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ORIGINAL ARTICLE

Biomarkers of bleeding and venous thromboembolism in patients with acute leukemia

Yohei Hisada[1] | Sierra J. Archibald[1] | Karan Bansal[2] | Yanjun Chen[2] | Chen Dai[2] | Sindhu Dwarampudi[2] | Nora Balas[2] | Lindsey Hageman[2] | Nigel S. Key[1] | Smita Bhatia[2] | Ravi Bhatia[3] | Nigel Mackman[1] | Radhika Gangaraju[2,3]

[1]UNC Blood Research Center, Division of Hematology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

[2]Institute for Cancer Outcomes and Survivorship, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA

[3]Department of Medicine, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA

Correspondence
Yohei Hisada, UNC Blood Research Center, Division of Hematology, Department of Medicine, University of North Carolina at Chapel Hill, 8004 Mary Ellen Jones Building CB#7035, 116 Manning Drive, Chapel Hill, NC 27599, USA.
Email: yohei_hisada@med.unc.edu

Radhika Gangaraju, Institute for Cancer Outcomes and Survivorship, School of Medicine, University of Alabama at Birmingham, 1600 7th Avenue South, Lowder 500, Birmingham, AL 35233, USA.
Email: rgangaraju@uabmc.edu

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Abstract

Background: Coagulopathy and associated bleeding and deep vein thrombosis (DVT) are major causes of morbidity and mortality in patients with acute leukemia. The underlying mechanisms of these complications have not been fully elucidated.

Objectives: To evaluate the associations between biomarker levels and bleeding and DVT in acute leukemia patients.

Methods: We examined plasma levels of activators, inhibitors, and biomarkers of the coagulation and fibrinolytic pathways in patients aged ≥18 years with newly diagnosed acute leukemia compared with those of normal controls. Multivariable regression models were used to examine the association of biomarkers with bleeding and DVT in acute leukemia patients. The study included 358 patients with acute leukemia (29 with acute promyelocytic leukemia [APL], 253 with non-APL acute myeloid leukemia, and 76 with acute lymphoblastic leukemia) and 30 normal controls.

Results: Patients with acute leukemia had higher levels of extracellular vesicle tissue factor (EVTF) activity, phosphatidylserine-positive extracellular vesicles, plasminogen activator inhibitor-1, plasmin-antiplasmin complexes, and cell-free DNA and lower levels of citrullinated histone H3-DNA complexes compared with normal controls. APL patients had the highest levels of EVTF activity and the lowest levels of tissue plasminogen activator among acute leukemia patients. There were 41 bleeding and 23 DVT events in acute leukemia patients. High EVTF activity was associated with increased risk of bleeding (subdistribution hazard ratio, 2.30; 95% CI, 0.99-5.31), whereas high levels of plasminogen activator inhibitor-1 were associated with increased risk of DVT (subdistribution hazard ratio, 3.00; 95% CI, 0.95-9.47) in these patients.

Conclusion: Our study shows alterations in several biomarkers in acute leukemia and identifies biomarkers associated with risk of bleeding and DVT.

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KEYWORDS
acute leukemia, biomarker, bleeding, deep vein thrombosis, disseminated intravascular coagulation

1 | INTRODUCTION

Acute leukemia is a rapidly progressing hematologic malignancy and includes 2 distinct major morphologic types: acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). While the 5-year survival rates are improving for acute leukemia, early morbidity and mortality from disseminated intravascular coagulation (DIC) and associated complications, such as bleeding and venous thromboembolism (VTE), remain high [1–11]. AML and acute promyelocytic leukemia (APL), a subtype of AML, have a similar incidence of severe bleeding at 7% to 29% [12–15], whereas 1 study found 11% incidence of severe bleeding in ALL patients [16]. Notably, APL patients have a high incidence of early mortality (7%-26%) within 30 days of leukemia diagnosis [4–7,17–21] compared with other types of acute leukemia. A recent study found that high white blood cell (WBC) count, presumably due to increased blast cell count, was associated with early hemorrhagic death in APL patients and indicated a role of leukemia cells in coagulopathy [22]. Another study reported that prolonged prothrombin time (PT)-international normalized ratio and low platelet count were associated with World Health Organization grade 4 bleeding in non-APL AML patients [13]. APL patients have a higher incidence of VTE (6%-43%) compared with AML (2%-12%) and ALL (2%-13%) patients. However, there is a lot of heterogeneity in the incidence and risk factors reported, and most of these studies had small sample sizes [8–10,14,23–25]. The presence of central venous catheter, older age, and chronic comorbidities were associated with VTE in acute leukemia patients [10,11].

Tissue factor (TF) is a transmembrane receptor for factor (F)VII/VIIa. The TF/FVIIa complex is the major physiological initiator of the coagulation protease cascade [26]. Leukemia cells express TF [27–30]. Peripheral blood mononuclear cells (PBMCs) that included leukemic blasts from AML patients were found to express higher TF procoagulant activity compared with PBMCs from healthy controls [27]; notably, TF procoagulant activity was higher in APL patients compared with non-APL AML patients [27]. In addition, PBMCs and blasts from AML patients with DIC had higher levels of TF activity compared with those from patients without DIC [27]. A human APL cell line, NB4, and a human AML cell line, HL-60, express TF, with higher levels in NB4 cells [28–30]. All-trans retinoic acid (ATRA) reduced TF expression in bone marrow cells (including blast cells) from APL patients and NB4 cells [28,31–35].

Many cells, including cancer cells and activated monocytes, release submicron vesicles called extracellular vesicles (EVs) that express TF and expose procoagulant phospholipids, such as phosphatidylserine (PS), on their surface [36,37]. NB4 and HL-60 cells have been shown to release TF + EVs [30]. Studies with small numbers of patients have shown high levels of circulating extracellular vesicle tissue factor (EVTF) activity in AML patients with DIC [38–40]. APL is also associated with hyperfibrinolysis [41]. APL patients have higher levels of urokinase-type plasminogen activator receptor and plasmin-antiplasmin complexes (PAP) compared with healthy controls [42]. In contrast, levels of plasminogen activator inhibitor-1 (PAI-1) antigen were lower in APL patients compared with controls [42]. Another study found that PAI-1 activity was lower in APL patients with DIC compared with healthy controls [43]. However, these studies were limited by small sample sizes (1-24 patients). To date, to our knowledge, there are no studies that have examined the association of biomarkers in coagulation and fibrinolytic pathways with bleeding and deep vein thrombosis (DVT) using a large number of samples from acute leukemia patients.

Leukemic blasts release cell-free (cf) DNA into the circulation [44]. Indeed, patients with acute leukemia have higher levels of cfDNA compared with healthy populations [27,45]. cfDNA can activate FXII, which leads to activation of the coagulation system [46]. Activated neutrophils release neutrophil extracellular traps (NETs) that consist of extracellular chromatin components, including DNA, citrullinated histone H3 (H3Cit), and neutrophil granule proteins [47]. NETs enhance VTE by capturing platelets and procoagulant EVs [48–51]. However, recent studies reported that neutrophils and granulocytes isolated from acute leukemia patients had reduced capacity to form NETs compared with those isolated from healthy controls [52–54].

The current study had 3 goals. The first was to compare levels of activators, inhibitors, and biomarkers of coagulation and fibrinolysis between patients with acute leukemia and controls. The second was to determine the prevalence of DIC in different types of acute leukemia. The third was to examine the association of routine and novel biomarkers in the coagulation pathway with bleeding and DVT in acute leukemia patients.

2 | METHODS

2.1 | Study population

This prospective cohort study included adult patients (aged ≥18 years at diagnosis) with newly diagnosed acute leukemia who provided blood samples for the University of Alabama at Birmingham biorepository between May 2016 and April 2021. Of the 826 subjects enrolled in the study, 570 had a diagnosis of acute leukemia. Of these, patients with relapsed/refractory disease at the time of sample collection, those with insufficient clinical data, and those who did not have adequate plasma samples were excluded (n = 212), yielding a final sample size of 358 (Figure 1). Blood samples were collected in 10-mL EDTA tubes at the time of leukemia diagnosis and before administration of chemotherapy. However, most patients with APL received at least 1 dose of ATRA prior to sample collection. Most of the patients in the study had a peripherally

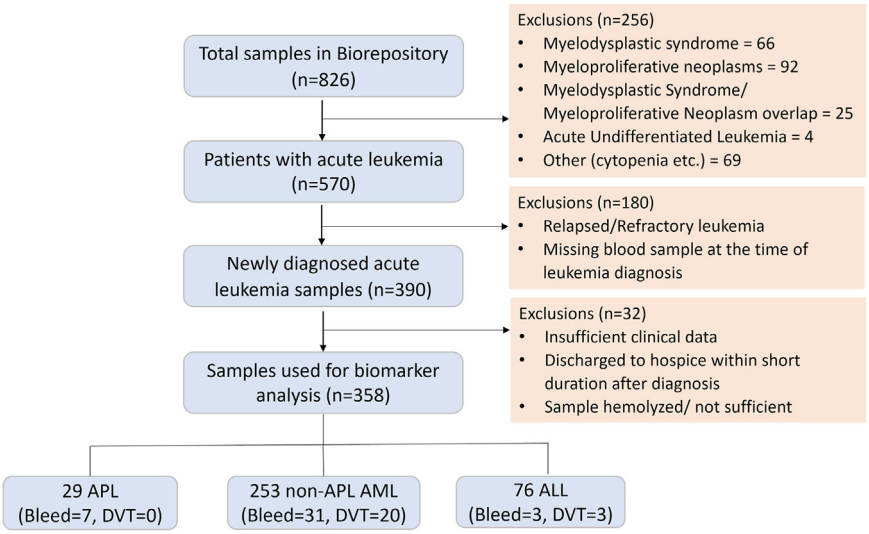


FIGURE 1 Flow chart to demonstrate inclusion and exclusion of patients in the study. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; APL, acute promyelocytic leukemia; DVT, deep vein thrombosis.

inserted central catheter line for administration of chemotherapy, and samples were collected from central lines for these patients. Blood samples were centrifuged at 2000 rpm for 5 minutes. The plasma was separated and stored at −80 °C.

Demographic information and clinical data regarding leukemia diagnosis, comorbidities, and outcomes (VTE and bleeding) were abstracted from the medical records until blood or marrow transplantation, last contact with patient, or death, whichever occurred first. Laboratory values collected as part of routine clinical care included WBC count, hemoglobin, platelet count, PT, partial thromboplastin time (PTT), D-dimer, fibrinogen, creatinine, lactate dehydrogenase (LDH), and peripheral blood blast cell percentage at leukemia diagnosis. This study was conducted with the approval of the institutional review board (IRB) at the University of Alabama at Birmingham, and informed consent was obtained according to the Declaration of Helsinki (IRB protocol number X160420008). The transfer of material to the University of North Carolina at Chapel Hill and measurement of biomarkers was approved by the IRBs of the respective institutions. IRB approval number for University of North Carolina was 21 0554.

2.2 | Outcomes

Outcomes and characteristics of interest for this study included overt DIC at the time of acute leukemia diagnosis, bleeding, and VTE. Overt DIC was defined based on the scoring system developed by the International Society on Thrombosis and Haemostasis (ISTH) using levels of D-dimer, fibrinogen, PT, and platelet count at the time of leukemia diagnosis [55,56]. Bleeding episodes were defined as major and clinically relevant nonmajor bleeding as per ISTH criteria [57]. VTE events included radiologically confirmed symptomatic or incidental pulmonary embolism, proximal or distal lower extremity DVT, upper extremity DVT, DVT at other sites, and superficial venous thrombosis, including catheter-related thrombosis [58]. Arterial thrombosis events were not included in the outcome. Detailed definitions of outcomes were provided in Supplementary Table S1. A trained reviewer (K.B.) performed abstraction of all radiology reports, clinical notes, and discharge summaries, and each outcome was independently assessed by a second reviewer (S.D.). Discrepancies were resolved by a third clinician reviewer with expertise in hematology (R.G.). VTE and bleeding outcomes were evaluated from the index date of leukemia diagnosis until blood or bone marrow transplantation, last follow-up, or death.

2.3 | Normal controls

Plasma samples were obtained from 30 normal individuals from whole blood drawn into EDTA tubes via venipuncture from Innovative Research (cat# IPLASK2E2ML). The samples tested negative for the FDA viral markers, and there was no known disease aside from that information. No further medical records or health screening data were obtained for controls. The donors were age- and sex-matched to APL patients.

2.4 | Biomarkers

Biomarkers in the coagulation and fibrinolytic pathways and NETs were measured at the University of North Carolina at Chapel Hill. EVTF activity was measured using an in-house 2-stage FXa generation assay as described previously [59]. PS+ EVs were measured using the ZYMUPHEN MP-ACTIVITY Assay Kit (cat# 521096, Aniara Diagnostica). H3Cit-DNA complexes were measured using an in-house enzyme-linked immunosorbent assay as described previously [60]. cfDNA was measured using the Quant-iT PicoGreen dsDNA Assay Kit

(cat# P11496, Thermo Fisher Scientific). PAP (cat# MBS2503292, MyBioSource), tissue plasminogen activator (tPA, cat# IHUTPAKTT, Innovative Research), and active PAI-1 (cat# HPAIKT, Innovative Research) were measured using commercial assays. EVTF activity data from 6 patients were used in a publication [61] for comparing our in-house EVTF activity assay with commercial TF enzyme-linked immunosorbent assays. We preselected these biomarkers because they have been evaluated in acute leukemia patients in previous studies, but the associations between their levels and bleeding and DVT have not been evaluated.

2.5 | Statistical analyses

The Shapiro–Wilk test was used for normality testing. For biomarker comparisons between leukemia patients and controls, we used the unpaired 2-tailed student *t*-test or the Mann–Whitney U-test depending on normality. The Kruskal–Wallis test with the Dunn's multiple comparisons test was used to compare biomarker levels in different groups. To compare bleeding and DVT rates between DIC and non-DIC patients, Fisher's exact test was used. These analyses were performed with PRISM version 7.03 (GraphPad Software).

We used SAS software version 9.4 (SAS Institute Inc) to analyze biomarker associations with bleeding, DVT, and VTE outcomes. Age at leukemia diagnosis and body mass index (BMI) were treated as continuous variables; the remaining variables were considered categorical. We calculated cumulative incidence of bleeding, DVT, or VTE using Fine and Gray methods, treating death as a competing risk [62]. We used 2-sample *t*-test or Wilcoxon rank sum test (for continuous variables) and chi-squared test (for categorical variables) to compare differences between groups. Using proportional subdistribution hazards multivariable regression analysis [63], we examined the association between biomarkers and the outcomes of interest. For this analysis, biomarkers were considered as tertiles and third and second tertile distributions were compared with the first tertile and adjusted for clinically relevant covariates in multivariable models. Due to collinearity between biomarkers, we developed multiple individual multivariable models for each biomarker, adjusting for the relevant covariates. For bleeding outcome, we created 2 multivariable models for each biomarker: model 1 adjusted for age at leukemia diagnosis, sex, and race/ethnicity and model 2 adjusted for age, sex, race/ethnicity, ISTH DIC score, number of comorbidities (hypertension, diabetes, dyslipidemia, congestive heart failure, valvular heart disease, rheumatologic disorder, peripheral vascular disease, peptic ulcer disease, inflammatory bowel disease, chronic obstructive pulmonary disease, arrhythmia, psychiatric disorder, liver disease, HIV infection, chronic kidney disease, history of prior transplant, coronary heart disease, and stroke), and history of bleeding. For DVT and VTE risk, multivariable models were adjusted for age at leukemia diagnosis, sex, race/ethnicity, acute leukemia type, BMI, history of VTE, and comorbidities. In a subanalysis, we examined the risk factors for bleeding and DVT in patients with non-APL AML and in patients with intracranial hemorrhage as this is the most severe major bleeding complication. Subanalyses were not performed for APL and ALL patients because the sample size was too small. Two-sided tests with $P < .05$ were considered statistically significant.

2.6 | Data sharing

For original data, please contact yohei_hisada@med.unc.edu.

3 | RESULTS

3.1 | Patient characteristics

Demographic and clinical characteristics of acute leukemia patients and controls as well as routine laboratory parameters at leukemia diagnosis are shown in Table 1. The study included 358 patients with acute leukemia (29 with APL, 253 with non-APL AML, and 76 with ALL) and 30 controls. The median age at diagnosis of acute leukemia was 59 (range, 19-89) years. The controls were enrolled at a median age of 58.5 (range, 23-76) years. There were 203 (56.7%) males and 264 (73.7%) non-Hispanic White individuals with acute leukemia. Over a median follow-up of 11.9 (range, 0.12-78.1) months, 41 acute leukemia patients developed bleeding (23 with major bleeding and 18 with clinically relevant nonmajor bleeding) and 37 developed VTE (23 with DVT and 14 with superficial vein thromboses). It is notable that 28 (75.7%) of these VTE events were catheter-related. There were 7 APL (24.1%), 31 non-APL AML (12.3%), and 3 ALL (3.9%) patients who developed bleeding and 3 APL (10.3%), 30 non-APL AML (11.9%), and 4 ALL (5.3%) patients who developed VTE. Characteristics of bleeding and VTE events are shown in Table 2.

3.2 | Biomarker levels in acute leukemia patients compared with controls

3.2.1 | Procoagulant EVs, cfDNA, and a biomarker of NETs

EVTF activity was higher in leukemia patients compared with normal controls (Figure 2A). APL patients had the highest levels of EVTF activity compared with non-APL AML or ALL patients. Higher levels of PS+ EVs were observed in all 3 leukemia types compared with normal controls; no significant differences were observed among leukemia types (Figure 2B). Levels of cfDNA were higher in leukemia patients compared with normal controls (Figure 2C), with the highest levels present in those with ALL. Interestingly, H3Cit-DNA complexes levels were significantly lower in all 3 leukemia types compared with normal controls (Figure 2D).

TABLE 1 Demographic characteristics of normal controls and acute leukemia patients and laboratory values for acute leukemia patients at diagnosis.

Characteristic	Normal controls (*n* = 30)	APL (*n* = 29)	Non-APL AML (*n* = 253)	ALL (*n* = 76)
Sex (male/female)	17/13	18/11	146/107	39/37
Age (median [range])	58.5 (23-76)	46 (22-81)	61 (19-89)	56 (19-80)
Race				
White	24	23	191	50
Black	3	5	55	21
Asian	0	1	3	0
Hispanic	3	0	1	4
American Indian	0	0	0	1
Unknown	0	0	3	0
Bleeding, *n* (%)	0	7 (24.1)	31 (12.3)	3 (3.9)
Major bleeding	0	7 (24.1)	14 (5.5)	2 (2.6)
Minor Bleeding	0	0 (0)	17 (6.7)	1 (1.3)
VTE, *n* (%)	0	3 (10.3)	30 (11.9)	4 (5.3)
DVT	0	0 (0)	20 (7.9)	3 (3.9)
SVT	0	3 (10.3)	10 (4.0)	1 (1.3)
Laboratory parameters (median [range])	Normal range			
D-dimer (ng/mL)[a]	<240	14 011 (222-20 000)	1049 (135-20 000)	1678 (233-20 000)
Fibrinogen (mg/dL)[b]	220-498	208 (60-636)	455 (60-1101)	387 (170-856)
PT (s)	12-14.5	16.5 (13.8-27.0)	15.3 (12.9-62.4)	14.8 (12.7-21.6)
PTT (s)	25-35	31 (24-200)	34 (19-88)	32 (24-43)
WBC count ($\times 10^3/\mu L$)	4-11	2.7 (0.6-79.8)	14.2 (0.4-435.9)	15.5 (0.1-357.0)
Peripheral blood blast cell (%)	0	73.0 (2.6-89.0)	58.5 (0-96.0)	62.8 (1.6-97.0)
Platelet count ($\times 10^3/\mu L$)	150-400	30.9 (7.1-131.6)	50 (3.4-862.4)	40.9 (7.9-377.3)
Creatinine (mg/dL)	0.4-1.2	0.9 (0.5-4.4)	0.9 (0.4-3.2)	0.9 (0.4-2.2)
Hemoglobin (g/dL)	13.5-17.5	8.6 (3.6-12.4)	8.8 (2.9-15.1)	8.4 (4.6-17.9)
LDH (Unit/L)	120-240	289 (113-1898)	426 (109-5469)	477 (119-6374)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; DVT, deep vein thrombosis; LDH, lactate dehydrogenase; PT, prothrombin time; PTT, partial thromboplastin time; SVT, superficial vein thrombosis; VTE, venous thromboembolism; WBC, white blood cell.

[a] Maximum value of detection for D-dimer is 20 000 ng/mL.

[b] Lowest limit of detection for fibrinogen was 60.

3.2.2 | Biomarkers of the fibrinolytic pathway

Levels of PAP were significantly higher in the leukemia patients compared with normal controls (Figure 2E). tPA levels were significantly lower in APL patients compared with normal controls as well as non-APL AML and ALL patients (Figure 2F). PAI-1 levels were higher in leukemia patients compared with controls, with significantly higher levels in the APL group compared with the non-APL AML group (Figure 2G).

3.3 | Comparisons between acute leukemia patients with high and mid to low EVTF activity

We determined if EVTF activity is associated with leukocytosis, high percentage of blast cells, D-dimer, and platelet count in acute leukemia patients by comparing their levels in patients with EVTF activity in the first tertile with those in patients with EVTF activity in the second and third tertiles. We found that both WBC count and peripheral blood blast cell percentage were significantly higher in the second and

TABLE 2 Characteristics of bleeding and venous thromboembolism events.

Bleeding events	Values, *n* (%)
Major bleeding	23 (56.1)
Clinically significant nonmajor bleeding	18 (43.9)
Site of bleeding	
Intracranial hemorrhage	12 (29.3)
Gastrointestinal bleeding	13 (31.7)
Hematuria	3 (7.3)
Hemoptysis	3 (7.3)
Vaginal bleeding	4 (9.8)
Epistaxis	3 (7.3)
Other sites	3 (7.3)
VTE type	
Deep vein thrombosis	23 (62.2)
Superficial vein thrombosis	14 (37.8)
Catheter-related thrombosis	28 (75.7)
Catheter-related superficial vein thrombosis	13 (46.4)
Catheter-related deep vein thrombosis	15 (53.6)
VTE site	
Lower extremity deep vein thrombosis	3 (8.1)
Upper extremity deep vein thrombosis	19 (51.4)
Upper extremity superficial vein thrombosis	13 (35.1)
Pulmonary embolism	1 (2.7)
Upper extremity deep vein thrombosis + pulmonary embolism	1 (2.7)

VTE, venous thromboembolism.

third tertiles compared with the first tertile (Supplementary Table S2). We also found that D-dimer was significantly higher in the second and third tertiles than in the first tertile (Supplementary Table S2). In contrast, we did not see a difference in platelet counts between the first tertile and the second and third tertiles (Supplementary Table S2).

3.4 | Comparisons between leukemia patients with and without overt DIC

We found that 90% of APL patients, 34% of non-APL AML patients, and 45% of ALL patients had overt DIC based on ISTH criteria at diagnosis. EVTF activity and WBC count were significantly higher in patients with APL with overt DIC compared with patients without DIC (referred to as no-DIC; Supplementary Table S3). In patients with APL, the ISTH overt DIC score components (D-dimer, fibrinogen, PT, and platelets) were significantly different between patients with and without overt DIC. In patients with non-APL AML, we found that EVTF activity, cfDNA, PTT, WBC count, peripheral blood blast cell percentage, creatinine, and LDH were significantly higher in patients with overt DIC compared with patients without DIC (Supplementary Table S4). In patients with non-APL AML, the 3 ISTH overt DIC score components (D-dimer, PT, and platelets) were significantly different between patients with and without overt DIC. The incidence of bleeding was significantly higher in non-APL AML patients with overt DIC compared with non-APL AML patients without DIC. In patients with ALL, we found significantly higher levels of PAP and lower levels of hemoglobin in patients with overt DIC compared with those in patients without DIC. PS+ EV levels were lower in ALL patients with overt DIC compared with those in patients without DIC (Supplementary Table S5). D-dimer and platelets were the only components of the ISTH overt DIC score that showed significant differences between ALL patients with overt DIC and those without DIC.

3.5 | Bleeding risk and associated biomarkers in acute leukemia patients

The characteristics of acute leukemia patients with and without bleeding are shown in Supplementary Table S6. Patients with bleeding had higher D-dimer and LDH levels and prolonged PT compared with those without. The cumulative incidence of bleeding was 6.0% at 1 month (SD, 1.3) and 12.6% at 1 year (SD, 1.9; Figure 3A). The majority of the bleeding events in APL patients occurred within 1 month after diagnosis, with a cumulative incidence of 25.0 ± 8.4% at 1 month, whereas the cumulative incidence was 5.3 ± 1.4% at 1 month and 14.6 ± 2.6% at 1 year in non-APL AML patients and 1.4 ± 1.3% at 1 month and 2.9 ± 2.1% at 1 year in ALL patients (Figure 3B).

When examining the associations between biomarkers and bleeding, we combined all leukemia types to ensure sufficient statistical power. Multivariable subdistribution hazards model with death as competing risk and adjusting for age, sex, and race/ethnicity showed that EVTF activity (subdistribution hazard ratio [sHR], 2.33; 95% CI, 1.08-5.04), PT (sHR, 2.55; 95% CI, 1.15-5.62), WBC count (sHR, 2.30; 95% CI, 1.03-5.13), and LDH (sHR, 2.80; 95% CI, 1.12-6.97) were associated with increased bleeding risk (Table 3). After further adjustment for the ISTH DIC score, comorbidities, and history of bleeding, only EVTF activity (sHR, 2.30; 95% CI, 0.99-5.31) was associated with increased risk of bleeding. The cumulative incidence of bleeding at 1 year was 15.6 ± 3.7%, 15.2 ± 3.7%, and 7.9 ± 2.7% for patients with EVTF activity in the third, second, and first tertiles, respectively (Figure 3C).

When restricting the analysis to only major bleeding events, none of the parameters were associated with major bleeding in multivariable subdistribution hazards model (Supplementary Table S7).

In addition, we set the median level of each biomarker as a cutoff value and calculated the percentage of acute leukemia patients with and without bleeding above the median value. Acute leukemia patients with bleeding had a higher percentage of above median levels of PT (without vs with bleeding: 48.9% vs 56.1%), WBC (without vs with bleeding: 47.6% vs 68.3%), LDH (47.5% vs 66.7%), and EVTF activity (49.0% vs 56.1%) compared with acute leukemia patients without bleeding (Supplementary Figures S1A–D).

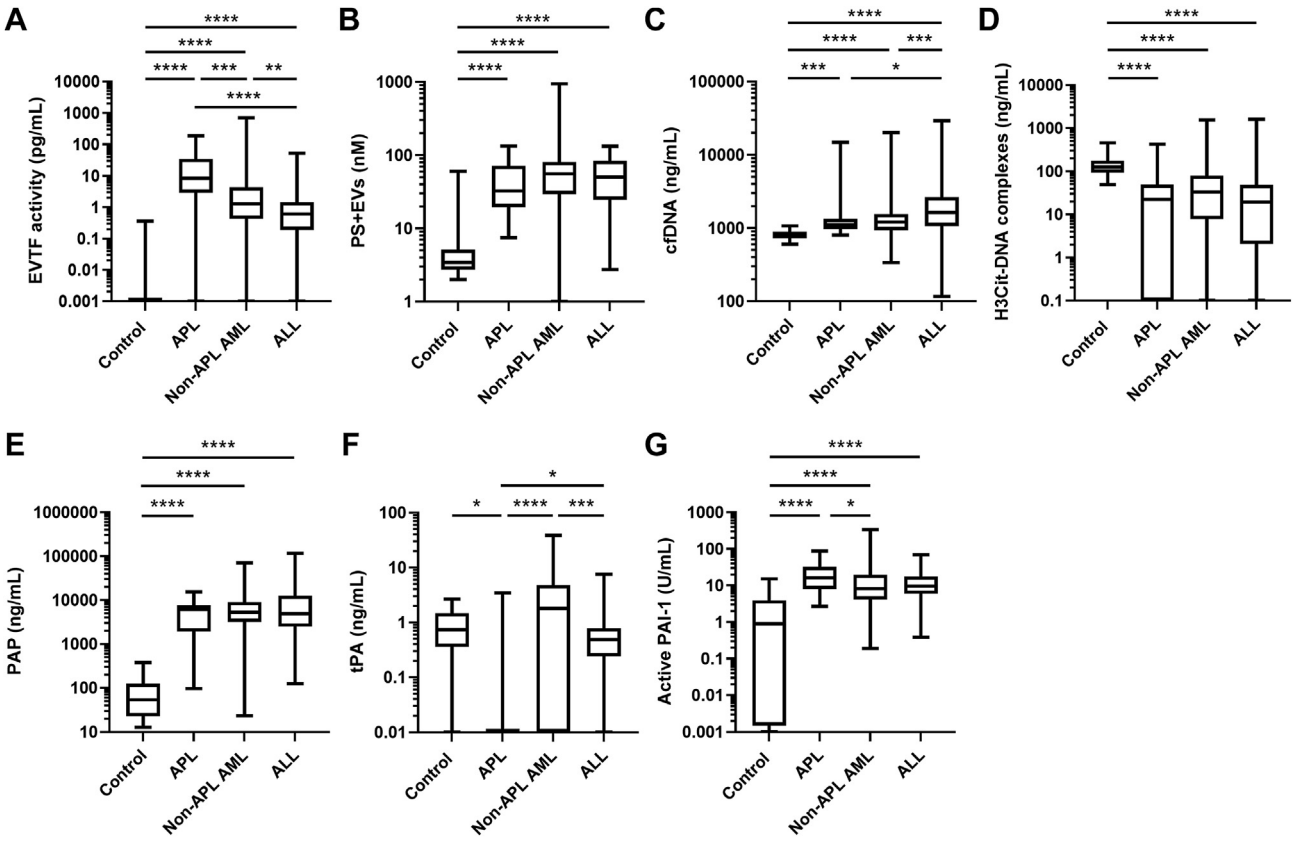
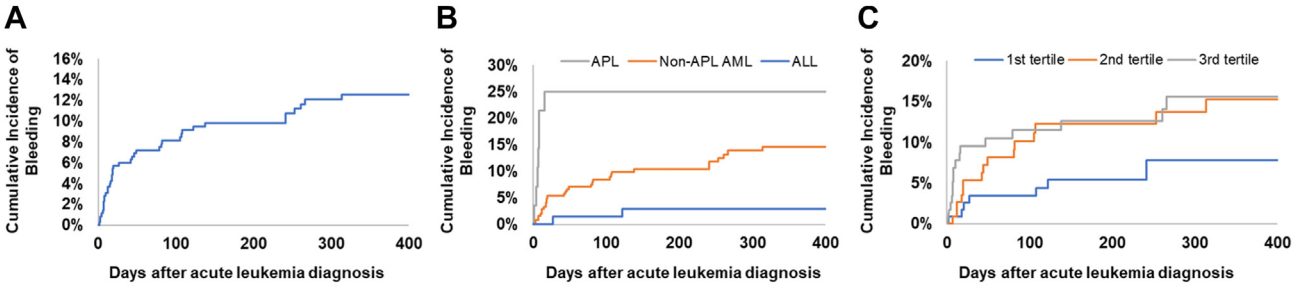


FIGURE 2 Levels of biomarkers in the coagulation pathway, neutrophil extracellular trap formation, and the fibrinolytic pathway in patients with different types of acute leukemia. Levels of (A) extracellular vesicle tissue factor (EVTF) activity, (B) phosphatidylserine-positive (PS+) extracellular vesicles (EVs), (C) cell-free (cf) DNA, (D) citrullinated histone H3 (H3Cit)-DNA complexes, (E) plasmin-antiplasmin complex (PAP), (F) tissue plasminogen activator (tPA), and (G) plasminogen activator inhibitor-1 (PAI-1) in patients with acute promyelocytic leukemia (APL), non-APL acute myeloid leukemia (AML), and acute lymphoblastic leukemia (ALL) were measured. Data are shown as box and whisker plots. The box ranges from the first quartile to the third quartile of the distribution. The median is indicated by a line across the box. The whiskers on box plots extend to the minimum and maximum data points. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.



Time point	Patients at risk	Cumulative Incidence (%)	Standard Error
1 month	300	6.0	1.3
12 months	113	12.6	1.9

Risk category	Time point	Patients at risk	Cumulative Incidence (%)	Standard Error
APL	1 month	19	25.0	8.4
	12 months	17	25.0	8.4
Non-APL AML	1 month	211	5.3	1.4
	12 months	68	14.6	2.6
ALL	1 month	70	1.4	1.3
	12 months	28	2.9	2.1

Risk category	Time point	Patients at risk	Cumulative Incidence (%)	Standard Error
1st Tertile	1 month	109	3.5	1.7
	12 months	42	7.9	2.7
2nd Tertile	1 month	97	5.4	2.1
	12 months	35	15.2	3.7
3rd Tertile	1 month	92	9.6	2.8
	12 months	35	15.6	3.7

FIGURE 3 Cumulative incidence of bleeding in patients with different types of acute leukemia. (A) Data from all patients, (B) cumulative incidence by acute leukemia type, and (C) cumulative incidence by tertiles of extracellular vesicle tissue factor activity are shown. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; APL, acute promyelocytic leukemia.

TABLE 3 Biomarkers associated with bleeding risk in acute leukemia.

	Univariable analysis	Multivariable analysis	
Biomarker	sHR (95% CI)	Model 1[a] sHR (95% CI)	Model 2[b] sHR (95% CI)
EVTF activity	**2.32 (1.08-4.99)**	**2.33 (1.08-5.04)**	**2.30 (0.99-5.31)**
PS+ EVs	1.11 (0.57-2.16)	1.11 (0.57-2.17)	1.20 (0.58-2.49)
cfDNA	1.62 (0.80-3.28)	1.64 (0.83-3.26)	1.80 (0.83-3.88)
H3Cit-DNA complexes	0.85 (0.45-1.61)	0.88 (0.46-1.68)	0.94 (0.47-1.88)
PAP	1.55 (0.76-3.18)	1.46 (0.71-3.00)	1.41 (0.66-3.02)
tPA	0.84 (0.44-1.59)	0.78 (0.40-1.53)	0.82 (0.40-1.68)
PAI-1	1.21 (0.62-2.36)	1.18 (0.59-2.36)	1.15 (0.57-2.35)
D-dimer	1.29 (0.64-2.57)	1.21 (0.60-2.47)	NA
Fibrinogen	0.75 (0.40-1.41)	0.72 (0.38-1.38)	NA
PT	**2.54 (1.14-5.68)**	**2.55 (1.15-5.62)**	NA
PTT	0.87 (0.45-1.66)	0.82 (0.42-1.58)	0.83 (0.41-1.65)
WBC count	**2.17 (0.99-4.71)**	**2.30 (1.03-5.13)**	2.30 (0.96-5.53)
Peripheral blood blast cell	1.91 (0.88-4.15)	1.96 (0.90-4.28)	2.20 (0.89-5.40)
Platelet count	0.56 (0.31-1.04)	**0.55 (0.30-1.02)**	NA
Creatinine	1.18 (0.59-2.38)	1.25 (0.53-2.96)	1.48 (0.57-3.85)
Hemoglobin	0.65 (0.35-1.21)	0.68 (0.37-1.25)	0.64 (0.35-1.18)
LDH	**2.89 (1.21-6.90)**	**2.80 (1.12-6.97)**	2.28 (0.92-5.63)

Biomarkers were considered as tertiles and third and second tertile distributions were compared with the first tertile and adjusted for clinically relevant covariates in multivariable models. Bold values indicate statistical significance.

cfDNA, cell-free DNA; EVTF, extracellular vesicle tissue factor; H3Cit-DNA, citrullinated histone H3-DNA; HR, hazard ratio; LDH, lactate dehydrogenase; NA, not applicable as these parameters are components of the disseminated intravascular coagulation score; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin-antiplasmin complex; PS+ EVs, phosphatidylserine-positive extracellular vesicles; PT, prothrombin time; PTT, partial thromboplastin time; sHR, subdistribution hazard ratio; tPA, tissue plasminogen activator; WBC, white blood cell.

[a] Model 1 was adjusted for age, sex, and race/ethnicity.

[b] Model 2 was adjusted for age, sex, race/ethnicity, ISTH disseminated intravascular coagulation score (<5 vs ≥5), comorbidities (0, 1, ≥2), and history of bleeding.

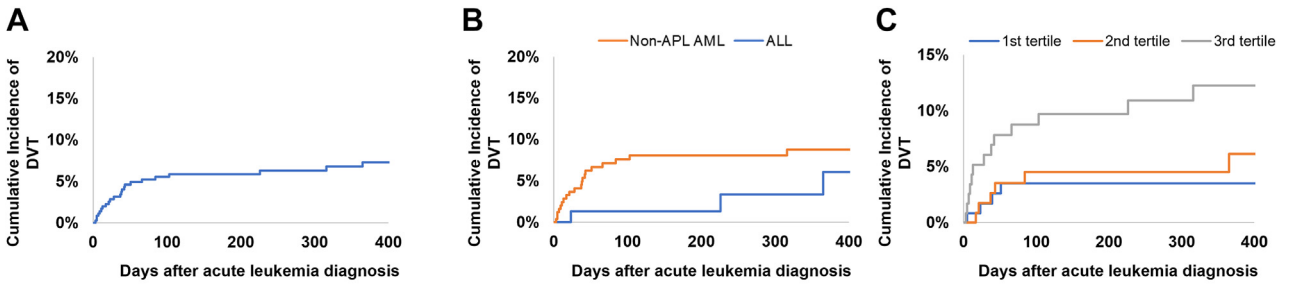
3.6 | Biomarkers of intracranial hemorrhage in acute leukemia patients

Although this subanalysis is underpowered due to small sample size, we found that low levels of tPA (sHR, 0.29; 95% CI, 0.09-0.90) and fibrinogen (sHR, 0.27; 95% CI, 0.09-0.84) were associated with intracranial hemorrhage, and high EVTF activity (sHR, 6.16; 95% CI, 0.82-46.34) was approaching statistical significance in univariate analysis (Supplementary Table S8).

3.7 | VTE risk and associated biomarkers in acute leukemia patients

The characteristics of acute leukemia patients with and without VTE are shown in Supplementary Table S9. Patients with VTE had higher PAI-1 levels compared with those without. The cumulative incidence of overall VTE was 6.5 ± 1.3% at 1 month and 11.3 ± 1.8% at 1 year (Supplementary Figure S2A). The cumulative incidence of VTE at 1 year was 10.4 ± 5.8% in APL, 12.9 ± 2.2% in non-APL AML, and 7.3 ± 3.8% in ALL patients (Supplementary Figure S2B). High PAI-1 levels were associated with increased risk of VTE (sHR, 3.79; 95% CI, 1.40-10.28) in multivariable models adjusting for age at leukemia diagnosis, sex, race/ethnicity, acute leukemia type, BMI, history of VTE, and comorbidities (Supplementary Table S10). The cumulative incidence of VTE at 1 year was 17.5 ± 3.7% in patients with PAI-1 levels in the third tertile of distribution compared with 12.2 ± 3.3% in the second tertile and 4.4 ± 1.9% in the first tertile (Supplementary Figure S2C). High levels of cfDNA (sHR, 0.53; 95% CI, 0.28-1.00) and tPA (sHR, 0.45; 95% CI, 0.24-0.86) were associated with a decreased risk of VTE in univariable analysis but did not reach statistical significance in a multivariable model (Supplementary Table S10).

When restricting the analysis to only DVT events (excluding superficial vein thrombosis), we found that the cumulative incidence of DVT was 3.1 ± 0.9% at 1 month and 7.3 ± 1.5% at 1 year (Figure 4A).



Time point	Patients at risk	Cumulative Incidence (%)	Standard Error
1 month	311	3.1	0.9
12 months	118	7.3	1.5

Risk category	Time point	Patients at risk	Cumulative Incidence (%)	Standard Error
Non-APL AML	1 month	215	4.1	1.3
	12 months	72	8.8	1.9
ALL	1 month	72	1.4	1.4
	12 months	27	6.1	3.6

Risk category	Time point	Patients at risk	Cumulative Incidence (%)	Standard Error
1st Tertile	1 month	107	1.7	1.2
	12 months	42	3.5	1.7
2nd Tertile	1 month	106	1.8	1.2
	12 months	45	4.5	2.0
3rd Tertile	1 month	98	6.1	2.2
	12 months	31	12.3	3.3

FIGURE 4 Cumulative incidence of deep vein thrombosis (DVT) in patients with different types of acute leukemia. (A) Data from all patients, (B) cumulative incidence by acute leukemia type (data of acute promyelocytic leukemia [APL] are not shown because there are no DVT patients in the APL group), and (C) cumulative incidence by tertiles of plasminogen activator inhibitor-1 levels are shown. AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

The cumulative incidence of DVT at 1 year was 0% in APL, 8.8 ± 1.9% in non-APL AML, and 6.1 ± 3.6% in ALL patients (Figure 4B). High PAI-1 levels were associated with increased risk of DVT (sHR, 3.00; 95% CI, 0.95-9.47; Table 4) in multivariable models adjusting for age at leukemia diagnosis, sex, race/ethnicity, acute leukemia type, BMI, history of VTE, and comorbidities. The cumulative incidence of DVT at 1 year was 12.3 ± 3.3% in patients with PAI-1 levels in the third tertile of distribution compared with 4.5 ± 2.0% in the second tertile and 3.5 ± 1.7% in the first tertile (Figure 4C).

3.8 | Biomarkers of bleeding and DVT risk in non-APL AML patients

In a subanalysis of patients with non-APL AML, EVTF activity (sHR, 2.35; 95% CI, 0.98-5.66), WBC count (sHR, 2.42; 95% CI, 1.04-5.62), and LDH (sHR, 3.50; 95% CI, 1.34-9.17) were associated with bleeding risk. High PAI-1 levels were associated with VTE (sHR, 4.18; 95% CI, 1.47-11.87) and DVT risk (sHR, 3.17; 95% CI, 0.93-10.79; Supplementary Table S11).

4 | DISCUSSION

In this large cohort study, we found high rates of ISTH overt DIC scores in non-APL AML and ALL patients, in addition to APL patients. We found alterations in levels of several activators and inhibitors of the coagulation and fibrinolytic pathways along with NET markers in patients with acute leukemia compared with normal controls. Elevated WBC, LDH, PT, and EVTF activity were associated with a high risk of bleeding, and PAI-1 was associated with a high risk of DVT in acute leukemia patients.

TF expression by PBMCs and TF-bearing EVs is central to the pathogenesis of DIC in AML, especially in APL subtype [27,38,39]. In a previous study, PBMCs (including blast cells) from AML patients had higher levels of TF procoagulant activity compared with PBMCs from ALL patients [27]. In addition, PBMCs from patients with APL had the highest levels of TF procoagulant activity compared with other subtypes of AML. These data are consistent with our findings that EVTF activity levels were highest in APL compared with other types of acute leukemia. In contrast to our results, Sakata et al. [43] observed lower PAI-1 activity in APL patients with DIC compared with that in normal controls. One possible explanation is that they used an in-house assay to measure PAI-1 activity, whereas we used a commercial assay for active PAI-1. The data need to be confirmed in a larger study. APL cells express annexin A2 [64] and S100A10 [65], which form a heterotetrameric complex [53,54]. This complex functions as a receptor for both tPA and plasminogen to effectively generate plasmin in a fibrin-free mechanism on the cell surface [66]. This may explain our results of significantly lower plasma levels of tPA in APL patients compared with those in normal controls and the APL and non-APL AML groups. More specifically, the majority of tPA may bind to the annexin A2/S100A10 complexes on APL cells, resulting in low plasma levels of tPA in APL patients. This hypothesis needs to be investigated in future studies.

We found high levels of cfDNA in acute leukemia patients, consistent with previous studies [27,45]. We also observed low levels of H3Cit-DNA complexes in leukemia patients. This is consistent with previous studies demonstrating that granulocytes and neutrophils from acute leukemia patients have reduced capacity to form NETs compared with those from normal controls [52–54]. It is also notable

TABLE 4 Biomarkers associated with deep vein thrombosis risk in acute leukemia.

Biomarker[a]	Univariable sHR (95% CI)	Multivariable analysis[b] sHR (95% CI)
EVTF activity	1.11 (0.46-2.71)	1.15 (0.46-2.92)
PS+ EVs	1.09 (0.45-2.64)	1.13 (0.47-2.71)
cfDNA	0.56 (0.25-1.26)	0.61 (0.26-1.41)
H3Cit-DNA complexes	0.75 (0.32-1.77)	0.66 (0.28-1.56)
PAP	0.75 (0.32-1.74)	0.87 (0.36-2.10)
tPA	0.53 (0.24-1.20)	0.48 (0.19-1.24)
PAI-1	2.43 (0.83-7.13)	**3.00 (0.95-9.47)**
D-dimer	0.72 (0.31-1.69)	0.98 (0.36-2.66)
Fibrinogen	0.81 (0.35-1.85)	0.60 (0.26-1.42)
PT	1.46 (0.58-3.68)	1.51 (0.62-3.65)
PTT	1.38 (0.55-3.48)	1.30 (0.49-3.44)
WBC count	0.96 (0.41-2.24)	0.80 (0.34-1.92)
Peripheral blood blast cell	0.57 (0.23-1.39)	0.80 (0.30-2.14)
Platelet count	1.46 (0.58-3.69)	1.49 (0.57-3.89)
Creatinine	1.11 (0.44-2.79)	1.39 (0.51-3.79)
Hemoglobin	1.50 (0.59-3.80)	1.38 (0.52-3.64)
LDH	0.90 (0.38-2.13)	0.79 (0.33-1.89)

Bold value indicate statistical significance.

cfDNA, cell-free DNA; EVTF, extracellular vesicle tissue factor; H3Cit-DNA, citrullinated histone H3-DNA; LDH, lactate dehydrogenase; PAI-1, plasminogen activator inhibitor-1; PAP, plasmin-antiplasmin complex; PS+ EVs, phosphatidylserine-positive extracellular vesicles; PT, prothrombin time; PTT, partial thromboplastin time; sHR, subdistribution hazard ratio; tPA, tissue plasminogen activator; WBC, white blood cell.

[a] Biomarkers were considered as tertiles and third and second tertile distributions were compared with the first tertile and adjusted for clinically relevant covariates in multivariable models.

[b] Multivariable model was adjusted for age, sex, race/ethnicity, leukemia type, body mass index, history of venous thromboembolism, and comorbidities.

that APL patients had lower WBC counts, which might also affect the levels of H3Cit-DNA complexes in APL patients.

We also found that acute leukemia patients with high EVTF activity had higher WBC count and peripheral blood blast percentage compared with patients with mid-to-low EVTF activity. These data suggest that WBC and blast cells are the sources of TF + EVs in these patients. Similarly, acute leukemia patients with high EVTF activity had higher D-dimer compared with patients with mid-to-low EVTF activity. These data suggest that EVTF activity is associated with activation of coagulation and fibrinolysis. The hypothesis regarding the association between EVTF activity and fibrinolysis is that TF + EVs activate coagulation that generates cross-linked fibrin, which provides a scaffold for tPA and plasminogen to enhance fibrinolysis and the generation of D-dimer. In contrast, there is no difference in platelet counts between acute leukemia patients with high EVTF activity and mid-to-low EVTF activity, suggesting that EVTF activity is not associated with platelet count.

In addition to the known high risk of DIC in APL, we also found high rates of DIC in non-APL AML and ALL patients. While the majority of bleeding and DVT complications occurred early in the clinical course of APL, we found a continued risk of these complications in non-APL AML beyond the first month. We also compared levels of biomarkers between patients with overt DIC and those without DIC. In APL and non-APL AML patients, EVTF activity was significantly higher in patients with overt DIC compared with patients without DIC. There are 2 possible interpretations of these data. The first is that TF + EVs are the major driver of overt DIC in these patients. The second is that EVTF activity may reflect more severe disease. In APL and non-APL AML patients, WBC count was significantly higher in overt DIC patients compared with patients without DIC, suggesting that leukocytosis is associated with overt DIC in APL and non-APL AML patients. In addition, peripheral blood blast cell percentage and LDH level were significantly higher in non-APL AML patients with overt DIC compared with patients without DIC. Collectively, these data suggest that leukemia cell burden and disease severity are associated with overt DIC in these patients. Interestingly, levels of cfDNA and PTT were significantly higher in non-APL AML patients with overt DIC compared with patients without DIC. This needs to be further investigated. Importantly, the incidence of bleeding was significantly higher in non-APL AML patients with overt DIC compared with those without DIC. We also found lower levels of PS+ EVs in ALL patients with overt DIC compared with ALL patients without DIC. In this regard, it is notable that WBC counts were lower and platelet counts were significantly lower in ALL patients with overt DIC compared with those in ALL patients without DIC. Altogether, we believe that the total number of PS+ EVs is low in ALL patients with overt DIC because the sources of PS+ EVs, such as WBC and platelets, are low. PAP levels and D-dimer were significantly higher in ALL patients with overt DIC compared with those in patients without DIC, supporting the conclusion that ALL patients with overt DIC have greater activation of fibrinolysis. In addition, we found that hemoglobin levels were significantly lower in ALL patients with overt DIC than those without DIC.

Libourel et al. [67] showed that the ISTH overt DIC score and D-dimer significantly predicted venous and arterial thrombosis in non-APL AML patients. In our study, we did not find an association between D-dimer and DVT in non-APL AML patients. One notable difference between the study by Libourel et al. [67] and our study is that they included both arterial and venous thrombosis, whereas we excluded arterial events. Our study also had a higher event rate, which may have contributed to the different results in the 2 studies.

To our knowledge, this is the largest cohort study evaluating the association between different biomarkers of activation and inhibition of the coagulation and fibrinolytic pathways with bleeding and DVT in acute leukemia patients. We found an association between levels of EVTF activity and risk of bleeding and between PAI-1 levels and DVT in acute leukemia patients. We hypothesize that TF + EVs contribute to overactivation of coagulation, which can ultimately result in

bleeding due to consumption of coagulation factors in acute leukemia patients. In contrast, PAI-1 inhibits fibrinolysis, which may contribute to DVT. Our data suggest that acute leukemia patients may have a sensitive balance between bleeding and DVT, which is driven by EVTF activity and PAI-1, respectively. It is also notable that in our study, acute leukemia patients showed much higher EVTF activity than levels reported in previous smaller studies [39,40]. One possible explanation is that different anticoagulants were used to draw blood. In our study, we used EDTA, whereas previous studies used sodium citrate [39,40].

There are some limitations in the current study. First, there were only 30 normal control plasma, although we had a total of 358 acute leukemia patients' plasma. Second, DVT and bleeding are dynamic events in the clinical course of leukemia patients, and this risk is determined by several patient- and treatment-related factors that change with time. Our study did not examine the role of chemotherapy, central venous catheters, and other complications, such as infection, which might contribute to DVT and/or bleeding risk. Since the majority of the patients included in the study had central venous catheters for administration of chemotherapy, this variable could not be included as a potential risk factor for VTE in analytic models. We were also not able to capture laboratory parameters at the time of DVT and bleeding. Smaller event rates also preclude examining the associations between biomarkers and outcomes in subtypes of leukemia. Third, 27 out of 29 APL patients received at least 1 dose of ATRA treatment before sample collection as ATRA is usually administered immediately upon suspicion of APL. Previous studies have shown that ATRA reduces TF protein levels in APL cells [28,31–34]. Therefore, EVTF activity in APL patients might have been affected by ATRA treatment in our study. However, blood sample was obtained before administration of other forms of chemotherapy. Fourth, the number of no-DIC patients in the APL group was only 3, which can skew the results. Fifth, only VTE and bleeding events occurring after leukemia diagnosis and blood collection were included. This resulted in the exclusion of 5 bleeding and 3 VTE events that could affect the results. Some of the bleeding and VTE events may have been missed if the patients were treated outside of our institution. However, we expect this to be a small number as we reviewed records from clinic visits and outside records for source documentation if they were scanned in our medical records.

In summary, patients with acute leukemia have high rates of DIC, irrespective of the subtype. They have several abnormalities in biomarkers of the coagulation and fibrinolytic pathways compared with normal individuals. Our data show that high EVTF activity, WBC, PT, and LDH were associated with increased risk of bleeding in acute leukemia patients. High levels of PAI-1 were associated with increased DVT risk. Routine laboratory results that were identified to be associated with bleeding risk can help in risk stratification of patients. In addition, TF and PAI-1 were identified as novel biomarkers of bleeding and DVT, respectively. Future studies measuring biomarkers in serial samples may give more insights into the impact of chemotherapy on these biomarkers and their role in the coagulopathy in acute leukemia because this will give a shorter time between the sample collection and the event compared with measurement of a single sample at diagnosis.

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AUTHOR CONTRIBUTIONS

Y.H., N.M., and R.G. designed the study; S.J.A. performed the experiments; K.B., S.D., N.B., and L.H. helped with data collection; Y.H., S.J.A., and Y.C. performed statistical analyses; Y.H. and R.G. wrote the article, which was reviewed and edited by all other authors.

DECLARATION OF COMPETING INTERESTS

R.G. has served as a consultant for Advisory Boards for Alexion, Takeda, and Sanofi and received an honorarium for presenting a webinar from Sanofi. The other authors declare no competing financial interests.

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SUPPLEMENTARY MATERIAL

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