# Factors associated with outcome of liver surgery and hepatocellular carcinoma

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# CHAPTER 6

## NO EVIDENCE FOR INCREASED PLATELET ACTIVATION IN PATIENTS WITH HEPATITIS B- OR C-RELATED CIRRHOSIS AND HEPATOCELLULAR CARCINOMA

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## ABSTRACT

BACKGROUND: Cancer is a major risk factor for developing venous thromboembolism (VTE). Plasma hypercoagulability is an established risk factor for cancer-related VTE. In addition, thrombocytosis and hyperreactive platelets have been implicated in VTE and cancer progression. Cirrhosis is associated with changes in platelet number and function. The platelet activation status of patients with cirrhosis and hepatocellular carcinoma has not yet been established. Here we assessed the platelet activation status in patients with hepatitis-related cirrhosis in presence or absence of HCC.

MATERIALS AND METHODS: We performed a cross-sectional study including thirty-eight consecutive patients with hepatitis B- or C- related liver cirrhosis in presence or absence of HCC. We studied basal and agonist-induced platelet activation using flow cytometry. In addition, we studied the plasma levels of von Willebrand factor (VWF) and the VWF-cleaving protease ADAMTS13. Twenty healthy volunteers served as controls.

RESULTS: We found no evidence of basal platelet activation in patients with cirrhosis compared to controls. However, we found reduced agonist-induced platelet activation in patients. No differences in the basal and agonist-induced platelets activation status between patients with or without HCC were detected. Plasma levels of VWF were increased and the levels of ADAMTS13 activity were decreased in patients compared to controls. No differences between the levels of VWF and ADAMTS13 in patients with or without HCC were detected.

CONCLUSIONS: HCC development or recurrence in patients with hepatitis B- or C-related cirrhosis does not appear to be associated with platelet activation and changes in pivotal proteins in primary hemostasis.

## INTRODUCTION

Cancer patients are in a hypercoagulable state and venous thrombotic event might be the first clinical sign of cancer.[1] In fact, twenty percent of all cases of venous thromboembolism (VTE) are related to cancer, and the risk of developing VTE is four to seven fold increased in cancer patients.[2] VTE in cancer patients is typically associated with plasma hypercoagulability that might be triggered by the cancer cells expressing procoagulant activity or by the host response to cancer (reviewed elsewhere[3-5]). Also, patient- and treatment-related factors such as chemotherapy, bed rest, infection, and surgery may play a contributory role.[6-8] In addition to changes in plasmatic coagulation, quantitative and qualitative changes in platelets have been reported in cancer patients.

Elevation of the circulating platelet count [9,10] (i.e. thrombocytosis) is frequently found in patients with cancer, and is associated with increased risk of VTE[11] and poor prognosis.[10] Moreover, platelet depletion or inhibition of platelet function has been shown to reduce or prevent cancer growth and distance metastasis in animal models.[12-14] In humans, aspirin intake has been shown to reduce death from cancer, and the incidence of metastases.[15-18] The detrimental effects of platelets in cancer may involve release of growth factors stored within platelets,[19,20] shielding of tumor cells from innate immune surveillance,[21] or facilitation of tumor extravasation resulting in metastasis.[22]

Enhanced activation of platelets has been demonstrated in human studies in multiple types of cancer.[23] However, little data on the platelet activation status in patients with hepatocellular carcinoma are available. In this context, this is an interesting tumor type as it frequently occurs in patients with cirrhosis. Cirrhosis is associated with complex changes in the hemostatic system, including quantitative and qualitative changes in platelets.[24,25]

Thrombocytopenia is common in cirrhosis as is a decreased platelet response in classical platelet aggregometry or the PFA-100.[24] It has, however, also been described that platelet functionality is preserved when tested under conditions of flow.[26] The in vivo activation status of platelets in patients with cirrhosis is still controversial, with older literature pointing towards a functional defect in platelets (reviewed in[24]), whereas more recent studies suggest in vivo platelet hyperreactivity.[27] Here we studied the in vivo platelet activation status in patients with cirrhosis in presence or absence of HCC. We compared the results with values obtained in healthy individuals.

## METHODS

### STUDY POPULATION

From February to April 2013, a total of thirty-eight consecutive patients with cirrhosis who visited the outpatient clinic or were admitted to the Hepatology ward of the Department of Gastroenterology of University Medical Center of Padua, Padua, Italy were enrolled into this study. We included all patients who had established cirrhosis, with or without HCC. Excluded were those who used platelet inhibitory drugs such as acetylsalicylic acid or P2Y12 inhibitors, patients who were undergoing eradication treatment for hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, patients with an active HCC who had a treatment-free interval less than two months at the time of inclusion, and patients who were on dialysis. The diagnosis of HCC was obtained by using the European Association for the Study of the Liver criteria, i.e., two imaging procedures (Spiral CT, MRI with paramagnetic contrast injection, or ultrasound with second generation intravenous contrast (Sonovue, Bracco, Italy)) confirming the presence of a lesion.

A control group used to establish reference values for the various tests consisted of 20 adult employees of the University Medical Center Groningen who voluntarily participated in this study. Exclusion criteria for the control group were platelet inhibitory drugs usage such as acetylsalicylic acid or P2Y12 inhibitors, documented history of congenital coagulation disorders, documented history of hepatic disease, and history of viral infection (<2 weeks). This study was performed in accordance with, and was approved by the local ethical committee. Written informed consent was obtained from each patient and control.

### BLOOD SAMPLES

Blood from patients and controls was drawn by clean venipuncture in 3.8% citrate tubes. A sample was processed directly for flow cytometry and the remainder of the blood was processed to plasma by centrifugation. First, samples were spun at 200 g for 15 min at ambient temperature to obtain platelet-rich plasma. The platelet-rich plasma was transferred to a clean tube and recentrifuged (500 g for 15 min at ambient temperature), in the presence of iloprost (2 ng/ml) purchased from Santa Cruz Biotechnology, Dallas, TX U.S.A. The supernatant, platelet poor plasma, was then collected in 2 ml tubes, snap-frozen, and stored at -80 °C until use.

### STUDY VARIABLES

Patient characteristics were obtained from patient charts. These included age, gender, the severity of cirrhosis (according to the Child-Pugh classification), platelet count, the international normalized ratio (INR), creatinine levels, and total bilirubin levels. For patients with HCC we also obtained the number of tumor lesions, the size of the tumors and the total

Tabel 1: Participant characteristics

| Demographic characteristics | Controls (n = 20) | Cirrhosis without HCC (n = 16) | Cirrhosis with HCC (n = 22) | *P* |
|---|---|---|---|---|
| Participant variables | | | | |
| Gender, Male | 11 (55%) | 10 (63%) | 20 (95%) | 0.01 |
| Age, mean (SD) | 29 (±5) | 55 (±14) | 70 (±10) | < 0.01 |
| Platelet count, (G/L), median (IQR) | - | 107 (48-159) | 102 (68-152) | 0.69 |
| INR, median (IQR) | - | 1.3 (1.2-1.6) | 1.2 (1.1-1.2) | 0.03 |
| MELD, median (IQR) | - | 12 (9-16) | 10 (8-11) | 0.09 |
| Etiology of liver Cirrhosis | - | | | 0.22 |
| HBV | | 3 (19%) | 7 (33%) | |
| HCV | | 13 (81%) | 12 (57%) | |
| HBV + HCV | | 0 | 2 (10%) | |
| Child Pugh, n (%) | - | | | 0.02 |
| A | | 4 (25%) | 11 (52%) | |
| B | | 9 (56%) | 6 (29%) | |
| C | | 3 (19%) | 2 (10%) | |
| AFP, median (IQR) | - | 6 (5-23) | 14 (3-24) | 0.55 |
| Tumor characteristics | - | - | | |
| Treated (TACE/RFA), yes | | - | 13 (59%) | |
| Number of lesions, median (IQR) | | - | 2 (1-3) | |
| Total size, mm (IQR) | | - | 40 (25-71) | |

AFP indicates Alpha fetoprotein; HBV, hepatitis B virus, HCC; Hepatocellular carcinoma, HCV, hepatitis C virus; INR, International Normalised Ratio; IQR, interquartile range; MELD: Model of End stage Liver Disease; RFA, radiofrequency ablation; SD, standard deviation, TACE, transarterial chemoembolization.

mass of HCC, whether or not the patients had a vasoinvasion, the presence of portal vein thrombosis, and if they were previously treated for the HCC. We calculated the Model for End-Stage Liver Disease score (0.957 × loge (creatinine)+0.378 × loge (bilirubin) +1.12 × loge (INR) +0.643] × 10) based on the last laboratory measurements just prior to the blood draw for the assays described in the present study. When necessary, computer-stored hospital files were reviewed for other relevant clinical parameters.

### Flow Cytometry

The platelet activation status was assessed using flow cytometry in whole blood. Five µl of blood was added to a 50 µl mixture in HEPES-buffered saline (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH2PO4, 1.7mMMgCl2, 5mMd-glucose, pH 7.4) containing vehicle or activators and a R-phycoerythrin (R-PE) labeled monoclonal antibody against human P-selectin (#555524, BD Pharmingen™, Franklin Lakes, NJ, 2 µl). Platelets were kept in a resting state or activated by adenosine diphosphate (ADP, 15 µM, Stago, Asnières, France), thrombin receptor activating peptide (TRAP6, 15 µM, Bachem, Bubendorf, Switserland), or a combination of both. After 20 min of incubation, the samples were fixed with 500 µL 0.2% formyl saline (0.2% formaldehyde in 0.9% NaCl) and kept at 4 °C in the dark until the analysis. A negativecontrol (mouse IgG1 monoclonal antibodies conjugated to R-PE (#555749, BD Pharmigen) was used.

The patient samples were analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) at the University of Padua. Control samples were analyzed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). All samples were analyzed within six hours after processing. Samples were gated on the basis of their forward and sideward scatter properties. The percentage of platelets expressing P-selectin were recorded. The proportion of platelets positive for P-selectin after activation were corrected for the proportion of platelet positive for P-selectin in the non-activated sample of each patient and control. The increase in the proportion of P-selectin positive platelets obtained in this manner represents the extent to which platelets in a given sample can be activated by a given activator (i.e., the platelet activatability).

### Plasma Levels of VWF, ADAMTS13 and sP-selectin

VWF antigen (VWF) levels were determined with an in-house enzyme-linked immunosorbent assay (ELISA) using commercially available polyclonal antibodies against VWF (DAKO, Glostrup, Denmark). Plasma a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) activity was assessed using the FRETS-VWF73 assay (Peptanova, Sandhausen, Germany) based on the method described by Kokame and associates. [28] VWF and ADAMTS levels in pooled normal plasma were set at 100%, and values obtained in test plasmas were expressed as a percentage of pooled normal plasma. Soluble P-selectin (sP-selectin) levels were determined using a commercially available sP-selectin ELISA (R&D Systems, Abingdon, UK), and values were both expressed as the sP-selectin concentration in plasma and as plasma concentrations corrected for the platelet count in platelet-rich plasma.

### Statistical Analysis

Statistical analyses were performed using the statistical software package SPSS 20 (IBM SPSS, Chicago, IL). Categorical variables are shown as numbers and percentages and groups were compared using Pearson's chi-squared test. Continuous variables are presented as means with standard deviation (SD) or as medians with interquartile range (IQR) based on their distribution. Continuous variables were compared using a standard t-test or the Mann Whitney U test, as appropriate. *P* values less than 0.05 were considered statistically significant.

## Results

### Study Population

Patient characteristics are summarized in Table 1. Included were thirty-eight patients with hepatitis-related cirrhosis of whom twenty-two (58%) had HCC. The patients that had HCC were more frequently male than patients that did not have HCC (95% vs 63%). There was no

significant difference between the patients with and without HCC regarding the type of hepatitis as underling cause of cirrhosis (HBV or HCV, 33% and 57% in patients with HCC vs 19% and 81% in the patients without HCC). Concomitant HBV and HCV infection was present in two patients (10%) who had HCC. Thirteen (59%) out of twenty-two patients that had an active HCC due to a recurrence or a residual tumor at the time of inclusion had previously been treated. Twelve patients were treated with trans-arterial chemoembolisation, one patient with radiofrequency ablation. The median time between treatment and the blood draw for the present study was 357 (IQR 127-608) days. None of the patients had evidence for vasoinvasion and none of the patients had portal vein thrombosis.

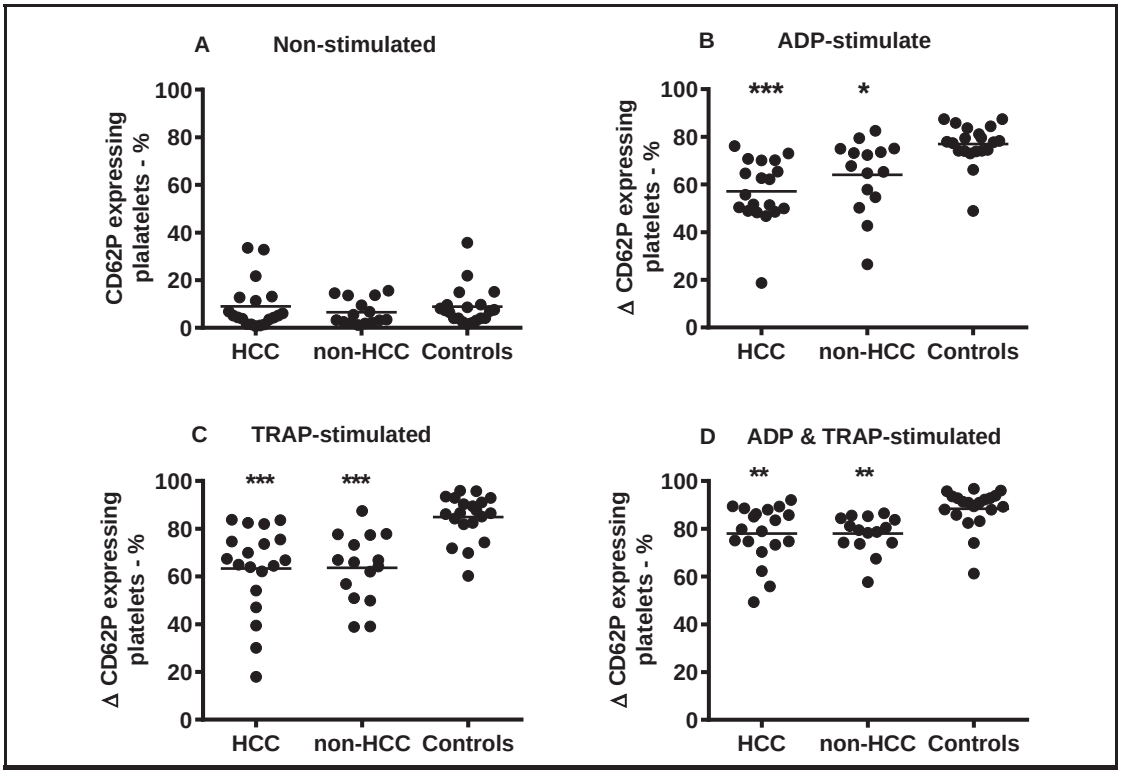


Figure 1: Basal and agonist-induced platelet activation in patients with cirrhosis with or without HCC. The basal platelet activation status (A) and the agonist-induced platelet activatability in patients and controls using ADP (B), TRAP (C), and the combination of ADP and TRAP (D) as assessed by flow cytometry for P-selectin in patients with or without HCC and healthy controls. Shown is the percentage of P-selectin positive platelets. Agonist-induced platelet activatability values were corrected for baseline values. Horizontal lines represent medians. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all v.s. control.

The patients were classified according to the Child-Pugh classification.[29] The patients who had HCC appeared to have slightly milder liver disease compared to those who did not have HCC. Furthermore, the patients with HCC were significantly older, had a lower international normalized ratio and MELD score, but had similar platelet counts compared to the patients without HCC. The patients with HCC had a median of 2 lesions (IQR, 1-3), a cumulative

tumor size of 4 cm (IQR, 3-7) and a serum alpha-fetoprotein (AFP) of 12.5 ng/ml (IQR, 3.8-23.5). One patient had an AFP level of 2.750 ng/ml, and a cumulative tumor size of 20 cm. No patients had portal vein thrombosis, extrahepatic manifestation of HCC, or macroscopic vasoinvasion.

### Is Basal or Agonist-Induced Platelet Activation Increased in Patients with HCC?

Figure 1 shows the basal platelet activation status and the agonist induced platelet activatability (defined as the increase in the proportion of P-selectin positive platelets after activation) in patients and controls. The number of p-selectin positive platelets at Baseline was not different between patients and controls. No differences were detected in the basal platelet activation status between patients that did or did not have HCC. When platelets were activated in vitro using ADP, TRAP or the combination of both, the percentage of platelets expressing P-selectin were significantly lower in patients than controls when corrected for baseline values. However, no differences were detected between patients that did or did not have HCC.

### Are Plasma Markers of Primary Hemostasis Increased in Patients with HCC?

We next assessed plasma levels of soluble P-selectin as a marker of in vivo platelet activation (Fig. 2). Plasma sP-selectin levels were similar between the patients and controls or between the patients with or without HCC. However, when the levels of sP- selectin were corrected for the circulating platelet count, levels of sP-selectin were significantly higher in patients without HCC. Plasma levels of VWF were significantly higher in patients compared to controls, but levels were similar between patients with and without HCC. ADAMTS13 activity was lower in patients compared to controls, although the difference between controls and patients without HCC did not reach statistical significance. ADAMTS13 activity was comparable between patients with or without HCC.

## Discussion

In this study we detected no differences in basal platelet activation status and platelet activatability between patients that had hepatitis-related cirrhosis and patients that had hepatitis-related cirrhosis and HCC. In addition the VWF/ADAMTS13 unbalance associated with cirrhosis, which we previously showed to promote platelet function,[30,31] was similar in patients with and without HCC. The basal platelet activation Status was similar between patients and controls. We did, however, detect substantially decreased platelet activatability in patients. Based on previous studies on the platelet activation status in different types of cancer, we anticipated that the platelet activation status in patients with cirrhosis who developed HCC would be increased in comparison to patients with cirrhosis without HCC.[32-38] However, our results indicate that patients with cirrhosis and HCC might not have a

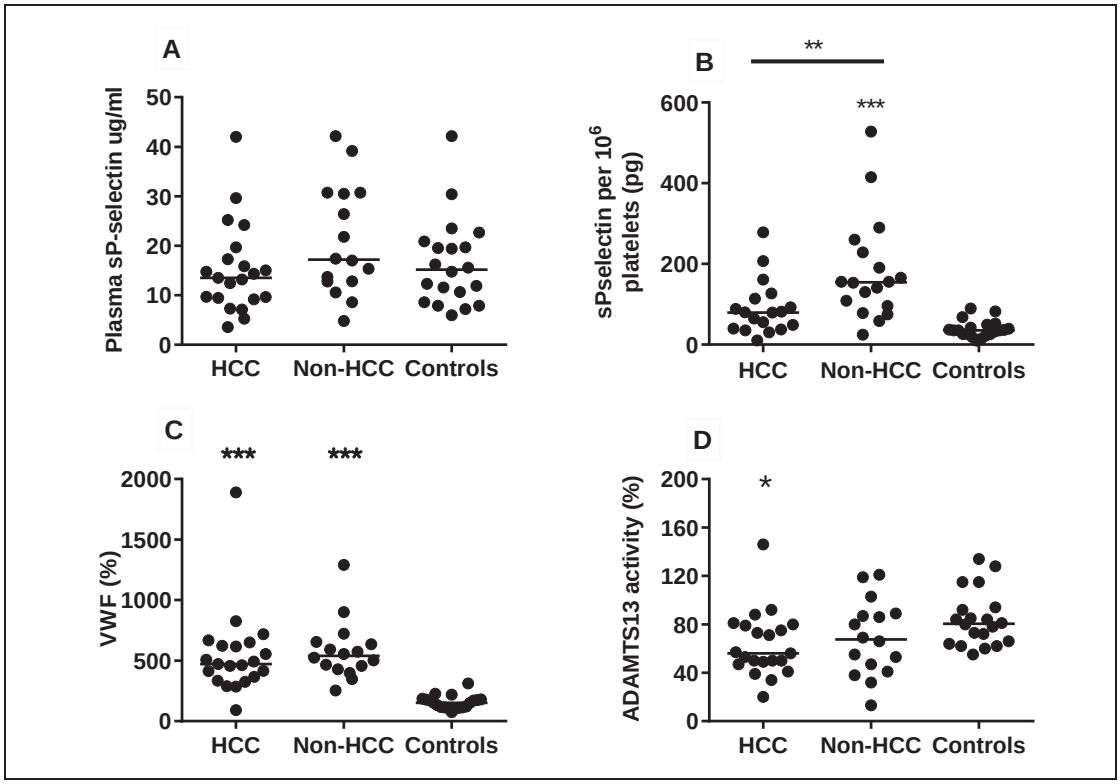


Figure 2: Plasma indices of primary hemostasis in patients with cirrhosis with or without HCC. Plasma levels of soluble P-selectin (A), soluble P-selectin corrected for platelet count (B), VWF (C), and ADAMTS13 (D) in patients with or without HCC and healthy controls. Horizontal lines represent medians. * $p < 0.05$, ** $p < 0.01$, *** $P < 0.001$, all v.s. control (except in panel B, ** $P < 0.01$ HCC vs non-HCC).

relative hyperreactivity of their primary hemostatic system compared to patients with cirrhosis without HCC. These results indicate that the complexity of the alterations in the primary hemostatic system of cirrhosis may overwhelm the changes in platelet function as a result of the HCC. Platelets are increasingly recognized as important players in cancer growth and metastasis.[12,13,39] Given the clinical benefit of aspirin in metastasis and cancer-related death, platelets may be an attractive therapeutic target for cancer in general. Recently, Sitia and colleagues [13] demonstrated that inhibition of platelet function prevents or delays the onset of HCC in a mouse model of chronic immune-mediated hepatitis B. The authors postulated that platelets are key players of immune-mediated necroinflammatory process that might be responsible for the onset of HCC in chronically infected patients. Given the role of platelets in inflammation and their multiple roles in liver diseases,[40] it may be that platelets are active players in disease progression by delivery of angiogenic proteins within the liver. We have recently shown that the intraplatelet levels of angiogenic proteins including VEGF, bFGF, and HGF were elevated in patients with hepatitis B or C related cirrhosis that did or did not have HCC.[41] However, as we have now demonstrated that the capacity to activate platelets in patients with HCC is decreased, the question arises whether secretion of such factors optimally occurs in this setting. In other words, would in vivo

secretion of these factors be decreased as a result of attenuated platelet activation, then the role of platelets in HCC development may be less than the role of platelets in other cancer types, and antiplatelet drugs may consequently be less effective in HCC patients compared to patients with different tumor types. On the other hand, our in vitro activation assay may not fully reflect physiology. We have previously demonstrated that platelet activation when studied under conditions of flow is similar in patients with cirrhosis compared to controls, despite decreased platelet activation capacity in assays performed under static conditions.[26]

Our data on the basal platelet activation status and in vitro activatability of patients with cirrhosis contrasts with recent findings in which platelet hyperactivity in cirrhosis was suggested.[27] Differences in methodology as outlined by us previously may be responsible for these discrepant results.[42] We demonstrate that basal platelet activation is not increased in cirrhosis, and that platelets cannot be fully activated, which is in line previous findings.[43-45] Although the increased sP-selectin levels corrected for the platelet count (Fig. 2B) may suggest enhanced in vivo platelet activation it should be noted that sP-selectin is likely cleared by the liver, which, in patients with cirrhosis, may lead to falsely elevated levels.

Our patients had significantly higher plasma levels of VWF than controls. This finding is in concordance with our previous study showing increasing levels of VWF in patients with cirrhosis that correlated with increasing severity of liver disease.[30] Notably, the levels of VWF were comparable in patients in presence or absence of HCC. It has been suggested that VWF plasma levels may be of prognostic significance in patients with solid tumors,[46-48] but the prognostic significance of VWF in HCC is absent, perhaps because of the effects of the underlying cirrhotic disease on VWF levels.

We found lower levels of ADAMTS13 compared to controls although the difference between the controls and patients without HCC did not reach statistical significance. Lower levels of ADAMTS13 activity have been reported in patients with liver disease.[49] ADAMTS13 levels have been reported as an independent risk factor of development of HCC within a year.[50] This is in contrast to our finding, since we found no difference in the levels of ADAMTS13 activity between the patient in presence or absence of HCC.
In conclusion, we found no evidence of increased platelet activation and altered activatability in patients with hepatitis B- or C related cirrhosis that developed HCC compared to patients without HCC. In addition, no changes in the VWF/ADAMTS13 axis were detected. HCC development or recurrence in hepatitis B or C-related cirrhosis may thus not be associated with changes in primary hemostasis.

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