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

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 doi:10.3324/haematol.2018.204990

Received: August 21, 2018.

Accepted: February 22, 2019.

Pre-published: February 28, 2019.

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

	PSD patients N=14*	**Internal reference range****
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)

****** (5^{th}-95^{th} percentiles)

Table S2. Single-nucleotide variants (n=107) identified in 14 PSD patients by WES followed by two prioritizing approaches, according to Leo et al.[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	rs34515106	p.K108Q	.	0.0007	0.0016	T	P	D	22.4	+	-	LB
C696	MERTK	c.A2305G	rs147899488	p.I769V	.	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.K8325E	0.008	0.0076	0.0093	T	B	D	13.41	+	-	LB
C696	TTN	c.A15563C	rs72648930	p.Q5188P	0.001	0.0021	0.0015	T	D	D	15.15	+	-	LB
C696	CSRNP1	c.G401A	rs757921966	p.R134H	.	.	1.5E-05	T	B	N	23.5	-	-	LB
C696	MMRN1	c.G1546T	rs141872900	p.V516L	0.007	0.0084	0.0073	T	B	N	0.575	+	-	LB
C696	DGKI	c.G553A	rs779164061	p.V185I	.	.	0.000015	D	P	D	23	+	-	LB
C708	QSOX1	c.G1060A	rs148353050	p.V354M	.	0.0008	0.0003	T	P	N	10.24	+	-	LB
C708	TTN	c.G106955A	rs200497615	p.R35652Q	.	0.0007	0.0003	T	B	D	24.6	+	-	VUS
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.V208L	.	0.0002	1.5E-05	T	B	N	0.135	+	+	LB
C708	COL4A4	c.G2630A	rs150979437	p.R877Q	.	0.0033	0.0038	T	B	N	9.424	-	-	LB
C708	ITPR1	c.C5098T	rs540818757	p.P1700S	.	.	.	T	.	D	9.375	+	+	LB

C708	CSRNP1	c.C1389G	.	p.S463R	.	.	.	D	D	N	25.9	-	-	LB
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	0.0009	T	D	D	24.8	+	-	LB
C708	NRP1	c.G620A	rs148308681	p.R207H	.	0.0001	0.0001	D	D	D	33	-	-	VUS
C729	FCGR2A	c.A836C	rs146883516	p.D279A	0.002	0.0017	0.002	D	B	N	22.6	+	+	LB
C729	TTN	c.C104564A	.	p.S34855Y	.	.	.	D	D	D	19.45	+	-	VUS
C729	TTN	c.A13364G	rs142304137	p.K4455R	0.002	0.0001	0.0003	D	P	D	8.924	+	-	LB
C729	ITGA2	c.G305A	rs41392746	p.S102N	.	.	3.01E-05	T	B	N	19.85	+	+	LB
C729	ITPR3	c.G2056A	.	p.E686K	.	.	.	T	P	D	23.7	+	-	LB
C729	MYO3A	c.T1525C	rs150793986	p.Y509H	.	0.0003	0.0002	D	D	D	27.4	-	+	VUS
C729	MUC2	c.G6931A	rs200823008	p.V2311I	.	0.0001	0.0008	.	.	.	.	-	+	VUS
C729	ARHGAP1	c.C787T	rs144801476	p.L263F	0.006	0.01	0.0076	D	P	D	23.3	+	+	LB
C732	COL24A1	c.C314T	rs372813075	p.P105L	.	0.0001	1.5E-05	T	P	D	13.79	-	-	LB
C732	LEFTY2	c.A613G	rs770500519	p.T205A	.	.	3.22E-05	D	P	D	23.5	-	-	LB
C732	ITPR1	c.C4236G	rs61757110	p.H1412Q	0.003	0.0015	0.0012	D	D	D	22.9	+	+	LB
C732	EXOC1	c.G2009A	rs35001804	p.G670E	0.003	0.0086	0.0086	D	D	D	32	+	+	VUS
C732	AP3S1	c.A368G	rs199536113	p.N123S	.	.	0.0005	T	B	D	13.77	+	+	LB
C732	BRPF3	c.A3055G	rs145016452	p.S1019G	0.001	0.0031	0.003	T	B	D	17.5	-	-	LB
C732	PLG	c.T1380A	rs116573785	p.S460R	0.001	0.0017	0.0027	T	B	N	7.855	+	-	LB
C732	GNB2	c.A367G	rs771355621	p.I123V	.	.	1.53E-05	T	B	D	11.45	+	+	LB
C739	RAP1GAP	c.A1904G	rs147394161	p.Y635C	0.0099	0.013	0.014	T	P	D	27.8	+	+	B
C739	ABCG5	c.A1567G	rs140899003	p.I523V	.	0.0024	0.002	T	B	N	0.001	-	+	LB
C739	TTN	c.G21202C	.	p.A7068P	.	.	.	T	B	D	20.5	+	-	LB
C739	DGKQ	c.C2596G	rs376714052	p.R866G	.	0.0001	3.37E-05	T	B	D	27.7	-	-	LB

C739	APC	c.A398G	.	p.Y133C	.	.	.	D	D	D	23.9	-	+	LB
C739	DIAPH1	c.T3227G	rs143763573	p.F1076C	.	.	0.0001	D	D	D	26	+	+	VUS
C739	ITPR3	c.C5720T	.	p.T1907M	.	.	.	D	D	D	33	+	-	VUS
C740	TTN	c.C72358T	rs372309164	p.L24120F	.	0.0002	0	T	D	D	17.65	+	-	VUS
C740	TTN	c.G1895A	rs150231219	p.G632D	.	0.0002	0	D	B	N	18.85	+	-	VUS
C740	STXBP5L	c.G3430A	rs139176240	p.D1120N	.	0.0001	0	T	D	D	25.1	+	+	VUS
C740	SLC2A7	c.C670T	rs35776221	p.R224C	0.006	0.01	0.008	D	D	D	27	-	-	VUS
C740	LCN1	c.G298C	rs117638349	p.G100R	0.006	0.008	0.004	D	D	B	23	-	-	VUS
C740	APC	c.C6821T	rs34919187	p.A2274V	.	0.0015	0	T	B	N	16.24	-	+	LB
C740	DNAH11	c.A9935T	rs72657389	p.D3312V	0.008	0.004	0	T	P	D	23.6	-	+	LB
C740	PRKACG	c.C280T	.	p.R94C	.	.	.	D	D	N	22.6	+	+	VUS
C740	ADRA2A	c.G116A	rs539511086	p.R39Q	.	.	0	D	B	N	22.6	+	+	LB
C740	MUC2	c.G2594A	.	p.S865N	.	.	.	.	.	.	.	-	+	VUS
C740	MUC2	c.A5038G	rs371137719	p.T1680A	0.0099	0.0024	0	.	.	.	.	-	+	VUS
C749	F5	c.C3438G	rs6005	p.H1146Q	.	0.0003	6E-05	D	P	N	1.962	+	-	LB
C749	LYST	c.G8806A	rs2753327	p.V2936I	0.001	0.0009	0.0009	T	B	D	22	-	+	VUS
C749	LYST	c.A8224C	rs766760874	p.M2742L	.	.	1.51E-05	T	B	N	16.27	-	+	LB
C749	TTN	c.G49413T	rs202094100	p.W16471C	.	0.0008	0.0006	D	D	D	23.5	+	-	VUS
C749	COL4A3	c.T4421C	rs200302125	p.L1474P	0.003	0.0041	0.0046	D	D	D	23.4	-	-	LB
C749	DGKG	c.T1524G	.	p.F508L	.	.	.	T	B	N	0.172	+	-	LB
C749	PDGFC	c.A113G	rs139145392	p.Q38R	0.008	0.0066	0.007	T	B	D	0.016	+	-	LB
C749	CSF1R	c.T2876C	.	p.I959T	.	.	.	T	B	N	0.001	-	-	LB
C749	PHF14	c.G298T	.	p.E100X	.	.	.	.	.	D	38	-	-	VUS
C749	PTPN12	c.C1066T	rs752211731	p.P356S	.	.	0	D	D	D	27	+	+	VUS
C783	PLAT	c.G1481C	rs61755432	p.G494A	.	0.0007	0.001	T	D	D	23.4	-	-	LB

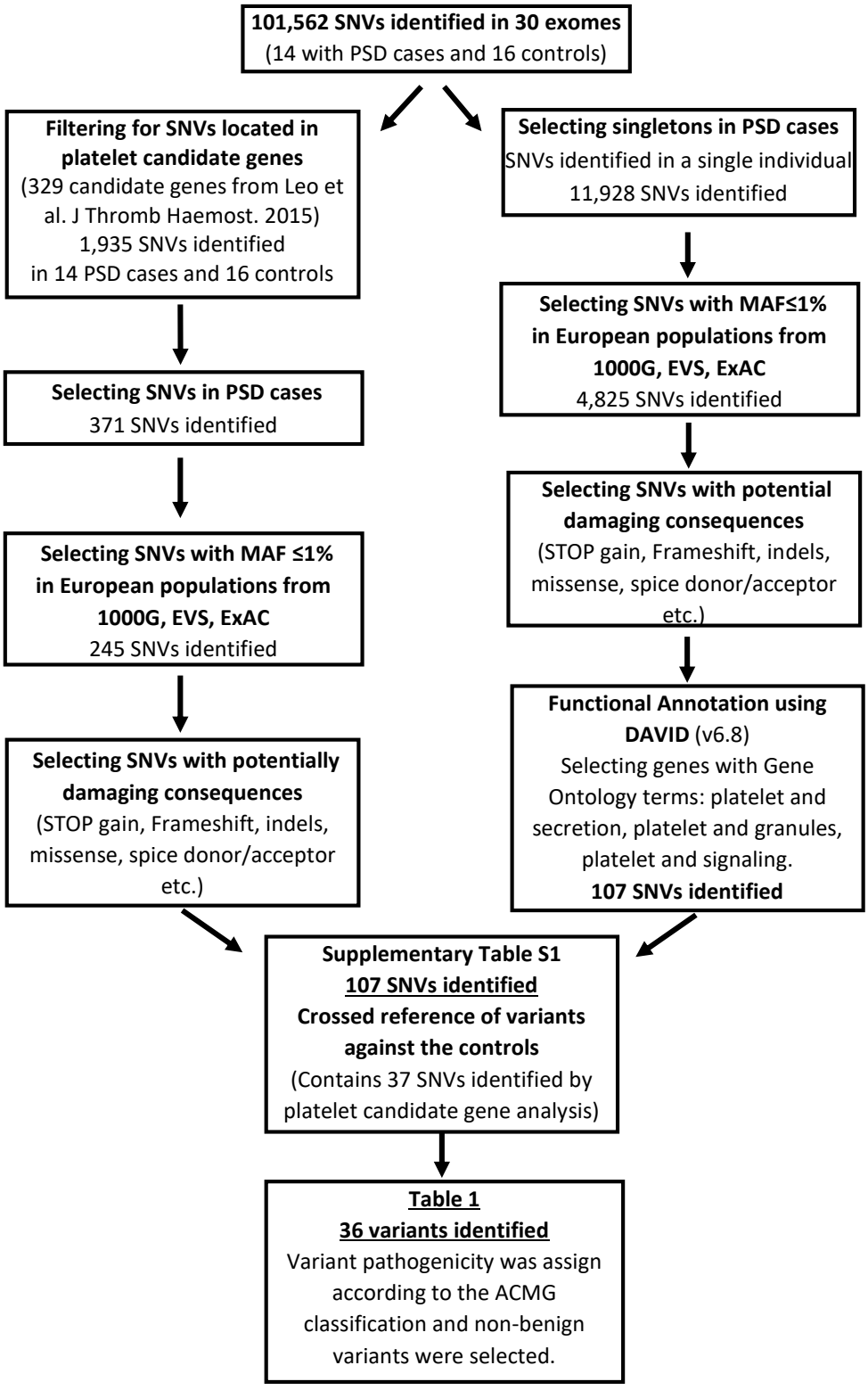
C797	TTN	c.C88394T	rs146181116	p.S29465F	0.007	0.0045	0.0039	T	D	D	22.5	+	-	LB
C797	TTN	c.T62996G	.	p.F20999C	.	.	.	T	P	N	2.844	+	-	LB
C797	TTN	c.C17T	rs201490999	p.P6L	.	.	.	D	D	D	23.8	+	-	VUS
C797	EGF	c.G3073A	.	p.A1025T	.	.	.	T	B	D	15.3	+	-	VUS
C797	PDGFRB	c.G946A	rs41287112	p.V316M	0.003	0.0046	0.0088	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	FN1	c.A751T	rs55822567	p.N251Y	0.001	0.0017	0.0026	D	P	N	23.7	+	-	LB
C831	FARP2	c.G1552A	rs746757859	p.G518R	.	.	9.36E-05	T	P	N	7.731	-	+	LB
C831	MMRN1	c.A3251G	rs201761344	p.N1084S	.	.	0.0001	D	P	N	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	.	.	.	D	D	D	26	+	+	VUS
C847	CASP9	c.A220G	rs145118493	p.M74V	.	0.0014	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	.	0.0001	0.0002	T	B	D	10.55	+	+	LB
C847	APC	c.G3949C	rs1801166	p.E1317Q	0.006	0.0093	0.0057	T	B	A	7.737	-	+	LB
C847	F13A1	c.G1861T	rs145180358	p.A621S	.	.	0.0007	T	B	D	23.8	+	-	LB
C847	PHACTR2	c.G1360C	.	p.D454H	.	.	.	D	D	D	25.8	+	-	VUS

C847	NOS3	c.C3385T	rs774447524	p.R1129C	.	.	2.31E-05	D	D	D	34	-	-	VUS
C847	PDGFRL	c.C1046A	rs146087994	p.T349K	.	.	1.5E-05	T	D	D	27.8	-	-	LB
C862	APC	c.C3511T	rs201830995	p.R1171C	0.001	0.0002	0.0003	D	B	N	24.1	-	+	LB
C1075	TTN	c.A53717G	rs727503606	p.K17906R	.	.	0.000015	T	B	N	7.856	+	-	B
C1075	TTN	c.T14477G	.	p.L4826R	.	.	.	D	P	N	1.837	+	-	LB
C1075	PRKCD	c.A1043G	rs33911937	p.N348S	.	0.0015	0.0016	T	B	D	15.06	+	+	VUS
C1075	STX11	c.G799A	rs45574234	p.V267M	0.0089	0.0092	0.0079	D	D	D	24	+	+	LB
C1107	COL11A1	c.G3847T	rs150669855	p.V1283L	0.001	0.0014	0.0013	T	B	N	0.012	-	-	LB
C1107	PTPN7	c.G425A	rs115136927	p.R142Q	0.003	0.0072	0.0062	T	D	D	26.9	+	+	VUS
C1107	TTN	c.T40931C	rs770248490	p.V13644A	.	.	1.57E-05	T	B	N	17.24	+	-	LB
C1107	PRKCD	c.G868T	.	p.A290S	.	.	.	T	D	D	24.5	+	+	VUS
C1107	MMRN1	c.G3680T	rs147451161	p.R1227L	0.003	0.0031	0.0036	D	D	D	27.8	+	-	VUS
C1107	ADCY2	c.C3167T	rs779183904	p.T1056M	.	.	6E-05	T	B	N	18.2	-	-	LB

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.

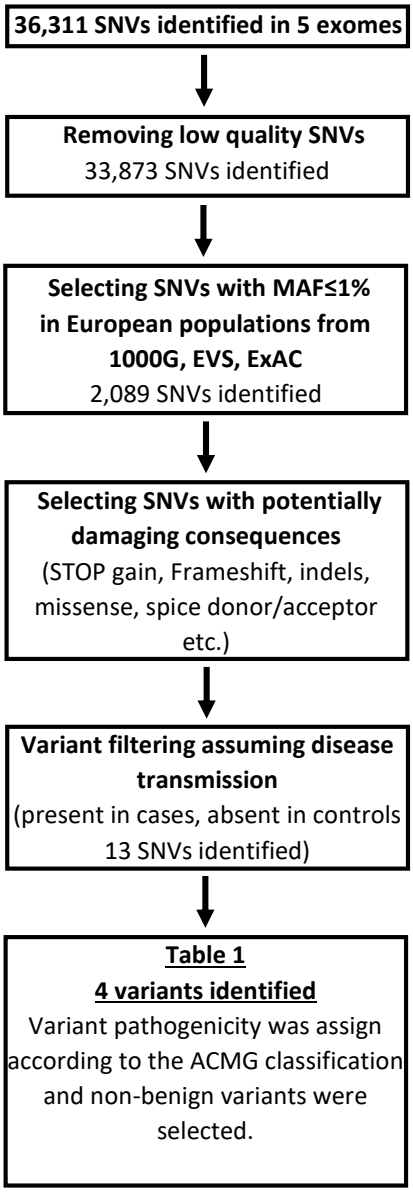
Supplementary Figures

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.



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;[2] ***PolyPhen2*** – Polymorphism Phenotyping v2.[3] ***Mutation Taster*** (www.mutationtaster.org); ***CADD*** C- score – Combined Annotation Dependent Depletion score.[4]

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.[5, 6] 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.[7]

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.[5] 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.[8] Platelet serotonin (5-HT) content was measured by the o-phthaldialdehyde method.[8] Fibrinogen was measured in washed platelets by a home-made enzyme-linked immunosorbent assay, using a polyclonal anti-fibrinogen antibody as previously reported.[8]

Whole-exome sequencing and variant annotation

Details of DNA extraction and preparation methods have been described elsewhere.[9]

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,[10] ClinVar,[11] Sorting Intolerant FromTolerant (SIFT),[2] Polymorphism Phenotyping v2 (Polyphen-2),[3] Mutation Taster,[12] and the Combined Annotation Dependent Depletion (CADD).[4] 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)[13] and the Exome Aggregation Consortium (ExAC).[14] In addition, functional annotation of each variant identifying synonymous, non-synonymous, intronic, and spice region variants etc. was performed using the Variant Effect Predictor.[15]

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.[16]

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),[17] 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.[1] 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[18] 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.