([3,21]\).

Despite the fact that both the Thrombodynamics assay and the thrombomodulin-modified thrombin generation assay showed similar results in patients with cirrhosis, correlation between test results was poor. Especially in the controls, we observed no significant correlation

Figure 1. Thrombodynamics data in plasma from controls and patients with Child A, B, and C cirrhosis. A: lag time, B: initial rate of clot growth (Vi), C: stationary rate of clot growth (Vst), D: clot size, and E: clot density. *P < 0.05 compared to controls.
between any of the Thrombodynamics data and the PT, APTT, or thrombin generation data. This may be explained by the fact that this novel Thrombodynamics assay uses a fundamentally different principle to assess the coagulation cascade than both the thrombin generation assay and the conventional coagulation tests. Whereas in these tests clotting is activated by homogeneously dissolved TF, in the Thrombodynamics test clotting is activated by a surface with immobilized TF, which better resembles in vivo clot formation. The correlations observed between Thrombodynamics test results and PT, APTT, or thrombin generation test results in patients were confusing and conflicting. For example, the negative correlation between the PT and clot density is not in line with the negative correlation between the ETP and clot density. Also the positive correlation between the PT and the initial rate of clot growth is difficult to explain.

The preserved clot formation in plasma from patients with cirrhosis observed with the novel Thrombodynamics assay in this study together with similar conclusions drawn from thrombomodulin-modified thrombin generation tests supports the notion that hemostasis in cirrhosis is rebalanced and even has hypercoagulable features [2,19,21]. The clinical consequences of this include a restrictive use of prophylactic prohemostatic agents in patients undergoing invasive procedures [3,22]. Furthermore, routine thrombosis prophylaxis should

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**Table 2.** PT, APTT, Thrombodynamics, and thrombin generation data in patients with cirrhosis and healthy subjects. Data are expressed as mean [SD] or median [range]. *P<0.05; **P<0.01; ***P<0.001 versus controls. PT: prothrombin time, APTT: activated partial thromboplastin time, Vi: initial rate of clot growth, Vst: stationary rate of clot growth, a.u.: arbitrary units, ETP: endogenous thrombin potential, TM: thrombomodulin.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls</th>
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<th>Child A</th>
<th>Child B</th>
<th>Child C</th>
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<tr>
<td>APTT</td>
<td>33.4 [27.6-39.1]</td>
<td>38.7 [33.3-48.7]**</td>
<td>38.7 [35.7-48.7]**</td>
<td>39.8 [33.3-47.0]**</td>
<td>37.5 [33.8-48.1]**</td>
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### Thrombin generation

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<tr>
<td>ETP</td>
<td>908.5 [684.1-1813.4]</td>
<td>771.7 [419.0-1262.3]**</td>
<td>692.0 [419.0-906.1]**</td>
<td>889.5 [641.1-1262.3]</td>
<td>822.6 [585.6-1030.6]</td>
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<tr>
<td>ETP TM</td>
<td>355.4 [168.3-1251.4]</td>
<td>616.4 [144.6-1046.7]**</td>
<td>370.9 [181.8-687.5]</td>
<td>726.0 [144.6-980.7]</td>
<td>745.3 [556.1-1046.7]**</td>
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### Peak

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<th>Child C</th>
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<tbody>
<tr>
<td>Peak</td>
<td>166.6 [83.7-278.2]</td>
<td>155.7 [55.7-241.1]</td>
<td>139.7 [55.7-188.1]</td>
<td>183.0 [98.0-241.1]</td>
<td>155.9 [115.2-186.5]</td>
</tr>
<tr>
<td>Peak TM</td>
<td>94.1 [45.8-221.3]</td>
<td>145.3 [34.5-252.1]**</td>
<td>97.6 [46.7-162.0]</td>
<td>170.6 [34.5-225.1]**</td>
<td>150.3 [113.1-184.7]**</td>
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</table>

### Velocity index

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<th>Child B</th>
<th>Child C</th>
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</thead>
<tbody>
<tr>
<td>Velocity index</td>
<td>67.9 [16.7-165.7]</td>
<td>76.6 [8.8-121.4]</td>
<td>55.7 [8.8-89.8]</td>
<td>83.4 [27.8-121.4]</td>
<td>78.9 [54.9-98.2]</td>
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### Lag time

<table>
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<th>Child C</th>
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<tr>
<td>Lag time TM</td>
<td>1.4 [1.0-2.0]</td>
<td>1.4 [1.1-2.0]</td>
<td>1.7 [1.3-1.7]</td>
<td>1.4 [1.2-2.0]</td>
<td>1.3 [1.1-1.7]</td>
</tr>
</tbody>
</table>
not be withheld in these patients, even though routine coagulation tests are abnormal and prolonged [3,23].

Since patients with cirrhosis can suffer from both thrombotic and bleeding complications, there is a frequent clinical need for predicting the risk of bleeding or thrombotic events before, during, or after procedures in these patients. Currently, two tests may be useful in assessing overall hemostasis, thrombin generation assays and thromboelastography. However, the thrombin generation test is not widely available, the addition of thrombomodulin is not standardized yet, and currently the test is too complicated for routine use in diagnostic laboratories [3,24]. Thromboelastography also has some major drawbacks, including its unique set of pre-analytic and analytic variables that impact test reliability and reproducibility. In addition, thromboelastography does not allow quantification of the contribution of individual components of the hemostatic system to abnormalities in the thromboelastographic tracing. Finally, there are various methods to perform the test and these results poorly correlate [25-27].

The Thrombodynamics assay is a global coagulation assay with a fundamentally new test principle. Given the stability of the assay, provided a strict blood processing protocol is followed, this test may have merit in the clinical setting [17]. It may hold promise in the prediction of bleeding or thrombotic risk in patients with a chronic liver disease, which awaits clinical validation studies.

In conclusion, we observed preserved clot formation in plasma from patients with cirrhosis using the novel Thrombodynamics assay, which is in line with the results of the thrombin generation assay in this study and previously reported by others [4,6,7]. The Thrombodynamics assay may be promising in assessing the hemostatic imbalance in patients with liver diseases, as it uses a fundamentally different principle to measure the coagulation than the other coagulation tests, which may better resemble the in vivo clot formation. However, future studies are needed to assess the clinical value of the test in predicting hemostatic abnormalities and to further standardize it for routine use.
References


17. Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV, Ataullakhanov FI. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thromb Haemost 2007;97:425-34.


