Thrombin Generation in Patients With Cirrhosis: The Role of Platelets

Armando Tripodi, Massimo Primignani, Veena Chantarangkul, Marigrazia Clerici, Alessandra Dell’Era, Federica Fabris, Francesco Salerno, and Pier Mannuccio Mannucci

Coagulation factor defects, thrombocytopenia, and thrombocytopathy are associated with cirrhosis. However, bleeding in patients who have cirrhosis does not entirely correlate with abnormal coagulation tests. Recently, it was shown that because of the concomitant abnormalities of the procoagulant and anticoagulant drives, thrombin generation in plasma patients with cirrhosis is normal when assessed with assays that include thrombomodulin (the main protein C activator). However, thrombin is also generated in vivo as a function of platelets, suggesting that thrombocytopenia and thrombocytopathy might affect thrombin generation in patients with cirrhosis. We addressed this issue using an assay that accounts for the contribution of plasma and platelets. The study showed that platelet-rich plasma with platelets adjusted by dilution of autologous platelet-rich into autologous platelet-poor plasma to a standard count (100 x 10^9/L) generates as much thrombin in patients with cirrhosis as in controls (1,063 nmol/L vs. 1,167 nmol/L; P value not significant). When platelets were adjusted to correspond to whole-blood counts, patients with cirrhosis generated significantly less thrombin than controls (949 nmol/L vs. 1,239 nmol/L; P < .001). Furthermore, thrombin generation correlated with platelet numbers (r = 0.50; P < .001). In addition, the amount of thrombin generated as a function of the whole-blood patients’ platelet counts increased significantly when the numbers were adjusted to 100 x 10^9/L (953 nmol/L vs. 1,063 nmol/L; P < .001). In conclusion, severe thrombocytopenia may limit thrombin generation in patients with cirrhosis. These findings might justify platelet transfusion in patients with low platelet counts when they bleed spontaneously or before undergoing surgery or liver biopsy. Controlled clinical trials supporting this indication are warranted.

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Multiple coagulation factor defects,1 thrombocytopenia,2,3 and thrombocytopathy4,5 are usually associated with liver disease. Because of this association, cirrhosis has long been regarded as the epitome of the acquired hemostatic defects. Accordingly, patients with cirrhosis are investigated by means of conventional tests for coagulation and primary hemostasis such as prothrombin time, activated partial thromboplastin time, and skin bleeding time.6 However, bleeding in patients with cirrhosis does not entirely correlate with the abnormalities of these tests, and their predictive value is rather poor.7 Recently, we postulated that because of the concomitant abnormalities of the procoagulant drive (coagulation factors) and the anticoagulant drive (protein C, antithrombin, and tissue factor pathway inhibitor), which characterize cirrhosis, thrombin generation might be normal in these patients. Indeed, a thrombin generation assay modified to include thrombomodulin, the main protein C activator operating in vivo (but not in vitro in prothrombin time and activated partial thromboplastin time), provided evidence that the balance of coagulation is normal in plasma from patients with cirrhosis despite the prolongation of conventional coagulation tests.8 However, thrombin is generated in vivo not only as a function of pro- and anticoagulant plasmatic factors, but also as a function of platelets9 and other cells.10 The occurrence of thrombocytopenia (and thrombocytopathy) might have profound implications on thrombin generation; this prompted us to address this issue in patients with cirrhosis with a thrombin generation assay that takes into account plasma and platelets.
**Patients and Methods**

**Patients.** Eighty-seven adult patients with cirrhosis (71 men, 16 women) with a median age of 63 years (range, 42-82) were enrolled in the study after we received approval from our institutional review board and obtained informed consent from the patients. Cirrhosis was diagnosed on the basis of clinical, laboratory, and ultrasonographic evidence. Criteria for exclusion were the use of drugs known to interfere with blood coagulation and platelet function, bacterial infections, hepatocellular carcinoma, extrahepatic malignancy, and known hemostatic disorders other than cirrhosis. The severity of the disease was estimated according to Child-Turcotte-Pugh classification.11 Sixty-two healthy individuals volunteered to be enrolled in the study as controls. Blood was drawn by clean venepuncture and collected in vacuum tubes (Becton and Dickinson, Meylan, France) containing 105 mmol/L trisodium citrate as anticoagulant in the proportion of 1/9 parts of anticoagulant/blood. Blood was centrifuged within 30 minutes at controlled room temperature using 2 different procedures.

**Procedure A: Centrifugation for 15 Minutes at 2,000g and Separation of Platelet-Poor Plasma Into 2 Portions.** The first portion was centrifuged for 5 minutes at 12,000 rpm in a microcentrifuge (centrifuge 5415C, Eppendorf, Hamburg, Germany) to minimize residual platelets. Platelet-free plasma was then harvested, quick-frozen in liquid nitrogen, and stored at −70°C until tested for thrombin generation, which was performed no later than 6 months after blood collection. The second portion of platelet-poor plasma was aliquoted, kept frozen as outlined above, and used for conventional coagulation tests (see below).

**Procedure B: Centrifugation for 15 Minutes at 150g to Obtain Platelet-Rich Plasma.** Supernatant plasma was harvested, and platelets were counted (Micros 60, Abx Diagnostics, Montpelier, France). Platelet numbers from each patient or control subject were then adjusted by appropriate dilutions of the autologous platelet-rich plasma into autologous platelet-poor plasma to a standard platelet count of 100 × 10⁹/L. Platelet numbers were also adjusted to correspond to the individual patient or control subject whole blood count. Platelet-rich plasmas were eventually tested for thrombin generation within 2 hours of the preparation. Aliquots of blood were also collected from patients and control subjects to prepare serum samples for measurement of the parameters to assess liver function.

**Measurements.** Thrombin generation was assessed as endogenous thrombin potential (ETP) according to Hemker et al.12 as described in detail by Chantarangkul et al.13 The test is based on the activation of coagulation in platelet-free or platelet-rich plasma after addition of human recombinant tissue factor (Recombinplastin; Instrumentation Laboratory, Orangeburg, NY) which acts as a coagulation trigger in the presence (only for the measurement in platelet-free plasma) of synthetic phospholipids 1,2-dioleoyl-sn-glycero-3-phosphoserine, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, and 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids Inc., Alabaster, AL) in the proportion of 20/20/60 (M/M). The concentrations of tissue factor and phospholipids in the test system were 1 pmol/L and 1.0 μmol/L, respectively. In some experiments, soluble thrombomodulin (ICN Biomedicals, Aurora, OH) was added in the reaction mixture at a final concentration of 4 nmol/L. Continuous registration of the generated thrombin was achieved with a fluorogenic synthetic substrate (Z-Gly-Gly-Arg-AMC HCl, Bachem, Switzerland) added to the test system at a final concentration of 2.5 mmol/L. The procedure was performed with an automated fluorometer (Fluoroskan Ascent; ThermoLabSystem, Helsinki, Finland). Readings from the fluorometer were automatically recorded and calculated using a dedicated software (Thrombinscope; Synapse b.v., Maastricht, The Netherlands) that displays thrombin generation curves (time vs. generated thrombin) and calculates the area under the curve, defined as ETP and expressed as thrombin concentration as function of an internal standard for thrombin (Synapse b.v.). This is the whole amount of thrombin which can be generated by the platelet-free or platelet-rich plasma under the specified conditions and represents the balance between the action of procoagulants and anticoagulants. All other parameters to assess procoagulant factors (prothrombin time, activated partial thromboplastin time, and factor II) and anticoagulant factors (antithrombin and protein C) as well as liver function were measured as previously reported.8

**Statistical Analyses.** Continuous variables were expressed as medians and ranges and tested for statistical significance with the nonparametric Mann-Whitney U and Wilcoxon tests. The Spearman coefficient was used to assess correlation between different variables, and a P value of ≤.05 was considered statistically significant. Statistical analyses were performed with SPSS version 13.0 software (SPSS Inc., Chicago, IL).

**Results**

Information on the patient population with regard to demographic characteristics, severity of cirrhosis and plasma levels of procoagulant and anticoagulant factors is given in Tables 1 and 2. As expected patients had statistically significant prolonged prothrombin time and acti-
vated partial thromboplastin time and reduced levels of antithrombin, protein C and factor II.

**Thrombin Generation in Platelet-Free Plasma.** Table 3 shows results of the thrombin generation expressed as ETP when measured on platelet-free plasma. The median ETP value for the patient population (1,398 nmol/L [range, 630-2,517]) was significantly lower \((P < .001)\) than the control value (1,872 nmol/L [range, 982-2,682]) when the test was performed without thrombomodulin, but the 2 values were not significantly different when the test was performed with thrombomodulin (866 nmol/L [range, 175-1,939] vs. 795 nmol/L [range, 75-1,473]).

**Thrombin Generation in Platelet-Rich Plasma.** The median platelet count for patients and controls was 80 \(\times 10^9/\text{L}\) (range, 20-196) and 198 \(\times 10^9/\text{L}\) (range, 141-343). Fig. 1 shows the distribution of ETP value for patients and controls when the test was performed in platelet-rich plasma with the numbers of platelets adjusted to \(100 \times 10^9/\text{L}\). As shown for platelet-free plasma (see Table 3), there was a statistically significant difference (patients, 1,358 nmol/L [range, 535-2,092] vs. controls, 1,883 nmol/L [range, 1,103-2,992]; \(P < .001\)) when the test was performed without thrombomodulin, but this difference was no longer evident (patients, 1,063 nmol/L [range, 341-1,784] vs. controls, 1,167 [range, 530-1,845]; \(P = .25\)) after the addition of thrombomodulin (Fig. 1).

When the platelet numbers in platelet-rich plasma were adjusted to correspond to whole blood counts in patients or controls, the differences (controls vs. patients) were statistically significant regardless of the addition of thrombomodulin to the assay system (Fig. 2). The above

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<th>Table 1. Demographic Characteristics of the Study Population Separated According to Child-Pugh Classification</th>
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<td>Leukocytes (10^9/L)</td>
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<td>Platelets (10^9/L)</td>
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NOTE. All values are expressed as the median (range) unless noted otherwise. Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; PT, prothrombin time.

*Patient-to-normal coagulation time.

<table>
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<th>Table 2. Hemostatic Parameters in Patients With Cirrhosis and in Controls</th>
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<td><strong>Parameters</strong></td>
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<td>PT ratio*</td>
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<td>APTT ratio*</td>
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<tr>
<td>Protein C (%)†</td>
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<td>Antithrombin (%)†</td>
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Abbreviations: PT, prothrombin time; APTT, activated partial thromboplastin time.

*Patient-to-normal coagulation time.
†Percent activity relative to the reference normal plasma set at 100%.
results taken together suggest that platelet function is suitable to support adequate thrombin generation (Fig. 1) and that platelet numbers play a role (Fig. 2).

Correlation Between Thrombin Generation and Platelet Counts. The ETP for the whole population (patients and controls) when measured in platelet-rich plasma (platelet numbers adjusted to correspond to whole blood counts) in the presence of thrombomodulin correlated with platelet counts ($r = 0.50; P < 0.001$) (Fig. 3). The analysis of the linear regression of the above results allows a rough estimate of the platelet numbers (i.e., $5.6 \times 10^9/L$) that can generate $875 \text{ nmol/L}$ thrombin (i.e., the 10th percentile of the distribution of values recorded in the control population).

Thrombin Generation as a Function of Platelet Count. The ETP for the patient population when measured in the presence of thrombomodulin in platelet-rich plasma with platelet numbers adjusted to $100 \times 10^9/L$ ($1,063 \text{ nmol/L} \text{ [range, 341-1,784]}$) was significantly higher ($P < .001$) than that measured in platelet-rich plasma with platelet numbers adjusted to correspond to whole blood patient counts ($953 \text{ nmol/L} \text{ [range, 55-2,252]}$) (Fig. 4). As might be expected, values for the control population measured under the same conditions were slightly but significantly higher when platelet numbers were adjusted to whole blood counts ($1,253 \text{ nmol/L} \text{ [range, 417-1,900]}$) than the corresponding value measured in platelet-rich plasma with platelet numbers adjusted to $100 \times 10^9/L$ ($1,182 \text{ nmol/L} \text{ [range, 530-1,845];} P < .05$) (Fig. 4).

Discussion

Thrombin generation assays are very convenient laboratory tools for assessing the endogenous thrombin potential in plasma after activation of coagulation. These assays

![Fig. 1](image1.png)  
**Fig. 1.** ETP expressed as thrombin values for controls ($n = 60$) (black circles) and patients ($n = 71$) (white circles) without and with thrombomodulin in platelet-rich plasma. Platelet numbers prior to ETP measurement were adjusted to $100 \times 10^9/L$. Horizontal bars represent median values. ETP, endogenous thrombin potential; nM, nmol/L; N.S., not significant.

![Fig. 2](image2.png)  
**Fig. 2.** ETP expressed as thrombin values for controls ($n = 51$) (closed circles) and patients ($n = 87$) (open circles) without and with thrombomodulin in platelet-rich plasma. Platelet numbers prior to ETP measurement were adjusted to the individual patient (median $80 \times 10^9/L$ [range, 20-196]) or control ($198 \times 10^9/L$ [range, 141-343]) whole blood counts. ETP, endogenous thrombin potential; nM, nmol/L.

![Fig. 3](image3.png)  
**Fig. 3.** Correlation of ETP with thrombomodulin in platelet-rich plasma from patients ($n = 87$) (closed circles) and controls ($n = 50$) (open circles) versus platelet count. Platelet numbers prior to ETP measurement were adjusted to the patient or control whole blood counts. ETP, endogenous thrombin potential; nM, nmol/L.
provide a unique opportunity to investigate mechanistically the coagulation balance under standardized conditions. However, until recently, these investigations were limited only to plasma, because the presence of cells makes the reaction medium too turbid and therefore unsuitable for chromogenic measurements. The introduction of fluorogenic substrates has overcome this problem, because the signal from the fluorophore is not quenched by turbidity, thus opening new venues for understanding the role played by platelets and possibly other cells in thrombin generation. We employed this assay to investigate the coagulation balance in patients with cirrhosis who are often thrombocytopenic. To our knowledge, this is the first attempt ever made to investigate blood coagulation in patients with cirrhosis by using assay conditions that mimic as closely as possible those presumably operating in vivo—that is, the presence of plasmatic coagulation factors, the optimal activation of protein C (mediated by thrombomodulin), and the presence of platelets. The numbers of platelets were adjusted to correspond to a standard count (i.e., $100 \times 10^9/L$) and also to the patient counts as measured in whole blood.

The results obtained in platelet-free plasma confirm those of the previous study\(^8\) showing that plasma from patients with cirrhosis when triggered with tissue factor in the presence of exogenous phospholipids generate as much thrombin as the control population, provided that protein C is activated by thrombomodulin (see Table 3). The implicit assumption for the translational application of these findings to the in vivo situation is that thrombomodulin from endothelial cells in patients with cirrhosis is functionally normal and that soluble thrombomodulin added to our assay acts similarly to that which is physiologically located on the endothelial cells.

A pattern of thrombin generation similar to that obtained in platelet-free plasma was obtained when the investigation was performed on platelet-rich plasmas from the same patients with numbers of platelets adjusted to correspond to a standard count of $100 \times 10^9/L$ (see Fig. 1). This finding suggests that platelets from patients with cirrhosis are suitable to support adequate thrombin generation. When the numbers of platelets were adjusted to correspond to the whole blood patients’ counts (reflecting the presence of thrombocytopenia), the differences between patients and controls were statistically significant, and there were patients who generated low levels of thrombin (see Fig. 2). Interestingly, these patients had the lowest platelets counts, indicating that the number of platelets plays a key role in thrombin generation in patients with cirrhosis. This conclusion is supported by 3 lines of evidence. First, there was a statistically significant difference between patients (low platelet counts) and controls (normal platelet counts) (Fig. 2). Second, the amount of generated thrombin correlated with platelet numbers (Fig. 3). Third, the amount of thrombin generated as a function of the whole blood patients’ platelet counts increased significantly when the platelet numbers were adjusted to $100 \times 10^9/L$ (Fig. 4). The degree of this increase is statistically significant, but its in vivo relevance has not yet been established. Although it should be sufficient to secure optimal thrombin generation because it is similar to that attained in the control population tested under the same conditions, clinical studies are needed to ascertain the clinical effect in the control of bleeding.

Numerous studies have addressed the issue of thrombocytopenia in cirrhosis.\(^{14,15}\) A complex defect has been described that ranges from an impaired synthesis of thromboxane A2 and defective signal transduction to the presence of storage pool disease and defects of glycoprotein Ib.\(^{16-21}\) However, the contribution of each of these defects to the hemostatic derangement in patients with cirrhosis has not been fully elucidated. Platelets support hemostasis in vivo through a dual mechanism. The first of these mechanisms is the adhesion to the subendothelium and subsequent aggregation, both being mediated by von Willebrand factor and fibrinogen.\(^{22}\) The second is to provide suitable negatively charged phospholipid surfaces for assembly of macromolecular enzymatic (tenase and prothrombinase) complexes needed for thrombin generation and fibrin formation.\(^{23}\) The observations stemming from this study suggest that the latter mechanism is intact in patients with cirrhosis because thrombin generation was normal provided that autologous platelets are adjusted to a standard count of $100 \times 10^9/L$ (see Fig 1). Although we did not specifically investigate thrombocy-
topathy in this setting, it is possible that the defect in platelets in our patients with cirrhosis does not concern the exposure of the negatively charged phospholipids or other membrane glycoproteins needed for platelets to exert their procoagulant activity. An alternative explanation would be that the assay used in this study is not sensitive enough to detect the impact that moderate thrombocytopenia in cirrhosis may have on thrombin generation.

In conclusion, the above findings support the hypothesis that thrombocytopenia plays a key role in thrombin generation and possibly bleeding tendency in patients who have cirrhosis. This conclusion—together with the observation that thrombin generation is normal in the plasma of individuals with cirrhosis8 (Table 3)—might justify platelet transfusion or treatment with recombinant human thrombopoietin24 in those patients with severe thrombocytopenia when they bleed spontaneously or before undergoing surgery or liver biopsy. Platelets would provide suitable phospholipid surfaces to complement the normal thrombin generation elicited by plasma. Although neither platelet transfusion nor treatment with thrombopoietin have been evaluated in controlled clinical trials of patients with cirrhosis, this approach would be particularly relevant and worthy of investigation as an alternative to the treatment with such other procoagulant agents as the recombinant-activated factor VII, which has demonstrated a relatively small efficacy in controlling variceal bleeding in patients with cirrhosis25 or in reducing the number of red blood cell units transfused perioperatively in patients undergoing orthotopic liver transplantation.26-28 Although the clinical use of transfused platelets may be limited by their relatively short survival in cirrhosis, it is unknown whether the same is true for treatment with thrombopoietin. Controlled clinical trials are needed to investigate these issues.

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References