Platelet functional abnormalities and clinical presentation in pediatric patients with germline *RUNX1*, *ANKRD26*, and *ETV6* mutations

Familial platelet disorder with propensity to acute myelogenous leukemia (FPD/AML) associated with mutations in the *RUNX1* gene, *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT), and *ETV6*-related thrombocytopenia (*ETV6*-RT) are inherited thrombocytopenias characterized by a moderate decrease in platelet number, normal mean platelet size and the predisposition to hematologic malignancies.[1-3] The assessment of bleeding risk in inherited thrombocytopenias is sometimes controversial because these conditions are associated not only with low platelet counts, but also with platelet functional abnormalities.[1] The mechanisms of platelet dysfunction in FPD/AML, *ANKRD26*-RT and *ETV6*-RT require further investigation. The risk of clonal evolution in inherited thrombocytopenias is another unresolved problem. This manuscript describes the clinical and hematologic phenotypes and platelet functional characteristics of 24 children with FPD/AML, *ANKRD26*-RT and *ETV6*-RT.

We performed a retrospective analysis of pediatric patients referred to our Center between 2013 and 2020. Peripheral blood samples from patients, their family members, and age-matched healthy controls were obtained with written informed consent. The study was approved by the local ethics committee (approval number 12э/4-21, 21.12.21) and conducted in accordance with the Declaration of Helsinki.

We used the International Society on Thrombosis and Hemostasis Bleeding Assessment Tool (ISTH BAT) scale[4] and Pediatric Bleeding Questionnaire (PBQ)[5] to assess bleeding tendency. Genetic studies were performed by Sanger sequencing or next-generation sequencing with MiSeq/NextSeq (Illumina, USA) using a custom target panel "Hemostasis". Multiplex ligation-dependent probe amplification analysis was used to detect gross deletions and duplications in *RUNX1* and *ETV6* genes. Light transmission aggregometry,[6] as well as endpoint (platelet functional activity assay[7-10]) and continuous (platelet signaling study[9,11]) flow cytometry were performed as described previously.

Fourteen children from 12 families were found to have *RUNX1* mutations (Table 1). Seven mutations were novel variants. In eight cases from five families, mutations were detected in the *ANKRD26* 5' untranslated region domain (Table 1). Two children had *ETV6* mutations. The new variants were considered to be causative according to American College of Medical Genetics criteria, segregation of the identified variants in the pedigrees and clinical data. Our patients had isolated mild-to-moderate thrombocytopenia with mild bleeding phenotypes, consistent with previously published findings.[2,12]

Twelve of 14 patients with FPD/AML had a family history of thrombocytopenia. There were four cases of acute myelogenous leukemia, one case of acute lymphoblastic leukemia and five cases of acute leukemia of unknown phenotype in the patients' pedigrees. In our pediatric cohort, there was one case of acute myelogenous leukemia. Patient 5 was a girl with a c.388delG variant in exon 5 of *RUNX1*. At the age of 10 years, she developed acute myelogenous leukemia with *BCR-ABL1* and co-expression of CD19, CD22 and an additional somatic *FLT3* mutation. She received chemotherapy and achieved complete remission. After an early relapse, she underwent hematopoietic stem cell transplantation, but died from the second relapse.

In four of the five families with *ANKRD26*-RT, relatives with thrombocytopenia were known. In these pedigrees thrombocytopenia was observed in two (1 family), three (2 families), and four (1 family) generations. There were no cases of acute leukemia in these families.

One of the patients with *ETV6*-RT had a family history of thrombocytopenia without known cases of acute leukemia. The second patient had several features of immunodeficiency (2 episodes of pneumonia before the age of 1 year, IgA <0.15 g/L, IgG 1.74 g/L). His mother had a confirmed *ETV6* mutation with adequate platelet count. This patient developed B-cell acute lymphoblastic leukemia at the age of 2 years. He responded to chemotherapy and achieved complete remission. While a germline *ETV6* mutation was revealed after completion of chemotherapy, this patient received the standard treatment regime. At the time of writing, he is still in complete remission for more than 5 years without having undergone allogeneic hematopoietic stem cell transplantation.

Aggregometry data were available for seven patients with FPD/AML and revealed diminished platelet aggregation upon stimulation with collagen (Figure 1A), protease-activated receptor 1 activating peptide (PAR1-AP), with no second wave (Figure 1B), and adrenaline (Figure 1C), predominantly decreased aggregation upon stimulation with adenosine diphosphate (ADP) (Figure 1D), and variably normal aggregation upon stimulation with ristocetin (Figure 1E). Aggregometry was performed in four patients with *ANKRD26*-RT and revealed decreased maximum aggregation in response to both PAR1-AP (Figure 1B) and adrenaline (Figure 1C), while aggregation upon stimulation with collagen (Figure 1A) was predominantly normal. A signifi-

Table 1. Characteristics of patients with *RUNX1* (n=14), *ANKRD26* (n=8) and *ETV6* (n=2) mutations.

Pt #	Sex	Mutation	Age,[a] years	Platelet count x10^9/L, median (range)	ISTH BAT/PBQ	Family history	At last follow-up	
							Age, years	Status
Patients with germline *RUNX1* mutations								
1	M	Exon 9 c.1087_1088dup p.I364Afs*231	Birth	128 (20-210)	0/0	Father (thrombocytopenia, AML)	4.8	Alive
2	F	Exon 5 c.496C>G p.R166G	0.5	139 (113-159)	4/4	Father (asymptomatic mutation carrier)	4.0	Alive
3	F	Exon 4 c.292C>T p.L98F	2	107 (53-140)	0/0	Father (thrombocytopenia), grandfather (thrombocytopenia, AL)	13.5	Alive
4	F	Gross duplication of exons 4–6	Birth	106 (100-150)	4/4	Grandmother (thrombocytopenia, AL); mother (thrombocytopenia, ALL, HSCT)	7.9	Alive
5	F	Exon 5 c.388delG p.V130Sfs*3	Birth	63 (50-70)	1/1	Mother (thrombocytopenia, cervical cancer), grandmother (thrombocytopenia), great-uncle (thrombocytopenia, AL), great-grandfather (thrombocytopenia), great-great-grandmother (thrombocytopenia, AL)	11.1	Dead[c]
6	F	Intron 6 c.613+1delG p.?	Birth	121 (81-170)	1/1	-	3.7	Alive
7	F	Gross deletion of exons 1–6	Birth	89 (50-113)	1/1	-	2.6	Alive
8	M	Exon 6 c.520A>C p. T174P	3.5	142 (80-180)	0/0	Sibling (Pt #9); mother and grandfather (thrombocytopenia), great-grandfather (AL), uncle (AML at age 5 years, died), aunt (AML at age 19 years, HSCT, CR)	13.1	Alive
9	M	Exon 6 c.520A>C p. T174P	Birth	116 (80-160)	1/1	Sibling (Pt #8)	3.7	Alive
10	M	Exon 5–Intron 5 c.497_508+10del p.?	Birth	64 (36-128)	1/1	Father (thrombocytopenia)	2.1	Alive
11	M	Exon 6 c.601C>T p.R201*	Birth	67 (45-192)	2/2	Sibling (Pt #12),[b] mother and father (mutation not detected)	3.3	Alive
12	M	Exon 6 c.601C>T p.R201*	Birth	62 (50-151)	2/2	Sibling (Pt #11)[b]	3.3	Alive
13	M	Exon 8 c.921delC p.S308Afs*3	Birth	98 (76-200)	2/2	Father (thrombocytopenia)	9.6	Alive
14	M	Exon 5 c.497 G>A p.R166Q	2	106 (60-140)	0/0	Father, aunt, grandmother (thrombocytopenia)	14.7	Alive
Patients with germline *ANKRD26* mutations								
1	F	Exon 1 (5' UTR) c.-118C>A regulatory	4	56 (46-173)	5/5	Sibling (Pt #2), father, grandmother (thrombocytopenia)	8.8	Alive
2	M	Exon 1 (5' UTR) c.-118C>A regulatory	1.5	52 (30-71)	3/3	Sibling (Pt #1)	4.7	Alive
3	M	Exon 1 (5' UTR) c.-119C>A regulatory	Birth	69 (53-226)	0/0	Mother (thrombocytopenia), grandfather (thrombocytopenia)	3.3	Alive

Continued on following page.

Pt #	Sex	Mutation	Age,[a] years	Platelet count x10^9/L, median (range)	ISTH BAT/ PBQ	Family history	At last follow-up	
							Age, years	Status
Patients with germline *ANKRD26* mutations								
4	F	Exon 1 (5' UTR) c.-128G>A regulatory	0.9	68 (58-100)	0/0	Sibling (Pt #5), father, grandmother (thrombocytopenia)	5.6	Alive
5	F	Exon 1 (5' UTR) c.-128G>A regulatory	0.1	77 (60-152)	2/1	Sibling (Pt #4)	1.1	Alive
6	F	Exon 1 (5' UTR) c.-134G>A regulatory	13	24 (18-57)	6/6	-	15.3	Alive
7	M	Exon 1 (5' UTR) c.-126T>C regulatory	2	28 (25-76)	3/3	Sibling (Pt #8), father (thrombocytopenia)	15.1	Alive
8	M	Exon 1 (5' UTR) c.-126T>C regulatory	0.2	42 (18-94)	1/1	Sibling (Pt #7), father (thrombocytopenia)	9.0	Alive
Patients with germline *ETV6* mutations								
1	M	Exon 6 c.1148A>G p.His383Arg (p.H383R)	1.3	106 (40-120)	2/2	Mother (asymptomatic carrier)	9.3	Alive
2	M	Exon 5 Gross deletion	0.5	115 (100-140)	2/2	Father (thrombocytopenia)	8.7	Alive[d]

RefSeq transcript: *RUNX1* - NM_001754.5, *ANKRD26* - NM_014915.3, *ETV6* - NM_001987.5. [a]At first time of documented thrombocytopenia. [b]Monozygotic twins. [c]Acute myeloblastic leukemia, co-expression of *CD19*, *CD22*, *FLT3* mutation (at the age of 10 years). [d]B-cell acute lymphoblastic leukemia at the age of 2 years; in complete remission for more than 5 years after standard treatment without allogeneic hematopoietic stem cell transplantation. M: male; F: female; ISTH BAT: International Society on Thrombosis and Haemostasis Bleeding Assessment Tool; PBQ: Pediatric Bleeding Questionnaire; AL: acute leukemia; ALL: acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; HSCT: hematopoietic stem cell transplantation; CR: complete remission.

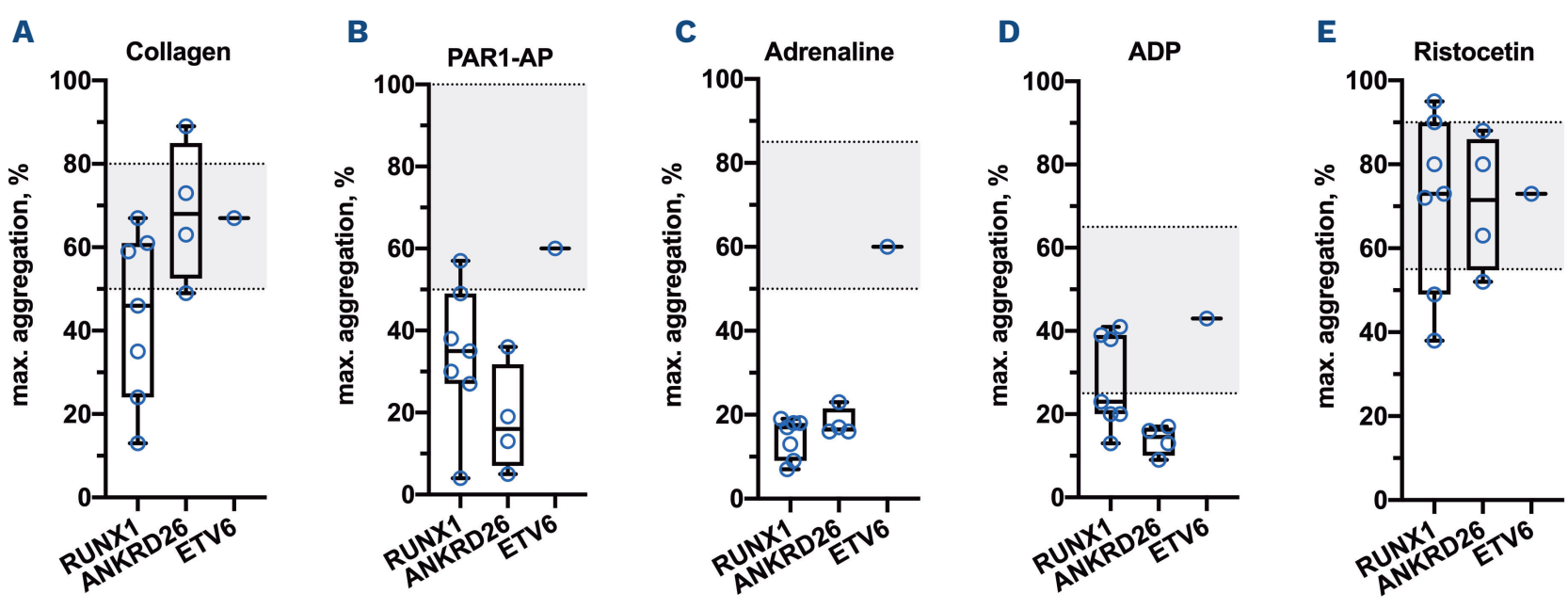

Figure 1. Light transmission aggregometry in patients with *RUNX1*, *ANKRD26*, or *ETV6* mutations upon stimulation with different agonists. (A) Platelet aggregation upon stimulation with 2 mg/mL collagen was predominantly decreased in patients with *RUNX1* mutations. (B, C) Platelet aggregation upon stimulation with 32 μM protease-activated receptor 1 activating peptide (B) and 5 μM adrenaline (C) was diminished in patients with *RUNX1* and *ANKRD26* mutations. (D) Platelet aggregation upon stimulation with 5 μM adenosine diphosphate was decreased in patients with *ANKRD26* mutations. (E) Platelet aggregation upon stimulation with 15 mg/mL ristocetin was within the normal range. The data points are the circle symbols, horizontal lines are medians, boxes show 25th-75th percentiles, error bars show 5-95% intervals, gray color indicates normal ranges. ADP: adenosine diphosphate; PAR1-AP: protease-activated receptor 1 activating peptide.

cantly diminished aggregation to weak stimulation with ADP was also observed in these patients (Figure 1D). In the patient with *ETV6*-RT, platelet aggregation was within normal ranges (Figure 1).

Platelet functional activity flow cytometry was performed in all 14 patients with FPD/AML. Platelet forward scatter was predominantly diminished (Figure 2A), while side scatter (SSC) was within normal ranges (Figure 2B). Flow cytometry revealed significantly impaired annexin V-positive (procoagulant) platelet formation (Figure 2C) upon dual stimulation with collagen-related peptide and PAR1-AP in comparison with that of age-matched, healthy controls. While platelet P-selectin expression in both resting and stimulated states was within normal

ranges (Figure 2D), reduced mepacrine loading (Figure 2E) and decreased dense granule secretion (Figure 2F) indicated defects of the platelet dense granule storage pool in these patients. Flow cytometry also revealed increased platelet GPIb expression (Figure 2G) in both resting and stimulated states. The expression of platelet GPIIb/IIIa (Figure 2H) and its active form (assessed by PAC1 binding) (Figure 2I) was comparable to that of the control cohort.

Platelet functional activity was assayed in seven patients with *ANKRD26*-RT. In resting platelets, no differences were observed in forward scatter (Figure 2A) or side scatter

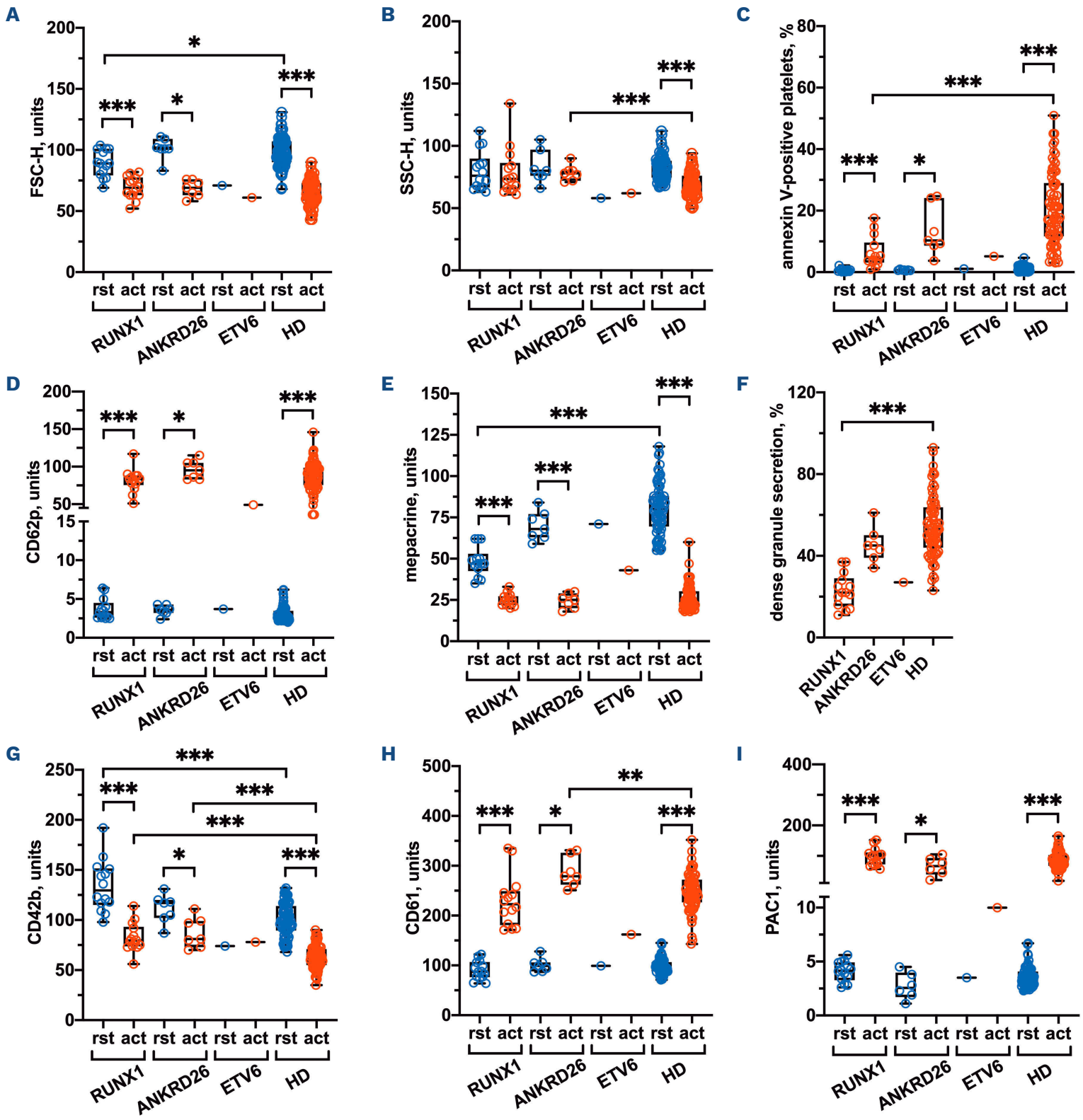

Figure 2. Platelet flow cytometry (platelet functional activity assay) in patients with *RUNX1*, *ANKRD26*, or *ETV6* mutations, and healthy children (in resting state and upon dual stimulation with 20 ?g/?L collagen-related peptide plus 12.5 ?M protease-activated receptor 1 agonist peptide. (A) Diminished forward scatter in patients with *RUNX1* and *ETV6* mutations. (B) Increased side scatter upon platelet stimulation in patients with *ANKRD26* mutations, decreased side scatter of platelets in the resting state in the patient with an *ETV6* mutation. (C) Decreased annexin V-positive (procoagulant) platelet percentage upon stimulation in patients with *RUNX1* mutations. (D) Normal platelet P-selectin expression in both resting and stimulated states. (E) Decreased mepacrine loading of resting platelets in patients with *RUNX1* mutations. (F) Decreased dense granule release upon platelet stimulation in patients with *RUNX1* and *ETV6* mutations. (G) Increased platelet CD42b expression in the resting state in patients with *RUNX1* mutations, increased platelet CD42b expression upon stimulation in patients with *RUNX1* and *ANKRD26* mutations. (H) Increased CD61 expression upon platelet stimulation in patients with *ANKRD26* mutations. (I) Decreased PAC1 binding upon activation in the patient with an *ETV6* mutation. The data points are the circle symbols, horizontal lines are medians, boxes show 25^{th}-75^{th} percentiles, error bars show 5-95% intervals. Blue color represents resting platelets, red color represents stimulated platelets. Statistical significance is shown by asterisks: **P*<0.05, ***P*<0.01, ****P*<0.001, no marking corresponds to non-significant differences. The Mann-Whitney U test was used to compare two independent samples and the Wilcoxon signed-rank test was used to conduct a paired difference test of repeated measurements. act: activated platelets; FSC: forward scatter; HD: healthy donors; rst: resting platelets; SSC: side scatter.

(Figure 2B) between patients and healthy controls, while side scatter upon platelet activation was significantly higher in patients (Figure 2B). These results indicate possible impairment in platelet shape changes upon activation. We observed normal GPIb expression in resting platelets and significantly increased GPIb expression upon platelet activation (Figure 2G). This may indicate impaired platelet GPIb shedding upon activation. We also observed that total GPIIb/IIIa density upon stimulation was significantly higher in patients than in healthy controls (Figure 2H), while the difference in active GPIIb/IIIa (Figure 2I) was insignificant. Procoagulant platelet formation (Figure 2C), dense-granule secretion (Figure 2F) and α-granule secretion (Figure 2D) upon platelet activation seem to be unimpaired in patients with *ANKRD26*-RT.

In the patient with *ETV6*-RT, for whom data were available, flow cytometry revealed decreased forward scatter (Figure 2A) and side scatter (Figure 2B) in resting platelets, diminished GPIIb/IIIa activation (Figure 2I), decreased dense granule secretion (Figure 2F), and impaired GPIb shedding (Figure 2G) upon platelet stimulation.

Platelet signaling studies (continuous flow cytometry) were performed in seven patients with FPD/AML. Cytosolic calcium concentration in resting platelets was increased in three patients, while it was normal in four others (*Online Supplementary Figure S1A*). Platelet cytosolic calcium mobilization (*Online Supplementary Figure S1B*) and fibrinogen binding (*Online Supplementary Figure S1C*) in response to ADP were significantly diminished. Impaired calcium mobilization and fibrinogen binding upon stimulation with PAR1-AP were less pronounced, yet clearly detectable (*Online Supplementary Figure S1D, E,* respectively). Increased cytosolic calcium concentration may indicate platelet pre-activation in these patients. Moreover, platelet pre-activation may explain dense granule storage pool deficiency (Figure 2E) due to premature dense granule release and overall platelet refractoriness in response to both weak (ADP) and strong (PAR1-AP) stimuli.

Platelet signaling studies were performed in only two patients with *ANKRD26*-RT and revealed diminished calcium responses to both ADP (*Online Supplementary Figure S1B*) and PAR1-AP (*Online Supplementary Figure S1D*) as well as a decreased fibrinogen response to PAR1-AP (*Online Supplementary Figure S1E*). In the patient with *ETV6*-RT, we observed normal platelet calcium signaling (*Online Supplementary Figure S1A, B*) and a diminished fibrinogen response upon stimulation with ADP (*Online Supplementary Figure S1C*).

Interestingly, we did not observe significant correlations between bleeding (ISTH BAT and PBQ scores) and platelet count in patients with germline *RUNX1* (Spearman r=0.01, *P*=0.98) (*Online Supplementary Figure S2*) or *ANKRD26* (r=-0.71, *P*=0.09) (*Online Supplementary Figure S3*) mutations. Moreover, we found no correlations between bleeding and maximum aggregation in response to any of the studied agonists (*data not shown*). However, several platelet functional characteristics assessed by flow cytometry correlated with bleeding. In patients with FPD/AML, we observed strong negative correlations between the percentage of procoagulant platelets upon stimulation and both the bleeding scores (r=-0.64, *P*=0.02), strong negative correlations between GPIb expression upon platelet activation and both the scores (r=-0.63, *P*=0.02), and moderate negative correlations between GPIIb/IIIa activation assessed by PAC1 binding and both the scores (r=-0.59, *P*=0.03). Strong negative correlations between the severity of thrombocytopenia and platelet side scatter, both in a resting state (r=-0.86, *P*<0.01) and upon stimulation (r=-0.74, *P*<0.01), were also found. In patients with *ANKRD26*-RT, we observed strong positive correlations between platelet side scatter in an activated state and both the scores (r=0.83, *P*=0.03). Statistical analysis was not performed in patients with *ETV6*-RT because of the small sample size.

Here we have described the clinical and hematologic phenotypes, history of neoplastic progression, and the results of platelet functional studies in pediatric patients with FPD/AML, *ANKRD26*-RT, and *ETV6*-RT. Our observations provide some new data on the pathogenesis of platelet dysfunction in inherited thrombocytopenias. While the results require validation in a larger number of patients, our findings indicate correlations between the severity of platelet function abnormalities and bleeding tendency in these patients. The development of protocols for bleeding risk assessment and management is a promising direction for further studies.

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Disclosures

No conflicts of interest to disclose.

Contributions

GSO concieved and designed the study, GSO, DVF and IPT wrote and revised the manuscript. DVF and PAZ evaluated and followed up the patients. IPT, AAM, AAI and EAP performed platelet function testing. IPT and AAM analyzed and interpreted data. AVP and EVR provided genetic testing. MAP, NSS and ANS reviewed the manuscript and contributed to study conception. MAM and GAN provided administrative, technical, and material support.

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Data-sharing statement

Raw data supporting the findings of this study are available from the author for correspondence (DVF) on request.

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