Exome sequencing in 116 patients with inherited thrombocytopenia that remained of unknown origin after systematic phenotype-driven diagnostic workup

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Supplementary Methods

Exome sequencing

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Exome enrichment and capture was carried out with the BGI Exome kit (BGI TECH SOLUTIONS, Hong Kong) in cases. Regarding control samples: 150 samples were captured with the BGI exome kit (BGI TECH SOLUTIONS), 139 with the Nextera DNA Library Preparation Kit, 18 with the TruSeq Exome Kit (Illumina), 139 with the SeqCap EZ Exome + UTR Kit or the SeqCap EZ MedExome Kit (Roche, Basel, Switzerland). All samples were sequenced as 100/150 bp paired-end reads on Illumina sequencing platforms. Single nucleotide variants (SNVs) and small insertions/deletions (InDels) were annotated with the Ensembl tool Variant Effect Predictor v.76 on Gencode v19 transcripts against the GRCh37 reference genome.

Qualifying variants criteria for known IT genes analysis

Criteria for qualifying variants selection for known IT genes analysis are the following:

1. Minor Allele Frequencies (MAF) from the ExAC (http://exac.broadinstitute.org/)[1].Non-Finnish European sub-population and across our in-house exomes dataset
   - ≤ 0.01 for genes with biallelic or X-linked mechanisms;
   - ≤0.001 for genes with monoallelic mode of inheritance;
2. Variant consequence. For *ANKRD26*, only variants in the 5'UTR were taken into account, while for all other genes, variants predicted to impact on the protein-coding sequence (nonsynonymous, splice-site and small insertions/deletions) were considered.

Classification following ACMG guidelines was done exploiting the VarSome utility (https://varsome.com/) [2].

Copy Number Variants (CNVs) identification through EXCAVATOR2

We used EXCAVATOR2 [3] as follows. A window of 50Kb was used and samples from female and male individuals were analyzed separately. Each group was analyzed in the "pooled" mode, against 20 sex-paired healthy controls. CNVs called by Excavator2 and overlapping for at least 1 bp any of the 43 known IT genes were retained as candidate and confirmed by real-time PCR through the Universal Probe Library (UPL) system (Roche), using the TaqMan Universal PCR Master Mix (ThermoFisher Scientific). Primer pairs were designed (Assay Design Center, http://lifescience.roche.com/) to amplify a genomic region mapping within each CNV (sequences available on request). The DNA of two healthy individuals was processed along with patients' DNA. Reactions were performed in triplicate for each primer pair, simultaneously amplifying the RPPH1 gene as diploid reference (TaqMan CopyNumber Reference Assay; ThermoFisher Scientific). The ΔΔCt method was applied to qPCR data to obtain a relative quantification of the copy number of analyzed genomic regions.

The SurePrint G3 Unrestricted CGH ISCA 8x60K platform (Agilent Technologies), with a 60 kb median probes spacing, was used to confirm *RUNX1* deletions. Scanned images were analyzed by the Cytogenomics v.5.0 software, and aberrations were called with the ADM1 algorithm with a sensitivity threshold of 6.0.

Variants selection in the gene-based collapsing analysis

Well covered genes were defined as having 80% of the target regions covered by ≥10 reads in at least 80% of the cohort (both cases and controls).

Sample selection in the gene-based collapsing analysis

The Principal Component Analysis (PCA) was done exploiting the EIGENSTRAT software [4] and restricted to 70,000 HapMap SNP sites with MAF >= 5% and residing within or near exons (+-100 bp). Non-European individuals were defined as those whose value on one of the top two axes of variation was at least 5 standard deviation from the mean values of 909 Non-Finnish European individuals (including in-house samples and individuals from TSI, GBR, IBS and CEU populations of the 1000 Genomes).

Statistical data analyses were performed in R3.4.2 (https://www.R-project.org/).

Supplementary Results

Pseudo-dominant inheritance of ABCG8 variant

In Family.43, where thrombocytopenia segregates in an apparent autosomal-dominant fashion (Figure 1A), no candidate variants were found to segregate in a dominant manner. Conversely, we found that the proband II-2 (Figure 1A) and both her affected children (III-1 and III-2) were homozygous for the *ABCG8* c.1234C>T, p.Arg412* variant, reported as pathogenic in ClinVar (RCV000005260.5). The apparently unrelated proband's healthy husband (II-1) was found to be heterozygous for the same variant. We identified a large ROH (about 11 Mb) encompassing *ABCG8* in II-2 and III-1. We estimated a coefficient of inbreeding of 0.028 for II-2 and 0.029 for III-1, compatible with relatedness between II-1 and II-2 of cousins of $2^{nd}/3^{rd}$ degree as well as between I-1 and I-2, who, unfortunately, were not available for genetic testing.

Bi-allelic *ABCG8* mutations cause sitosterolemia (OMIM #210250), a rare disorder characterized by increased plasma and tissue levels of plant sterols due to intestinal hyperabsorption and poor bile excretion. Clinical spectrum of sitosterolemia is extremely variable: possible features include thrombocytopenia with large platelets, hemolytic anemia, tendon and tuberous xanthomas, hypercholesterolemia, and premature atherosclerosis; asymptomatic individuals have been reported.[5,6] Our patients were a 51-year-old female and her 16- and 13-year-old children presenting with mild thrombocytopenia (90-100 $x10^9$/L) with large platelets as the only clinical manifestation of the disease. Apart platelet macrocytosis, platelet morphology was normal in the three affected individuals. Platelet aggregation in response to ADP, collagen, ristocetin and arachidonic acid was tested in II-2 and III-1 and resulted within the normal range.

WAS mutation inherited from heterozygous female

In Family.41 (Figure 1B), we identified the *WAS* c.134C>T, p.Thr45Met variant, reported in ClinVar (VCV000011123.1) as causative of X-linked Thrombocytopenia (XLT, OMIM #313900), the allelic and mild variant of Wiskott-Aldrich Syndrome. The p.Thr45Met variant is present in hemizygous state in the two affected males IV-1 and IV-3, presenting severe thrombocytopenia (platelet count 19 and 22 $x10^9$/L, respectively) and is inherited from their mother III-4 who has a higher platelet count (80 $x10^9$/L). X chromosome inactivation test of III-4 showed a balanced profile (data not shown). The father of III-4 (II-1), having a platelet count below the normal threshold (120 $x10^9$/L), was initially considered to be affected and for these reasons, X-linked inheritance was not suspected in this family. However, based on the age- and gender-adjusted reference intervals for platelet count,[7] platelet count of II-1 actually falls within the normal range (112-361 $x10^9$/L). DNA sample was not available for II-1 and II-2, and it was therefore not possible to evaluate their carrier status. However, the fact that the other daughter, III-3, does not carry the variant indicates that II-1 is not a variant carrier. Patients III-4, IV-1 and IV-3 have no other relevant clinical features in addition to thrombocytopenia. Analysis of platelet size on peripheral blood smears according to a previously reported method[8] revealed that thrombocytopenia is associated with small platelets in individual IV-3 (mean platelet diameter, MPD, 2.10 µm), whereas III-4 and IV-1 presented normal platelet size (MPD 2.55 and 2.51 µm, respectively). Platelet aggregation in response to ADP, collagen, ristocetin and arachidonic acid was investigated in subject III-4 and resulted normal. Flow cytometry analysis showed that the patients III-4, IV-1 and IV-3 had normal expression of platelet glycoprotein complexes GPIb-IX-V, GPIIb-IIIa, and GPIa-IIa on the platelet surface.

As reported in the literature, female carriers of *WAS* mutations are usually healthy because of a skewed inactivation of the mutated X chromosome[9,10] and the few affected heterozygous females have total inactivation of the wild type allele.[11-13] Of note, in our female patient (III-4) thrombocytopenia is much less severe than in the affected males of the family, suggesting a mild effect of this variant in non-skewed female carriers.

GATA1 mutation in heterozygous female

In the female proband of Family.40 (Figure 1.C), we identified the heterozygous c.146delC, p.P50Rfs*86 variant in *GATA1,* a gene mapping to the X chromosome, for which we observed a balanced inactivation (data not shown). *GATA1* mutations cause a spectrum of disorders from either dyserythropoietic anemia or hemolytic anemia resembling beta-thalassemia (OMIM #314050) in males to mild isolated thrombocytopenia rarely reported in heterozygous females.[14,15]

Our proband is a 16-year-old female presenting with isolated thrombocytopenia (platelet count 110 x10^9/L), mild platelet macrocytosis, mild alpha-granule defect, and slight reduction of bone marrow megakaryocytes. Platelet aggregation studies showed normal response to ADP, collagen, and ristocetin. Her mother also has a lifelong history of mild thrombocytopenia, but was not available for genetic analysis to confirm the status of carrier.

To the best of our knowledge, few cases of heterozygous females affected with *GATA1*-associated disorders have been described.[16-19] In only one case, a non-skewed X chromosome inactivation was demonstrated.[20]

RUNX1* haploinsufficiency caused by heterozygous intragenic deletions involving *RUNX1

Table S7 and S8 summarize the main clinical and laboratory findings of the investigated patients with heterozygous intragenic deletions involving *RUNX1*. The details about the deletions and family pedigrees are reported in Figure 2.

Family 44. The proband (IV-1) was a 16-year-old boy referred for mild thrombocytopenia with easy bruising. Platelet count was stably around 100 x10^9/L with normal MPV. Examination of peripheral blood smears showed no abnormalities of platelet size and morphology. *In vitro* platelet aggregation demonstrated defective response to ADP and collagen, and normal response to ristocetin (Table S7). Platelet expression of GPIb-IX-V and GPIIb-IIIa was normal. The proband's 46 year-old-mother (III-3) reported a history of mild thrombocytopenia since childhood and was diagnosed with myelodysplastic syndrome with multilineage dysplasia at the age of 44. Of note, she reported a history of leukemia (not otherwise specified) in her paternal lineage (Figure 2C). Even the proband's maternal uncle (III-4) and a cousin (IV-3) presented a mild reduction in platelet count with normal platelet size and morphology and no significant bleeding tendency.

Family 66. The proband (I-1) was a 45-year-old female referred for mild chronic thrombocytopenia (platelet count around 100 x10^9/L) and easy bruising as the only bleeding manifestation. She reported a slight worsening of thrombocytopenia during her only pregnancy; however, the delivery proceeded without bleeding complications. Examination of peripheral blood smears did not reveal alterations of platelet size and morphology. The study of *in vitro* platelet aggregation showed normal response to ADP, collagen, and ristocetin; flow cytometry revealed normal platelet surface expression of GPIb-IX-V and GPIIb-IIIa. The proband's son (II-1) presented thrombocytopenia since birth and reported easy bruising as the only bleeding manifestation.

Family 72. The proband (I-1) was a 56-year-old male referred to our institution for a lifelong history of thrombocytopenia and easy bruising as the only bleeding manifestation. Platelet count ranged from 50 to 70 x10^9/L with normal mean platelet volume (MPV); no further alterations of blood cell counts were present. At examination of peripheral blood smears, no citomorphological abnormalities were observed, in particular platelet size and morphology were normal. The proband's 39-year-old son (II-2), his 38-year-old daughter (II-4), and 4 of his 5 grandchildren presented thrombocytopenia. Subject II-2 reported only very mild easy bruising; conversely, his sister II-4 had a lifelong history of easy bruising, frequent epistaxis, gum bleeding, and menorrhagia requiring hormonal therapy and iron supplementation. The proband's grandchildren presented no bleeding tendency (n=1) or only mild bleeding tendency consisting in easy bruising and/or prolonged bleeding after minor wounds (n=3). All the investigated proband's relatives had normal platelet size and morphology (Table S7). *In vitro* platelet aggregation was studied in 5 patients (Table S8). In summary, four patients showed defective response to ADP (n=4 to ADP 5 mM, and n=3 to ADP 20 mM as well); three of them presented also reduced aggregation after stimulation with collagen 4 and 20 µg/mL. One patient showed normal aggregation with all the tested agonists (Table S8). Flow cytometry analysis of platelets showed normal expression of the glycoprotein (GP) complexes Ib-IX-V and IIb-IIIa in all the 5 investigated patients (Table S7).

At age 59 years, the proband I-1 began to present progressive worsening of thrombocytopenia and of bleeding tendency, hyporegenerative anemia, and monocytosis; he was diagnosed with chronic myelomonocytic leukemia. He was treated with chemotherapy and allogeneic hematopoietic stem cell transplantation (HSCT). CMML relapsed a few months after HSCT and the patient died from progression of the hematological disease. No hematological malignancies have been reported in the other family members.

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Supplementary Tables

Table S1. Main features of the study population.

Table S2. Known IT genes included in this study. *Sub-optimal coverage for variant calling.

Table S3. Specific diagnoses made in 116 probands through the application of the phenotype-driven diagnostic algorithm (reference 2) to 255 unrelated probands with inherited thrombocytopenia.

Table S4. Known IT genes. VUS and (likely) benign variants identified in the 43 known IT genes.

Table S5. Gene-based enrichment analysis: qualifying variants. Qualifying variants detected in genes with a p value <= 0.0015. Only variants with segregation compatible with the disorder and found in families with no disease causative variants in known IT genes are reported. Variants in the genes *ACTN1* and *ETV6* are reported in Table 1 and Table S3.

Table S6. ACMG scoring details for variants in Table 1 and Table S3.

Table S7. Main clinical and laboratory features of 13 FPD-AML patients with *RUNX1* haploinsufficiency caused by heterozygous intragenic deletions involving *RUNX1*.

Table S8. In vitro platelet aggregation in 7 FPD-AML patients carrying heterozygous intragenic deletions involving RUNX1, maximal extent (percentages).

Supplementary Figures

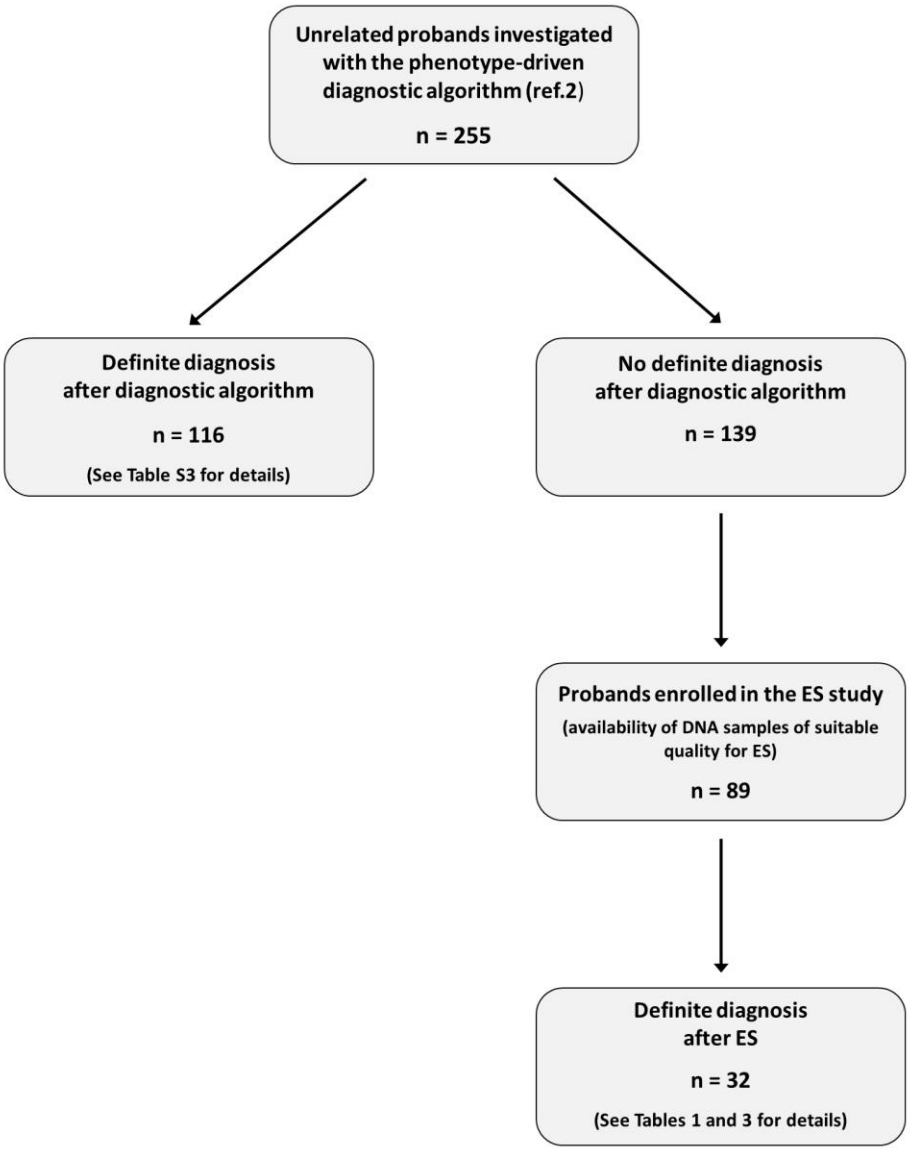

Figure S1. Flow-chart summarizing the total number of investigated probands, the results of the application of the phenotype-driven diagnostic algorithm, the recruitment and the results of the exome sequencing (ES) analysis.

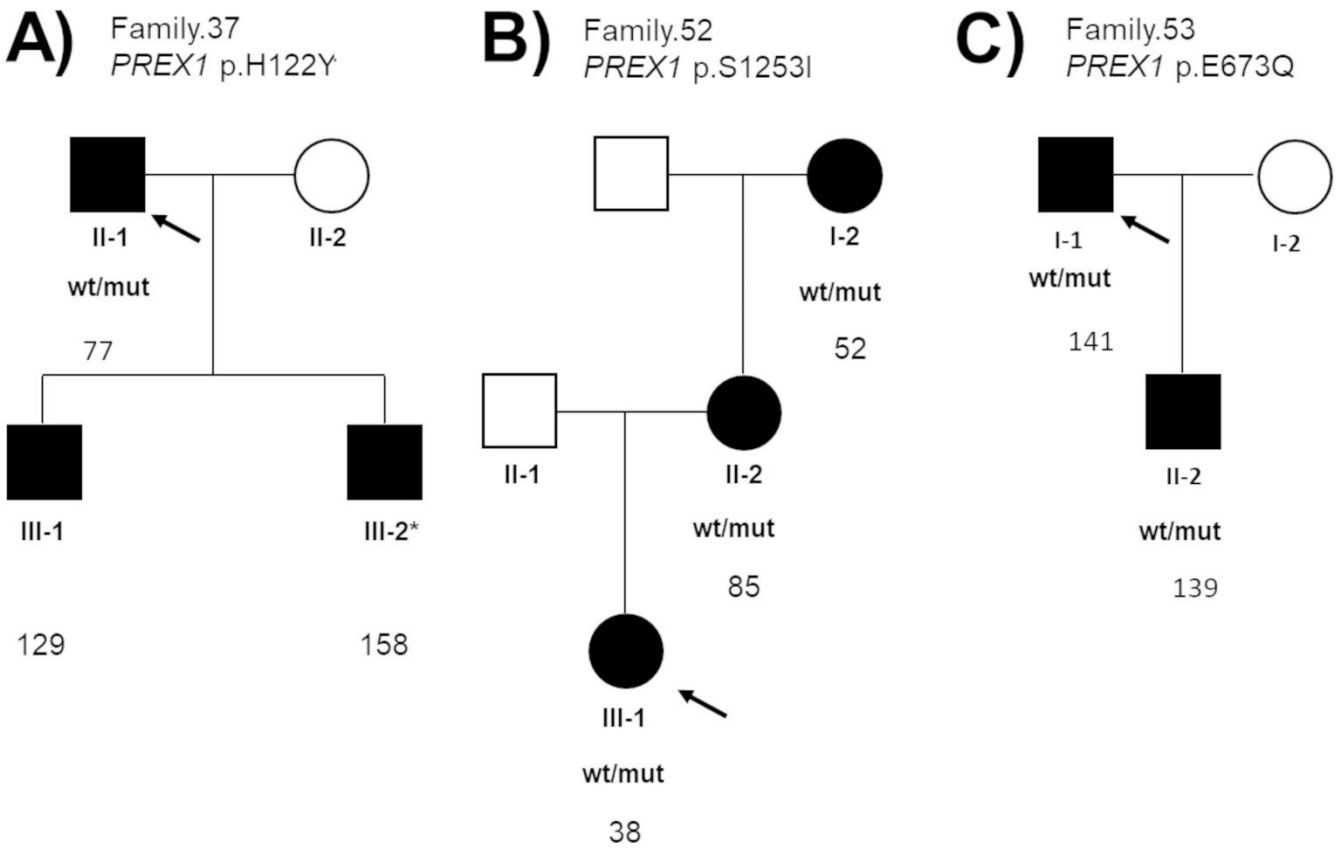

Figure S2 Families carrying candidate variants in *PREX1*. Platelet count ($x10^9$/L) and genotype are reported when available. wt=wt allele, mut=mutated allele.