Molecular phenotype and bleeding risks of an inherited platelet disorder in a family with a \textit{RUNX1} frameshift mutation

M. S. Badin,* J. K. Iyer,* M. Chong,* L. Graf,*1 G. E. Rivard,† J. S. Waye,* A. D. Paterson,‡ G. Pare¶ and C. P. M. Hayward**

*Department of Pathology and Molecular Medicine McMaster University, Hamilton, ON; †Hematology/Oncology Centre Hospitalier Universitaire Sainte-Justine, Montreal, QC; ‡Genetics and Genome Biology, The Hospital for Sick Children; ¶The Dalla Lana School of Public Health and Institute of Medical Sciences, University of Toronto, Toronto; **Hamilton Regional Laboratory Medicine Program and **Department of Medicine, McMaster University, Hamilton, ON, Canada

Introduction: Inherited defects in \textit{RUNX1} are important causes of platelet function disorders. Aim: Our goals were to evaluate \textit{RUNX1}-related platelet disorders among individuals evaluated for uncharacterized, inherited platelet function disorders and test a proof of concept that bleeding risks could be quantitatively estimated for typical families with an inherited platelet function disorder. Methods: Index cases with an uncharacterized inherited platelet function disorder were subjected to exome sequencing with confirmation of \textit{RUNX1} mutations by Sanger sequencing. Laboratory findings were obtained from medical records and persistence of platelet non-muscle myosin heavy chain IIB (MYH10), a biomarker of \textit{RUNX1} defects, was assessed by Western blotting. Bleeding histories were assessed using standardized assessment tools. Bleeding risks were estimated as odds ratios (OR) using questionnaire data for affected individuals compared to controls. Results: Among 12 index cases who had their exomes sequenced, one individual from a family with eight study participants had a c.583dup in \textit{RUNX1} that segregated with the disease and was predicted to cause a frameshift and \textit{RUNX1} haploinsufficiency. Unlike unaffected family members \((n=2)\), affected family members \((n=6)\) had increased bleeding scores and abnormal platelet aggregation and dense granule release responses to agonists but only some had thrombocytopenia and/or dense granule deficiency. This family’s mutation was associated with persistence of MYH10 in platelets and increased risks \((OR 11–440)\) for wound healing problems and mild bleeding symptoms, including bleeding interfering with lifestyle in women. Conclusion: Inherited platelet dysfunction due to a \textit{RUNX1} haploinsufficiency mutation significantly increases bleeding risks.

Keywords: bleeding risks, blood platelet disorders, exome sequencing, \textit{RUNX1} mutation, wound healing

Introduction
Inherited platelet function disorders (IPD) are important disorders with diverse causes that are often associated with increased bleeding symptoms and bleeding scores [1]. Separate from bleeding scores, the risks for experiencing different bleeding symptoms/problems in IPD have rarely been quantified, except for Quebec platelet disorder (QPD) [2]. Nonetheless, many IPD are considered to be mild bleeding disorders [3,4]. Recent studies indicate that dominantly inherited mutations in transcription factors expressed by megakaryocytes, such as \textit{RUNX1}, are important causes of IPD [1,3,5–7]. \textit{RUNX1} influences platelet formation and function [6,8] and some \textit{RUNX1} mutations are associated with hereditary predisposition to myelodysplastic syndrome/leukaemia, defective platelet aggregation and secretion, dense granule deficiency and mild thrombocytopenia [9–19]. Aberrant persistence of platelet MYH10 is a biomarker for IPD caused by \textit{RUNX1} and \textit{FLI1} mutations as non-muscle myosin heavy chain IIB (MYH10) is normally down-regulated during megakaryopoiesis [18–20].
A recent study that reported RUNX1 mutations in 3 of 13 index cases with IPD evaluated by exome sequencing [7] led us to review our findings for a similar cohort study. We uncovered a novel, RUNX1 mutation in 1 of 12 index cases without thrombocytopenia and evaluated family members to test an important proof of concept: that bleeding risks and molecular phenotype could be estimated for a typical family with an IPD.

Materials and methods

The study was conducted with the Hamilton Integrated Research Ethics Board approval in accordance with the recently revised Declaration of Helsinki. All subjects provided written informed consent and identities were anonymized.

Subject recruitment and selection

Index cases with uncharacterized IPD were recruited from consecutive patients seen at Hamilton Health Science (HHS) and tested by the Hamilton Regional Laboratory Medicine Program (HRLMP). Inclusion criteria were as follows:

1. Bleeding problems compatible with IPD, based on the recorded opinion of the patients’ haematologist(s), obtained by medical record review, plus
2. ≥1 of the following abnormalities:
   a. Impaired maximal aggregation (MA) responses to ≥2 agonists by light transmittance aggregation (LTA) that was not from a well-characterized disorder (e.g. Glanzmann thrombasthenia) [21]. Abnormalities were confirmed on another sample if dense granule deficiency was excluded.
   b. Confirmed dense granule deficiency based on whole mount electron microscopy quantification of average platelet dense granule numbers in 30 platelets [21].

Relatives of index cases were invited to participate. Control subjects [22] were recruited for bleeding assessment tool (BAT) and Western blot analyses.

Genomic DNA isolation

Genomic DNA from EDTA anticoagulated whole blood was isolated using Qiagen QIAmp DNA blood Maxi Kits (QIAGEN, Courtaboeuf, France).

Whole exome sequencing and variant annotation

Exome sequencing of index case samples was performed using 300 ng of DNA, Illumina TrueSeq Exome Enrichment Kits and a HiSeq1500 sequencer to obtain 100 bp paired end reads. Read pairs were mapped to the hg19 human reference genome using the Burrows Wheeler Aligner [23] and processed through the standard Genome Analysis Tool Kit (GATK) protocol (Unified Genotyper variant caller) to obtain calls for single nucleotide variants (SNV) and insertions/deletions (indel) [24]. Duplicate reads were removed using Picard Tools (http://picard.sourceforge.net), followed by base quality score recalibration, fine-tuning by local realignment, variant calling and variant quality score recalibration using GATK. Variant annotation and filtering were performed under the KGGSEQ v0.8 framework to examine only rare protein-altering mutations. RefSeq transcripts were used to define gene boundaries and mutation effects [25]. Protein-altering mutations were categorized as: missense, splice site, nonsense, stoploss, nonframeshift indels and frameshift indels. A minor allele frequency (MAF) threshold of 0.01 was applied to define ‘rare variants’ using frequencies from both internal (~350 exomes) and external (NHLBI Exome Sequencing Project 6500 [26], 1000G [27]), Exome Aggregation Consortium [http://exac.broadinstitute.org/ (August, 2015)] sequence databases. The effect of the mutation at the protein level was predicted with EMBOSS TRANSEQ software (http://www.ebi.ac.uk).

Genomic DNA analysis by PCR and Sanger sequencing analysis

Mutations were confirmed by polymerase chain reaction (PCR) amplification of the relevant region and Sanger sequencing. PCR was performed on a Biometra thermocycler (Biometa, Göttingen, Germany) using 0.1–1 μg of template DNA, 25 μL Thermo Scientific Dream Taq PCR master mix (Thermo Fisher Scientific, Burlington, Canada) and 1 μL of 10 μM primers (Mobix, Hamilton, Canada). Sets of forward (F) and reverse (R) primers used to verify RUNX1 mutations included: (i) For the c.583dupA mutation: F: 5′-TCT GAG ACA TGG TCC CTGAG T-3′ and R: 5′-TAT GTT CAG GCC ACC AAC CTC-3′; and (ii) For the C737T mutation: F: 5′-AGATGATCAGACAAGCCCG -3′ and R: 5′-CTCCATCGGTACCCTGC-3′. PCR products were purifed using MinElute PCR Purification Kits (QIAGEN) and assessed on a Nanodrop 2000c (Thermo Scientific, Boston, MA, USA) to confirm acceptable purity. Sequences obtained (from MOBIX Lab, Hamilton, ON, Canada) were compared to RUNXI transcript NM_001754 (isoform AML1c).

Bleeding history assessment

Subjects and general population controls (40 females, 20 males to obtain data for ≥40 subjects for each symptom/problem) with similar ages to affected subjects were evaluated using: (i) International Society for Thrombosis and
Haemostasis BAT (ISTH-BAT) and (ii) CHAT-P, a clinical history assessment tool for IPD (Appendix S1) that was completed by subjects (or parent if a young child) for review by a haematologist. Control subject ISTH-BAT data were described previously [28]. Subjects were contacted to clarify discrepancies.

Clinical laboratory data

Patient subjects’ blood counts, mean platelet volumes (MPV), bone marrow findings (if performed) and findings for validated assays for dense granule deficiency [21], LTA [29] and dense granule ATP release [22,30] were obtained from HRLMP records, including: (i) MA responses to 2.5 and 5.0 μM adenosine diphosphate (ADP), 1.25 and 5.0 μg mL⁻¹ Horm collagen, 6.0 μM epinephrine, 1.6 mM arachidonic acid, 1.0 μM thromboxane analogue U46619 and 0.5 and 1.25 mg mL⁻¹ ristocetin and (ii) n x of dense granule ATP release in response to: 1 U mL⁻¹ thrombin (IIa), 5.0 μg mL⁻¹ Horm collagen, 6 μmol L⁻¹ epinephrine, 1.6 mmol L⁻¹ arachidonic acid and 1.0 μmol L⁻¹ thromboxane analogue U46619. For dense granule EM, an updated RI of 4.9–10.0 dense granules/platelet was used, based on a non-parametric estimate of 99% confidence intervals (CI) for 124 tests on 33 control subjects.

Immunoblot analysis of platelet MYH10

Aberrant persistence of non-muscle myosin heavy chain IIB (MYH10) in platelets was evaluated using washed platelet samples (6 μL of 1 x 10¹⁰ platelets mL⁻¹) from subjects consenting to additional donations. Platelets were isolated, washed and solubilized in lysing buffer containing protease inhibitors (as described [20]) and 2% sodium dodecyl sulphate (SDS), reduced and separated on 6% SDS-polyacrylamide gels, before transfer to nitrocellulose membranes. Membranes were cut between 50 and 75 kDa markers before probing larger proteins for MYH10 (1:1000 dilution rabbit anti-human MYH10; Cell Signalling, Danvers, MA, USA), followed by 1:40 000 HRP-conjugated donkey anti-rabbit IgG; Jackson ImmunoResearch Inc., Baltimore, MD, USA, visualization using SuperSignal™ West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific, Waltham, MA, USA) and smaller proteins for β-actin (1:5000 dilution of HRP-conjugated rabbit anti-human β-actin; Cell Signalling, Danvers, MA, USA, visualization with Immobilon Western Chemiluminescent HRP Substrate; Millipore Corporation, Billerica, MA, USA).

Statistical analysis

Two-tailed Mann–Whitney tests were used to compare affected to unaffected family members. ANOVA were used to compare multiple groups. Odds ratios (OR) with 95% CI were used to estimate likelihoods for bleeding symptoms/problems [2], using CHAT-P data for individuals with RUNXI mutations and general population controls. OR were estimated using GRAPHPAD 6.0 (GraphPad Software Inc., San Diego, CA, USA) after adding 0.5 to all contingency table cells with 0 values, as recommended [31]. P-values <0.05 were considered statistically significant.

Results

Among the consecutive index cases (one declined participation) whose exome sequencing was completed by April 2016, one of the twelve (all without thrombocytopenia) had RUNXI sequence changes. This proband from a French Canadian family with eight study participants (median ages: 25.5 years; range 1–69) (Fig. 1a), had two RUNXI sequence changes:

1. single base pair duplication (A) in exon 6 (c.583dup) on chromosome 21 at base (http://www.ensembl.org/) (Fig. 1b), predicted to cause a frameshift beginning at position Ile195 and introduce a premature termination codon 17 positions downstream (p.Ile195Asnfs*18), truncating RUNXI at amino acid 211 instead of 480. AAAA insertion at this site was demonstrated to cause RUNXI haploinsufficiency [32]. The c.583dup variant was not found in the Exome Aggregation Consortium (ExAC) [33] or Catalogue of somatic mutations in cancer (COSMIC) [34] databases.

2. single base pair substitution (G>A) in exon 7 (21:34834478 within Genome Reference Consortium Human Build 38) (Fig. 1c), predicted to change amino acid 246 from Thr to Met (p.T246M), rs555366994 [35], with an allele frequency of <0.0001 based on Exome Aggregation Consortium (ExAC) data [33]. This mutation was identified in ClinVar (accessible at: http://www.ncbi.nlm.nih.gov/clinvar/variation/239054/) and was observed once in Luhya Webuye, Kenya from the 1000 genomes project, and in six heterozygotes from 60 642 in ExAC.

Sanger sequencing indicated that six family members were heterozygous for both c.583dup and rs555366994 (Fig. 1a,b) whereas the other two had neither (Fig. 1a,b), consistent with both being on the same haplotype. These data implicated c.583dup as the pathologic mutation as it introduces a stop codon upstream of rs555366994.

All family members with c.583dup (n = 6) had symptoms typical of an IPD, reflected by higher ISTH-BAT scores (median: 8.5, range 4–15) than unaffected family members (median: 0.5, range: 0–1) and unrelated controls (median: 0, range: 0–6) (P<0.01)
Their most severe ISTH-BAT scores were for epistaxis, menorrhagia, dental extractions, cutaneous and CNS (subdural haematoma at birth) bleeding (Fig. 2b). ISTH-BAT scores were similar for affected individuals with or without thrombocytopenia (median: 8 vs. 9) or dense granule deficiency (median: 9 vs. 8). Only the maternal aunt of the proband’s father (deceased, mutation status unknown) was known to have developed leukaemia or myelodysplasia.

Laboratory findings for the family with the RUNX1 mutation

Among affected family members \( (n = 6) \), one had mild anaemia \( (\text{subject I.1: haemoglobin 102–117 g L}^{-1}) \) and two had mild thrombocytopenia \( (\text{platelets} <150 \times 10^9 \text{ L}^{-1}) \); first HRLMP platelet counts, affected members, median: 164; range: 125–169) (Fig. 3a). Excluding counts that dropped during an infection, affected individuals’ platelet counts varied...
8–27% over time. All family members had low MPV (lower RI limit 7.4; ranges, affected: 6.0–7.3; unaffected: 6.9 and 7.0).

Three affected individuals had bone marrow examinations. The proband’s father had chronic mild anaemia and a hypercellular marrow with dysplastic erythropoiesis (ring sideroblasts: >50% of erythrocyte precursors). The proband had a hypercellular bone marrow with some dysplastic megakaryocytes and erythrocyte precursors, grade 3–4 haemosiderin staining (normal: 1–3) and a number of type II sideroblasts. Her brother (subject II.5, assessed months after transient pancytopenia) had a normocellular bone marrow with reduced megakaryopoiesis, increased promyelocytes and grade 3–4 haemosiderin staining.

Three of the six affected individuals had mild dense granule deficiency (RI: 4.9–10.0; range for affected: 4.0–6.0; unaffected: 7.5) (Fig. 3a). Affected individuals showed reduced MA with 1.25 µg mL⁻¹ collagen and 1.0 µM thromboxane analogue U46619 (S/5) and arachidonic acid (4/5). Only one showed reduced MA with ristocetin (Fig. 3b). Affected individuals had reduced dense granule ATP release with all evaluated agonists (5/6 tested with all agonists), unlike unaffected relatives (Fig. 3c) and they also had abnormal persistence of MYH10 in platelets (n = 4 evaluated, Fig. 3d).

**Bleeding risk estimates for individuals with the RUNX1 mutation**

Figures 4 and 5, and Table S1 and Figures S1 and S2 (contained within data Appendix S2) summarize CHAT-P findings. CHAT-P findings were similar for male and female controls except proportionately more females reported anaemia, iron deficiency and treatment with iron (9/40 vs. 0/20; P = 0.02) (Table S1).

Affected individuals (who completed questionnaires before genetic testing) had a higher likelihood of reporting first degree relatives with: bleeding problems (OR = 300; 95% CI, 13–7100; P < 0.0001) including bleeding problems causing death or serious complications (OR = 29; 95% CI, 3.4–240; P = 0.0041); thrombocytopenia (OR = 440; 95% CI, 16–12 000; P < 0.0001); and leukaemia/bone marrow problems (OR = 29; 95% CI, 3.4–240; P = 0.0041) (Fig. 4, Table S1).

Most affected individuals (5/6) reported abnormal bleeding before 18 years. Compared to controls, affected individuals had a higher likelihood for
experiencing symptoms (OR: 11–440) (Fig. 5, Table S1) including: bleeding requiring lifestyle changes in women (OR = 130; 95% CI, 4.2–4100; P = 0.004) but not men; prolonged bleeds from cuts/ minor wounds lasting >10 min (OR = 300; 95% CI, 16–5500; P < 0.001) or > an hour (OR = 120; 95% CI, 5.2–2800; P = 0.0004), none requiring medical attention; and prolonged nosebleeds (>15 min) (OR 19; 95% CI 2.6–140; P = 0.007), sometimes requiring packing or cauteryization (OR 121; 95% CI 5.2–2800; P = <0.001) but rarely hospital admission. They also had an increased likelihood for experiencing numerous bruises (>2 or 3 bruises at one time, OR 95 and 58 respectively) and bruises that were as follows: very large, appeared without reason, disproportionate to trauma, lumpy and/or left permanent marks (OR 11–67) (Fig. 5, Table S1). Their likelihood of experiencing wound healing problems after injuries/surgery/ dental procedures was increased (OR 38; 95% CI 4.9–300; P = <0.001) as were their likelihoods for experiencing: excessive oral or dental bleeding (OR 77; 95% CI 5.3–1100; P = 0.001), excessive surgical bleeding (OR 15; 95% CI 1.6–150; P = 0.04) and for being given/recommended medications to prevent bleeding (OR 220; 95% CI 9–5300; P = <0.001) (Fig. 5, Table S1). Most affected individuals who had undergone dental procedures (2/3) reported abnormal bleeding with every dental procedure that lasted longer than a day, with extensive bruising (Table S1). Both affected individuals who had experienced excessive surgical bleeding had not received prophylactic treatment (Table S1). Among the four affected family members who had received desmopressin prophylaxis for dental or surgical challenges, three reported it had worked (i.e. no abnormal bleeding occurred), whereas the other was unsure (Table S1).

Affected individuals did not have increased likelihoods for experiencing spontaneous haematuria, joint bleeds, muscle or central nervous system or gastrointestinal bleeds (Fig. 5, Table S1), but one affected family member reported a severe bleed from an ulcer, requiring admission and platelet transfusions (Table S1) and another had suffered a subdural haematoma at birth (Fig. 5, Table S1).

Although only two affected female family members were evaluated, their risks were increased for heavy menses limiting lifestyle during most periods (OR 32; 95% CI 1.4–770; P = 0.02) (Fig. 5, Table S1). The proband reported a postpartum haemorrhage after one of two uncomplicated vaginal deliveries and had menorrhagia requiring oral contraceptive treatment, and later, an endometrial ablation coupled with Mirena IUD insertion, with good effects (Table S1).

Affected family members reported more CHAT-P symptoms/problems with OR >1 (5–15, median 11) than unaffected family members (0–7, median 4) or
controls (0–8, median 0) \((P = <0.01)\) (Figure S1). The number of symptoms that they reported showed no association with age \((R^2 = 0.092)\) and were similar for those with or without thrombocytopenia (median: 9 vs. 11; \(P = 0.6\)), dense granule deficiency (median: 9 vs. 12; \(P = 0.5\)) or prior surgery (median: 11 vs. 10; \(P = 0.9\)) (Figure S2).

**Discussion**

With all bleeding disorders, it is important to assess for phenotypic variation and the health implications of disease-causing mutations [36]. *RUNX1* mutations represent an important IPD that has been associated with thrombocytopenia, aggregation/secretion defects, dense granule deficiency and familial leukaemia/myelodysplasia [7,16,37]. In our study, we found a *RUNX1* mutation in one out of twelve index cases with IPD who had exome sequencing performed. This individual (who was not thrombocytopenic) had a novel, single base pair duplication in exon 6 of the *RUNX1* (c.583dup) (Fig. 1), which introduces a frameshift and *RUNX1* truncation at a site that causes haploinsufficiency [32]. Three generations of affected individuals in the proband’s family had clear cut LTA and dense granule ATP release abnormalities (Fig. 3), whereas only some had thrombocytopenia and/or dense granule deficiency. While this family’s history of leukaemia/myelodysplasia was not striking, the persistence of MYH10 in their platelets (Fig. 3) was consistent with a pathological *RUNX1* mutation [1,6,7,11,14,15,19,20]. Furthermore, affected individuals had platelet aggregation abnormalities that were readily evident with thromboxane analogue U46619, collagen, and often arachidonic acid, illustrating that these agonists help detect *RUNX1*-related IPD and exclude aspirin-like defects, given the response to thromboxane analogue U46619. As only half of the affected family members had dense granule deficiency, we suggest that impairments in platelet signalling/release pathways are more important than dense granule numbers in *RUNX1*-related IPD. We succeeded with testing a proof of concept: that bleeding risks can be estimated for a typical family with an IPD. This family’s IPD increased risks for wound healing problems and for a variety of bleeding symptoms/problems (OR \(14\rightarrow400\)) including bleeding interfering with lifestyle in women, but few had ever sought emergency medical treatment for bleeding (Figs 5 and S2).

![Fig. 4. Responses to questions about general health and family history for individuals with the *RUNX1* mutation compared to general population controls. The proportions of affected family members (Affected) and general population controls (Controls) that reported each symptom/problem are summarized, along with the odds ratios (OR) and 95% confidence intervals (CI).](image-url)

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disorders due to RUNX1 mutations [7,10,37]. While an assessment of dense granule ATP release by lumia-gregometry can be useful for phenotyping an IPD, the test findings show significant variability and are generally not predictive of increased bleeding scores or the clinical diagnosis of a bleeding disorder [22]. It is possible that other tests could be useful for identifying IPD due to RUNX1 defects. Decreased α-granule numbers and P-selectin expression on activated platelets have been observed in familial platelet disorders due to RUNX1 mutations [17,37] but the usefulness of assessing these parameters for detection of RUNX1 defects is uncertain. Pathogenic RUNX1 mutations increase risks for malignant transformation [18], as does acquisition of additional RUNX1 mutations [38]. Dominant negative mutations that preserve RUNX1 binding to CBFβ [16] confer higher risks for leukaemia/myelodysplasia than haploinsufficiency mutations [17,39,40]. Given the increased risks for developing leukaemia/myelodysplasia in individuals with a RUNX1 mutation, long-term surveillance for the development of more serious blood disorders is warranted, with early consideration of bone marrow transplantation.

Affected members of the family that we studied had increased bleeding detected by ISTH-BAT and CHAT-P tools. The affected individuals’ elevated ISTH-BAT scores (median: 10.5, range 4–20 compared to 0–1 for unaffected relatives and 0–6 for general population controls) are consistent with the bleeding we have observed in others with RUNX1 mutations [39] but contradict a report that most individuals with

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Fig. 5. Bleeding symptoms experienced by individuals with the RUNX1 mutation compared to general population controls. The proportions of affected family members (Affected) and general population controls (Controls) that reported each symptom/problem are summarized, along with the odds ratios (OR) and 95% confidence intervals (CI).
RUNX1 mutations have normal ISTH-BAT scores [40]. Perhaps this reflects differences in molecular causes. We observed that CHAT-P tool (which was written in lay language) provided very complete details of a subject’s bleeding history for review by a haematologist, suggesting that it may be useful to help gather a person’s bleeding history for clinical purposes. While both the ISTH-BAT and CHAT-P tools documented the increased bleeding in our subjects with a RUNX1 mutation, we found CHAT-P more useful to evaluate a range of bleeding symptoms/problems, and assess bleeding risks and responses to therapy, which are important for developing evidence-based treatment plans. Because our ‘typical’ family had limited numbers of sibs and offspring, we could not quantify some risks that we had assessed for QPD [2]. Many studies have found overlap in bleeding scores for subjects that do, or do not, have a bleeding disorder [41] and this was also evident when we used CHAT-P data to determine a score. However, the logistic regression method that we used to estimate bleeding risks, using CHAT-P data, compensates for the mild bleeding symptoms reported by some controls [40] and it also censors data for subjects without exposures to some challenges (e.g. surgery or dental extractions), which is not considered when determining an ISTH-BAT score. In addition, we observed that risk estimates were more useful than ISTH-BAT scores for discussions with affected individuals, who wished to know how much more likely they were to experience different types of bleeding. The CHAT-P information on responses to prior treatments and bleeding risks also aided discussions with affected individuals about reducing bleeding risks with prophylactic treatments at times of dental and surgical challenges.

Now that we have illustrated that bleeding risks can be estimated for typical families with an IPD, it will be important to estimate bleeding risks for other families with IPD and translate the findings for improved care.

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Disclosures

All authors stated that they had no interests which might be perceived as posing a conflict or bias.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. CHAT-P, the clinical bleeding history assessment tool that was used to gather the detailed bleeding histories of study subjects.

Appendix S2. Summary of CHAT-P data for affected individuals with a RUNX1 mutation predicted to cause haploinsufficiency vs. healthy controls.