Complications of whole-exome sequencing for causal gene discovery in primary platelet secretion defects

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Table S1. ADP, ATP, serotonin, and fibrinogen platelet content.

|                             | PSD patients | Internal reference range** |
|-----------------------------|--------------|----------------------------|
|                             | N=14*        |                            |
| **Delta granules**          |              |                            |
| ADP (nmoles/10^8 platelets) | 2.26 (1.19-4.15) | 1.30-2.88                 |
| ATP (nmoles/10^8 platelets) | 5.00 (3.20-9.40) | 3.17-7.07                  |
| ATP/ADP                     | 2.42 (1.83-2.84) | 1.55-3.42                  |
| Serotonin (nmoles/10^8 platelets) | 0.37 (0.25-0.64) | 0.19-0.40 |
| **Alpha granules**          |              |                            |
| Fibrinogen (mg/10^9 platelets) | 0.06 (0.04-0.13) | 0.03-0.19                  |

*Median (min-max)

** (5¹⁰⁻⁹⁵¹⁰ percentile)


Table S2. Single-nucleotide variants (n=107) identified in 14 PSD patients by WES followed by two prioritizing approaches, according to Leo et al.\textsuperscript{1} classification or by selecting singletons.

All variants were heterozygous. Variant filtering steps are reported in Figure S1.

| ID  | Gene | Nucleotide change | dbSNP | Amino acid change | MAF 1000G EUR | MAF ESP EA | ExAC NFE | SIFT | Polyphen2 | Mutation Taster | CADD C-score | Platelet expression | Leo et al. (JTH, 2015) | ACMG |
|-----|------|-------------------|-------|-------------------|---------------|------------|----------|------|-----------|-------------------|--------------|---------------------|----------------------|------|
| C696| COL24A1 | c.G4673A | . | p.G1558E | . | . | . | D | D | D | 25.2 | - | - | VUS |
| C696| LTBP1 | c.G3011A | rs141080282 | . | p.R1004Q | 0.005 | 0.0067 | 0.0059 | D | P | D | 24.2 | + | + | LB |
| C696| PLEK | c.A322C | rs346515106 | . | p.K108Q | . | 0.0007 | 0.0016 | T | P | D | 22.4 | + | - | LB |
| C696| MERTK | c.A2305G | rs147899488 | . | p.L769V | . | 0.0001 | 0.0004 | T | B | D | 16.37 | - | - | LB |
| C696| TUBA3D | c.G331A | rs550660894 | . | p.G111S | . | 4.5E-05 | . | D | D | 27.5 | - | + | LB |
| C696| TTN | c.T99179C | rs763888823 | . | p.I33060T | . | . | . | T | B | D | 20.7 | + | - | LB |
| C696| TTN | c.G63309T | . | p.M21103I | . | . | . | T | B | D | 19.1 | + | - | LB |
| C696| TTN | c.A24973G | rs72648984 | . | p.K832E | 0.008 | 0.0076 | 0.0093 | T | B | D | 13.41 | + | - | LB |
| C696| TTN | c.A15563C | rs72648930 | . | p.V518P | 0.001 | 0.0021 | 0.0015 | T | D | D | 15.15 | + | - | LB |
| C696| CSRNP1 | c.G401A | rs75921966 | . | p.R134H | 1.5E-05 | 1.5E-05 | T | B | N | 23.5 | - | - | LB |
| C696| MMRN1 | c.G1546T | rs141872900 | . | p.V354M | 0.007 | 0.0064 | 0.0073 | T | B | N | 0.575 | + | - | LB |
| C696| DGKI | c.G553A | rs779164061 | . | p.Q185P | 0.0001 | 0.00015 | D | P | D | 23 | + | - | LB |
| C708| QSOX1 | c.G1060A | rs148353050 | . | p.R32587H | 0.003 | 0.0038 | 0.0056 | T | D | D | 10.24 | + | - | LB |
| C708| TTN | c.G104695A | rs200497615 | . | p.R35652Q | 0.007 | 0.0064 | 0.0073 | T | B | D | 24.6 | + | - | LB |
| C708| TTN | c.G97760A | rs55704830 | . | p.R32587H | 0.003 | 0.0038 | 0.0056 | T | D | D | 25.5 | + | - | LB |
| C708| SERPINE2 | c.G622C | rs375757013 | . | p.R748L | 0.0001 | 0.0001 | 0.0002 | T | B | N | 0.135 | + | + | LB |
| C708| COL4A4 | c.G2630A | rs150979437 | . | p.Q185P | 0.0001 | 0.0001 | 0.0002 | T | B | N | 9.424 | - | - | LB |
| C708| ITT1 | c.C5098T | rs540818757 | . | p.P1700S | . | . | . | T | . | D | 9.375 | + | + | LB |
| Gene   | Gene ID | Exon | SNP ID      | Allele | Effect | p-value | q-value | Beta | P-value | OR   | q-value | OR   |
|--------|---------|------|-------------|--------|--------|---------|---------|------|---------|------|---------|------|
| C708 CSRNP1 | c.C1389G |  | p.S463R |        | D      | 25.9    | -       | -    |         |      |         |      |
| C708 CSRNP1 | c.C673T | rs142034027 | p.R225W | 0.0007 | 0.0001 | D      | D      | D    | 32      | -    | -       | VUS  |
| C708 MYLK4 | c.A1286G | rs35211631 | p.Q429R | 0.0021 | 0.0011 | T      | B      | N    | 15.14  | -    | B       |
| C708 PLG   | c.T2045A | rs147175166 | p.I682N | 0.001  | 0.0008 | T      | D      | D    | 24.8   | +    | -       | LB   |
| C708 NRP1 | c.G620A | rs148308681 | p.R207H | 0.0007 | 0.0001 | D      | D      | D    | 33     | -    | VUS     |
| C729 FCGR2A | c.A836C | rs146883516 | p.D279A | 0.002  | 0.0017 | D      | B      | N    | 22.6   | +    | +       | LB   |
| C729 TTN   | c.C1389G | rs142034027 | p.R225W | 0.0007 | 0.0001 | D      | D      | D    | 32     | -    | -       | VUS  |
| C729 MYLK4 | c.A1286G | rs35211631 | p.Q429R | 0.0021 | 0.0011 | T      | B      | N    | 15.14  | -    | B       |
| C729 PLG   | c.T2045A | rs147175166 | p.I682N | 0.001  | 0.0008 | T      | D      | D    | 24.8   | +    | -       | LB   |
| C729 NRP1 | c.G620A | rs148308681 | p.R207H | 0.0007 | 0.0001 | D      | D      | D    | 33     | -    | VUS     |
| C729 FCGR2A | c.A836C | rs146883516 | p.D279A | 0.002  | 0.0017 | D      | B      | N    | 22.6   | +    | +       | LB   |
| C729 TTN   | c.C1389G | rs142034027 | p.R225W | 0.0007 | 0.0001 | D      | D      | D    | 32     | -    | -       | VUS  |
| C729 MYLK4 | c.A1286G | rs35211631 | p.Q429R | 0.0021 | 0.0011 | T      | B      | N    | 15.14  | -    | B       |
| C729 PLG   | c.T2045A | rs147175166 | p.I682N | 0.001  | 0.0008 | T      | D      | D    | 24.8   | +    | -       | LB   |
| C729 NRP1 | c.G620A | rs148308681 | p.R207H | 0.0007 | 0.0001 | D      | D      | D    | 33     | -    | VUS     |
| C729 FCGR2A | c.A836C | rs146883516 | p.D279A | 0.002  | 0.0017 | D      | B      | N    | 22.6   | +    | +       | LB   |
| C729 TTN   | c.C1389G | rs142034027 | p.R225W | 0.0007 | 0.0001 | D      | D      | D    | 32     | -    | -       | VUS  |
| C729 MYLK4 | c.A1286G | rs35211631 | p.Q429R | 0.0021 | 0.0011 | T      | B      | N    | 15.14  | -    | B       |
| C729 PLG   | c.T2045A | rs147175166 | p.I682N | 0.001  | 0.0008 | T      | D      | D    | 24.8   | +    | -       | LB   |
| C729 NRP1 | c.G620A | rs148308681 | p.R207H | 0.0007 | 0.0001 | D      | D      | D    | 33     | -    | VUS     |
| C729 FCGR2A | c.A836C | rs146883516 | p.D279A | 0.002  | 0.0017 | D      | B      | N    | 22.6   | +    | +       | LB   |
| C729 TTN   | c.C1389G | rs142034027 | p.R225W | 0.0007 | 0.0001 | D      | D      | D    | 32     | -    | -       | VUS  |
| C729 MYLK4 | c.A1286G | rs35211631 | p.Q429R | 0.0021 | 0.0011 | T      | B      | N    | 15.14  | -    | B       |
| C729 PLG   | c.T2045A | rs147175166 | p.I682N | 0.001  | 0.0008 | T      | D      | D    | 24.8   | +    | -       | LB   |
| C729 NRP1 | c.G620A | rs148308681 | p.R207H | 0.0007 | 0.0001 | D      | D      | D    | 33     | -    | VUS     |
| C729 FCGR2A | c.A836C | rs146883516 | p.D279A | 0.002  | 0.0017 | D      | B      | N    | 22.6   | +    | +       | LB   |
| Gene  | Symbol | Chromosome | Location | Mutation Type | p-value | Risk allele | OR        |
|-------|--------|------------|----------|---------------|---------|-------------|-----------|
| APC   | c.A398G| rs202094100| .        | .             | .       | T           | 2.01E-05 |
| DIAPO | c.T3227G| rs150231219| .        | .             | 0.0002  | T           | 0.52     |
| ITPR3 | c.C5720T| rs139176240| .        | .             | 0.0001  | T           | 0.65     |
| TTN   | c.C72358T| rs202094100| .        | .             | 0.0001  | T           | 0.65     |
| LCN1  | c.G1895A| rs150231219| .        | .             | 0.0002  | T           | 0.52     |
| APC   | c.A6821T| rs34919187 | .        | .             | 0.0001  | T           | 0.65     |
| DNAH1 | c.A9935T| rs72657389 | .        | .             | 0.0001  | T           | 0.65     |
| PRKACG| c.C280T| rs34919187 | .        | .             | 0.0001  | T           | 0.65     |
| ADRA2A| c.G116A| rs539511086| .        | .             | 0.0001  | T           | 0.65     |
| MUC2  | c.G2594A| rs150231219| .        | .             | 0.0001  | T           | 0.65     |
| MUC2  | c.A5038G| rs371137719| .        | .             | 0.0001  | T           | 0.65     |
| F5    | c.F3438G| rs6005     | .        | .             | .       | T           | 0.52     |
| LYST  | c.G8806A| rs2753327  | .        | .             | .       | T           | 0.52     |
| LYST  | c.A8224C| rs766760874| .        | .             | .       | T           | 0.52     |
| TTN   | c.G49413T| rs202094100| .        | .             | .       | T           | 0.52     |
| COL4A3| c.T4421C| rs200302125| .        | .             | .       | T           | 0.52     |
| DGKG  | c.T1524C| rs200302125| .        | .             | .       | T           | 0.52     |
| PDGFRC| c.A113G| rs139145392| .        | .             | .       | T           | 0.52     |
| CSF1R | c.T2876C| rs150231219| .        | .             | .       | T           | 0.52     |
| PHF14 | c.G298T| rs150231219| .        | .             | .       | T           | 0.52     |
| Chromosome | Gene    | Position | rs ID     | Protein Change | p-Value (1) | p-Value (2) | p-Value (3) | T | D | D | + | - | LB |
|------------|---------|----------|-----------|---------------|-------------|-------------|-------------|---|---|---|---|---|----|
| C797       | TTN     | c.C88394T| rs146181116| p.S29465F     | 0.007       | 0.0045      | 0.0039      | T | D | D | 22.5 | + | - | LB |
| C797       | TTN     | c.T62996G|           | p.F20999C     |             |             |             | T | P | N | 2.844 | + | - | LB |
| C797       | TTN     | c.C17T   | rs201490999| p.P6L         |             |             |             | D | D | D | 23.8 | + | - | VUS |
| C797       | EGF     | c.G3073A |           | p.A1025T      |             |             |             | T | B | D | 15.3 | + | - | VUS |
| C797       | PDGFRB  | c.G946A  | rs41287112 | p.V316M       | 0.003       | 0.0046      | 0.0088      | T | B | N | 14.92 | - | - | LB |
| C831       | PRKCZ   | c.G1109A | rs147033679| p.R370K       |             | 1.53E-05    |             | T | B | D | 7.976 | - | - | LB |
| C831       | RAP1GAP | c.G1390C |           | p.A464P       |             |             |             | T | B | N | 23.1 | + | + | LB |
| C831       | WNT3A   | c.G527A  | rs779729203| p.R176Q       |             | 1.58E-05    |             | T | D | D | 29.1 | - | - | LB |
| C831       | TTN     | c.T15768A| rs138826545| p.H5256Q      |             | 0.0002      | 0.0002      | T | B | D | 11.85 | + | - | VUS |
| C831       | PHF14   | c.G2431A | rs61996285 | p.V811I       |             |             |             | T | B | D | 19.69 | - | - | LB |
| C831       | MMRN1   | c.A3251G | rs201761344| p.N1084S      |             | 0.0001      |             | T | N | D | 22.8 | + | - | LB |
| C831       | EGF     | c.G1723A | rs115396821| p.G575R       | 0.008       | 0.0024      | 0.0027      | D | D | D | 26   | + | - | VUS |
| C831       | PHF14   | c.G2431A | rs61996285 | p.V811I       | 0.003       | 0.0016      | 0.0017      | T | B | D | 19.69 | - | - | LB |
| C831       | DNAH11  | c.A4282G | rs72657315  | p.T1428A      | 0.002       | 0.0028      | 0.0043      | D | B | N | 22.3 | - | + | LB |
| C831       | DGKI    | c.C457T  | rs61757580  | p.L153F       | 0.0099      | 0.0073      | 0.0078      | T | P | D | 14.82 | + | - | B  |
| C831       | TBXAS1  | c.151_152del|           | p.V51fs      |             |             |             | + | - |  |     |    |    | VUS |
| C831       | VWF     | c.G8171A |           | p.C2724Y      |             |             |             | + | + |  |     |    |    | VUS |
| C847       | CASP9   | c.A220G  | rs145118493| p.M74V        |             | 0.0015      | 0.0013      | T | B | N | 7.542 | + | - | LB |
| C847       | F5      | c.G43A   | rs9332485  | p.G15S        | 0.001       | 0.0002      | 0.0006      | D | D | D | 29.2 | + | - | LB |
| C847       | TTN     | c.C91384T| rs373623340| p.R30462W     |             | 3.01E-05    |             | D | D | D | 25.7 | + | - | VUS |
| C847       | TTC37   | c.C3253G | rs202214985| p.Q1085E      | 0.0011      | 0.0002      | 0.0006      | T | B | D | 10.55 | + | - | LB |
| C847       | APC     | c.G3949C | rs1801166  | p.E1317Q      | 0.006       | 0.0039      | 0.0057      | T | B | A | 7.737 | - | + | LB |
| C847       | F13A1   | c.G1861T | rs145180358| p.A621S       | 0.0002      | 0.0008      |             | T | B | D | 23.8 | + | - | LB |
| C847       | PHACTR2 | c.G1360C |           | p.D454H       |             |             |             | D | D | D | 25.8 | + | - | VUS |
| Position | Gene | Change | Reference SNP ID | Protein Change | dbSNP MAF | ESP MAF | ExAC MAF | 1000G MAF | PolyPhen2 | Mutation Taster | CADD C score | VUS/LB |
|----------|------|--------|-----------------|----------------|-----------|--------|----------|---------|-----------|---------------|-------------|--------|
| C847     | NOS3 | c.C3385T | rs774447524     | p.R1129C       | .         | .      | 2.31E-05 | D       | D         | D             | 34          | -      |
| C847     | PDGFRL | c.C1046A | rs146087994     | p.T349K        | .         | .      | 1.5E-05  | T       | D         | D             | 27.8        | -      |
| C862     | APC  | c.C3511T | rs201830995     | p.R1171C       | 0.001     | 0.0002 | 0.0003  | D       | B         | N             | 24.1        | -      |
| C1075    | TTN  | c.A53717G | rs727503606     | p.K17906R      | .         | .      | 0.000015 | T       | B         | N             | 7.856       | +      |
| C1075    | TTN  | c.T14477G | .              | p.L4826R       | .         | .      | .       | D       | P         | N             | 1.837       | +      |
| C1075    | PRKCD | c.A1043G | rs33911937      | p.N348S        | .         | 0.0015 | 0.0016  | T       | D         | D             | 15.06       | +      |
| C1075    | STX11 | c.G799A | rs45574234      | p.V267M        | 0.0089    | 0.0092 | 0.0079  | D       | D         | D             | 24          | +      |
| C1107    | COL11A1 | c.G3847T | rs150669855     | p.V1283L       | 0.001     | 0.0014 | 0.0013  | T       | B         | N             | 0.012       | -      |
| C1107    | PTPN7 | c.G425A | rs115136927     | p.R142Q        | 0.003     | 0.0072 | 0.0062  | T       | D         | D             | 26.9        | +      |
| C1107    | TTN  | c.T40931C | rs770248490     | p.V13644A      | .         | .      | 1.57E-05 | T       | B         | N             | 17.24       | +      |
| C1107    | PRKCD | c.G868T | .              | p.A290S        | .         | .      | .       | T       | D         | D             | 24.5        | +      |
| C1107    | MMRN1 | c.G3680T | rs147451161     | p.R1227L       | 0.003     | 0.0031 | 0.0036  | D       | D         | D             | 27.8        | +      |
| C1107    | ADCY2 | c.C3167T | rs779183904     | p.T1056M       | .         | 6E-05  | .       | T       | B         | N             | 18.2        | -      |

*dbSNP* – Database of Single Nucleotide Polymorphisms v.138. *MAF* – Minor allele frequency (MAF from European populations are shown). *1000G* – the 1000 Genomes Project phase 3 populations. *ESP* – the Exome Sequencing Project; *ExAC* – the Exome Aggregation Consortium; *SIFT* – Sorting Intolerant From Tolerant; *PolyPhen2* – Polymorphism Phenotyping v2; *Mutation Taster*: prediction scores: D – Damaging, B – Benign; *CADD C score* – Combined Annotation Dependent Depletion score; *VUS* – variant of uncertain significance; *LB* – likely benign.
Supplementary Figures

Figure S1. Filtering steps for single nucleotide variants (SNVs) identified by WES in 14 PSD patients and 16 healthy controls.

**SNV** – Single Nucleotide Variant; **MAF** – Minor Allele Frequency; **1000G** – the 1000 Genomes Project; **EVS** – the Exome Variant Server; **ExAC** – the Exome Aggregation Consortium; **DAVID** – the Database for Annotation, Visualization and Integrated Discovery; **ACMG** – the American College of Medical Genetics and Genomics.
Figure S2. Filtering steps for SNVs identified by WES in four family members of PSD patient C740.

36,311 SNVs identified in 5 exomes

- Removing low quality SNVs
  33,873 SNVs identified

- Selecting SNVs with MAF≤1% in European populations from 1000G, EVS, ExAC
  2,089 SNVs identified

- Selecting SNVs with potentially damaging consequences (STOP gain, Frameshift, indels, missense, splice donor/acceptor etc.)
  484 SNVs identified

- Variant filtering assuming disease transmission (present in cases, absent in controls)
  13 SNVs identified

Table 1
4 variants identified
Variant pathogenicity was assigned according to the ACMG classification and non-benign variants were selected.

SNV – Single Nucleotide Variant; MAF – Minor Allele Frequency; 1000G – the 1000 Genomes Project; EVS – the Exome Variant Server; ExAC – the Exome Aggregation Consortium; DAVID – the Database for Annotation, Visualization and Integrated Discovery; SIFT – Sorting Intolerant From Tolerant; PolyPhen2 – Polymorphism Phenotyping v2; Mutation Taster (www.mutationtaster.org); CADD C-score – Combined Annotation Dependent Depletion score.  

9
Supplementary Methods

Materials
Adenosine diphosphate (ADP), adenosine triphosphate (ATP), thromboxane/prostaglandin endoperoxide analogue 9,11-dideoxy-11,9-epoxymethano-prostaglandin F2 (U46619), thrombin receptor activating peptide (TRAP; Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe) were from Sigma Aldrich (St. Louis, MO, USA). Horm collagen was from Mascia Brunelli (Milano, IT). Commercial preparations of luciferin/luciferase reagent and protein kinase (Roche Diagnostic, Monza, IT) were used to measure the platelet ATP and ADP contents (ATP Assay Kit, Promega Italia, Milano, IT).

Commercial preparations of luciferin/luciferase (Chrono-lume; Chrono-log Corp, Havertown, PA, USA) were used to measure the platelet ATP released concurrently with platelet aggregation.

Blood sampling
Blood samples were drawn and 3 mL of blood were collected into commercial K-EDTA tubes for complete blood count analysis (ABX Micros 60, Horiba, Milano, IT). Platelet rich plasma (PRP) was prepared from trisodium citrate (129 mM, 1/9 v/v) anticoagulated whole blood samples by centrifugation at 200 x g at room temperature for 15 min. Platelet poor plasma (PPP) was obtained by centrifugation at 1400 x g at room temperature for 15 min of samples from which PRP had been removed. Native platelet count of PRP was not modified.

Platelet aggregation and secretion by lumiaggregometry
Platelet aggregation was measured in a lumi-aggregometer (Chrono-log, 560, Mascia Brunelli, Milano, IT) according to International Society on Thrombosis and Haemostasis recommendations. ATP secretion from platelet dense granules was assessed simultaneously with aggregation by using the luciferase/Luciferin reagent (Chrono-lume) added to the PRP. Secreted ATP levels were calculated by measuring the maximal amplitude of luminescence during the aggregation. Results were expressed as maximal increase (%) in light transmission for platelet aggregation and in ATP nmoli/10^8 plt for secretion within 3 minutes after platelet stimulation with the agonists: ADP (4 and 20 μM), collagen (2 μg/mL), thrombin receptor activator peptide (TRAP)-14 (10 μM), and thromboxane A2 analogue U46619 (1 μM).
Measurement of adenine nucleotides, serotonin and fibrinogen platelet content
Total platelet ADP and ATP content was measured with a luminometer (LKB 1250, Bio-Orbit Oy, Turku, Finland) by the firefly luciferin/luciferase method. Platelet serotonin (5-HT) content was measured by the o-phthalaldehyde method. Fibrinogen was measured in washed platelets by a home-made enzyme-linked immunosorbent assay, using a polyclonal anti-fibrinogen antibody as previously reported.

Whole-exome sequencing and variant annotation
Details of DNA extraction and preparation methods have been described elsewhere. Following variant alignment and calling, variants not meeting the following quality control criteria were removed: variants with more than 3 mismatches, variants-to-read ratio >0.1, variant reads mapping to single strand, total coverage <10 and Qual >30.

Next, variants were annotated onto dbSNPvs138, ClinVar, Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping v2 (Polyphen-2), Mutation Taster, and the Combined Annotation Dependent Depletion (CADD). Minor allele frequencies (MAFs) were obtained from the Exome Variant Server (EVS); (http://evs.gs.washington.edu/EVS/), the 1000 Genomes Project phase 3 populations (1KG) and the Exome Aggregation Consortium (ExAC). In addition, functional annotation of each variant identifying synonymous, non-synonymous, intronic, and splice region variants etc. was performed using the Variant Effect Predictor.

Variant filtering and candidate gene discovery
Exome sequencing of healthy controls was carried out to perform analysis-by-exclusion, which involves prioritizing of rare variants with potential damaging consequences henceforth referred to as deleterious (e.g. missense, STOP gain/loss, insertions/deletions [indels], exon-intron boundaries) that are present exclusively in PSD patients, assuming that if present in controls, by definition, they could not be causal. All variant filtering steps were carried out using VCFtools.

To select singletons, we filtered for private variants in PSD patients, followed by the selection of rare variants with minor allele frequency (MAF) ≤1% in the European populations from the 1KG, EVS and ExAC. Rare variants were further filtered by selecting those with putative functional consequences henceforth referred to as deleterious as described above. Next, functional annotation analysis was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID v.6.8; www.david.ncifcrf.gov), which allowed enrichment of genes carrying the Gene Ontology (GO) terms such as platelet secretion and signalling in biological
process, cellular component and molecular function, followed by identification of relevant annotation categories. Statistical significance of annotation terms was based on a DAVID Expression Analysis Systematic Explorer Score, which is based on a Modified Fisher Exact test. Gene clusters were considered significant with a Bonferroni P<0.05. GO terms such as platelets and secretion, platelets and granules, and platelets and signaling were used to select potential candidate genes.

The candidate platelet gene analysis was performed exploiting a list of 329 putative genes affected in individuals with platelet function disorders previously described. In this part of analysis, we selected all variants present in the coding regions, 100 base pairs (bp) of 5’ and 3’ untranslated regions and 10 bp exon-intron boundaries of the 329 candidate genes in PSD cases. Rare variants were selected on the bases of MAF ≤1% followed by selection of putatively deleterious variants as described above.

Variants identified in both filtering strategies were pulled together in one table and cross-referenced against the controls and only SNVs present in PSD patients were selected. Supporting information was gathered using the UniProt Consortium and the ClinVar (www.ncbi.nlm.nih.gov/clinvar/).

Sanger sequencing was performed to confirm NGS results.
Supplementary References

1. Leo VC, Morgan NV, Bem D, et al. Use of next-generation sequencing and candidate gene analysis to identify underlying defects in patients with inherited platelet function disorders. J Thromb Haemost. 2015;13(4):643-650.

2. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res. 2003;31(13):3812-3814.

3. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet. 2013; 76(1):7.20.01-41.

4. Kircher M, Witten DM, Jain P, O’Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nature genetics. 2014;46(3):310-315.

5. Cattaneo M, Cerletti C, Harrison P, et al. Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH. J Thromb Haemost. 2013;11(6):1183-1186.

6. Femia EA, Pugliano M, Podda G, Cattaneo M. Comparison of different procedures to prepare platelet-rich plasma for studies of platelet aggregation by light transmission aggregometry. Platelets. 2012;23(1):7-10.

7. Cattaneo M, Lecchi A, Zighetti M, Lussana F. Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. Haematologica. 2007;92(5):694-697.

8. Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML. Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores: further evidence that some cases of platelet 'primary secretion defect' are heterozygous for a defect of P2CYC receptors. Arterioscler Thromb Vasc Biol. 2000;20(11):101-106.

9. Lotta LA, Wang M, Yu J, et al. Identification of genetic risk variants for deep vein thrombosis by multiplexed next-generation sequencing of 186 hemostatic/pro-inflammatory genes. BMC Med Genomics. 2012;5(7):1-8.

10. Sherry ST, Ward M, Sirotkin K. dbSNP-database for single nucleotide polymorphisms and other classes of minor genetic variation. Genome Res. 1999;9(8):677-679.
11. Landrum MJ, Lee JM, Riley GR, et al. ClinVar: public archive of relationships among sequence variation and human phenotype. Nucleic Acids Res. 2014;42(Database issue):D980-D985.

12. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Met. 2014;11(4):361-362.

13. Abecasis GR, Auton A, Brooks LD, et al. An integrated map of genetic variation from 1,092 human genomes. Nature. 2012;491(7422):56-65.

14. Lek M, Karczewski KJ, Minikel EV, et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016;536(7616):285-291.

15. McLaren W, Pritchard B, Rios D, Chen Y, Flicek P, Cunningham F. Deriving the consequences of genomic variants with the Ensembl API and SNP Effect Predictor. Bioinformatics. 2010;26(16):2069-2070.

16. Danecek P, Auton A, Abecasis G, et al. The variant call format and VCFtools. Bioinformatics. 2011;27(15):2156-2158.

17. Huang DW, Sherman BT, Tan Q, et al. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol. 2007;8(9):183.181-183.116.

18. Pundir S, Martin MJ, O'Donovan C. UniProt Protein Knowledgebase. Methods Mol Biol. 2017;1558(2):41-55.