ARTICLE

Platelet proteome and function in X–linked thrombocytopenia with thalassemia and *in silico* comparisons with gray platelet syndrome

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Ferrata Storti Foundation

Haematologica 2021
Volume 106(11):2947-2959

ABSTRACT

In X-linked thrombocytopenia with thalassemia (XLTT; OMIM 314050), caused by the mutation p.R216Q in exon 4 of the *GATA1* gene, male hemizygous patients display macrothrombocytopenia, bleeding diathesis and a β-thalassemia trait. Herein, we describe findings in two unrelated Swedish XLTT families with a bleeding tendency exceeding what is expected from the thrombocytopenia. Blood tests revealed low P-PAI-1 and P-factor 5, and elevated S-thrombopoietin levels. Transmission electron microscopy showed diminished numbers of platelet α- and dense granules. The proteomes of isolated blood platelets from five male XLTT patients, compared to five sex- and age-matched controls, were explored. Quantitative mass spectrometry showed alterations of 83 proteins (fold change ≥±1.2, $q<0.05$). Of 46 downregulated proteins, 39 were previously reported to be associated with platelet granules. Reduced protein levels of PTGS1 and SLC35D3 were validated in megakaryocytes of XLTT bone marrow biopsies by immunohistochemistry. Platelet function testing by flow cytometry revealed low dense- and α-granule release and fibrinogen binding in response to ligation of receptors for ADP, the thrombin receptor PAR4 and the collagen receptor GPVI. Significant reductions of a number of α-granule proteins overlapped with a previous platelet proteomics investigation in the inherited macrothrombocytopenia gray platelet syndrome. In contrast, Ca^{2+} transporter proteins that facilitate dense granule release were downregulated in XLTT but upregulated in gray platelet syndrome. Ingenuity pathway analysis showed altered coagulation system and protein ubiquitination pathways in the XLTT platelets. Collectively, the results revealed protein and functional alterations affecting platelet α- and dense granules in XLTT, probably contributing to bleeding.

Introduction

The inherited platelet disorder, X-linked thrombocytopenia with thalassemia (XLTT; OMIM 314050) was first described in 1977 in a family where three men presented with macrothrombocytopenia, bleeding diathesis, splenomegaly and mild hemolysis of the β-thalassemia type.[1] Three additional families were reported[2-5] prior to our description of two Swedish XLTT families exhibiting a previously not reported grade 1–2/3 myelofibrosis.[6] Recently, a Danish–Swedish whole-exome sequencing study of 156 patients with bleeding tendency identified two additional

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Received: February 19, 2020.
Accepted: September 15, 2020.
Pre-published: September 28, 2020.

https://doi.org/10.3324/haematol.2020.249805

families (three individuals) with the disease.[7] All reported XLTT patients carried the same exon 4 *GATA1* p.R216Q mutation.[2-4,6-8] Similarities with the autosomal inherited disorder gray platelet syndrome (GPS)[9] have been noted regarding deficiency of platelet α-granules,[2,4,6,10] splenomegaly,[1,2,6,11] and, more recently, myelofibrosis.[6,11]

Bleeding diathesis beyond what could be expected from blood platelet counts was observed in several male XLTT patients, with occasional severe bleeds requiring platelet and/or erythrocyte transfusions[1-4,6,7] We, therefore, set out to evaluate hemostasis and platelet functions in members of our two Swedish XLTT families, as a complement to earlier investigations. Our approach was to map the XLTT platelet proteome in order to disclose anticipated platelet granule deficiencies and other abnormalities. Subsequently, we sought to validate alterations of selected proteins by immunohistochemistry (IHC) in bone marrow (BM) megakaryocytes and platelet functional reactions related to granule deficiencies by flow cytometry. Finally, we compared our platelet proteomic findings to those of a published dataset from a *NBEAL2* mutated patient diagnosed with GPS (by courtesy of Dr. Meral Gunay-Aygun, Johns Hopkins University, e-mail, 24 October 2018), aiming to disclose similarities and differences.[12]

Methods

Patients and healthy controls

Five male XLTT patients from two unrelated Swedish families (A and B) were recruited for the study. Subjects gave written informed consent in accordance with institutional guidelines and the Declaration of Helsinki. The Regional Ethical Review Board (Uppsala, Sweden) approved the studies (reference 2010/294). Pyrosequencing confirmed hemizygosity of the p.R216Q *GATA1* mutation, corresponding to the amino acid change Arg216Gln, in the five investigated males.[2,4] The phenotypical aspects, including bleedings, of the four adult patients were described previously.[6,13] Additionally, an 8-year old boy (at sampling) from family B,[6] was now included for proteomics and routine investigations. The control material for proteomics consisted of platelets from five sex- and age-matched healthy blood donors.

Platelet proteomics

For proteomic analysis, platelets from the five male XLTT patients and the five sex- and age-matched healthy volunteers were isolated. Whole blood samples were collected into EDTA Vacutainer® tubes and processed as described in the *Online Supplementary Methods* in order to obtain lysed platelet pellets.

Quantitative mass spectrometry (QMS) was performed at the Proteomics Core Facility (PCF), Sahlgrenska Academy, University of Gothenburg, Sweden. Equal amounts of total protein from each sample were trypsin digested, alkylated, and peptides subjected to the isobaric mass tagging reagent TMT® and further acidified. Peptides were purified, fractionated and analyzed on Q Exactive™ or Orbitrap Fusion Tribrid mass spectrometers (Thermo Scientific™, Waltham, MA, USA). For the identification of proteins, a database search was performed using the Mascot search engine (Matrix Science, Boston, MA, USA) followed by protein quantification based on TMT reporter ion intensities (see the *Online Supplementary Methods* for details).

Flow cytometry

Platelet activation responses to stimulation of the platelet receptors for ADP, thrombin (PAR1 and PAR4) and collagen (GPVI) were investigated using flow cytometry (see the *Online Supplementary Methods* and *Online Supplementary Figure S1* for details). In summary, diluted whole blood was incubated for 10 minutes with specific receptor agonists, and platelet activation was detected as follows: (i) a conformational change in the platelet fibrinogen receptor GPIIb/IIIa was detected as binding of a chicken anti-human fibrinogen antibody; (ii) exocytosis of platelet α-granules was detected as binding of a mouse anti-human P-selectin (CD62P) antibody; (iii) exocytosis of platelet lysosomes was detected as binding of an antibody towards human LAMP1 (CD107a); and (iv) exposure of the procoagulant phospholipid phosphatidylserine (PS) was detected as binding of annexin V. Capacity of platelet dense granule release of ADP was detected by an indirect method, where the effect of addition of apyrase to samples to degrade released ADP was investigated as previously described.[14,15] Compared to e.g., CD63 or LAMP2 exposure, which only proves granule exocytosis but does not give information on granule contents, this alternative approach was used as it illustrates the functional consequences of released dense granule contents and not only the possibility to exocytose the granules.

For additional methods regarding hematological and other blood tests, transmission electron microscopy (TEM), platelet proteomics, bioinformatics, immunohistochemistry, flow cytometry, and statistical analyses, see the *Online Supplementary Methods*.

Data sharing statement

The complete set of quantified platelet proteins that was uploaded to Ingenuity Pathway Analysis (IPA)[16] is listed in the *Online Supplementary Appendix*.

Results

Bleeding and laboratory tests

The five male patients (including one child) included in the proteomics investigation had bleeding diathesis with recurring nose bleeds and spontaneous hematomas. One of the adult patients (who had stable platelet counts between 50–90×10^9/L) had on one occasion, after a minor trauma during sports activity, severe thigh muscle bleeding with compartment syndrome, necessitating transfusions. Pedigrees, case reports including bleeding information, hematological indices and BM fibrosis grades of members of the A and B XLTT families were given previously.[6] Platelet and hemoglobin values as well as hemostasis related laboratory characteristics are shown in Table 1. The patients displayed mild hemolytic anemia and moderate macrothrombocytopenia (*Online Supplementary Figure S2A* and *B*), the latter despite increased numbers of CD61-positive megakaryocytes in the BM (*Online Supplementary Figure S2C* and *D*).[6] Among routine coagulation tests, APTT and PK-INR were slightly elevated in three and two adults, respectively, from family B. Plasma levels of plasminogen activator inhibitor 1 (P-PAI-1) were consistently low. P-Factor 8 levels were normal, but P-Factor 5 (P-F5) levels were below the normal range in the three adults from family B, whereas two patients had P-F5 levels in the low normal range (Table 1). Thus, the previously noted low P-F5 value in one XLTT patient[6] was also found in other family members, probably contributing to prolonged APTT and bleeding tendency. No mutation was detected in the *F5* gene from the individual with the lowest P-F5 using a TruSight One Expanded sequencing panel (Illumina, Inc., San Diego, CA, USA).

Table 1. Age at samplig for proteomics and laboratory characteristics of the five investigated male X-linked thrombocytopenia with thalassemia patients (with normal range for individual biomarkers in parentheses).

ID*	I	II	III	IV	V
Age, years	59	37	33	8	39
Hemoglobin† (134-170 g/L)	115-131	130-137	125-141	116-126	116-147
Platelet count† (145-387×10⁹/L)	25-67	44-64	57-87	96-115	22-101
MPV (7-9 fL)	11.6	11.3	11.1	11.3	11.8
APTT (29-42 s)	47	43 (ref 28-40)	48	29 (ref 26-33)	38
PK-INR (<1.2 INR)	1.0	1.2	1.2-1.3	1.1	1.0
P-Fibrinogen (2.0-4.0 g/L)	2.4-2.7	1.6	2.2	2.5	2.3
P-Factor 5 (0.6-1.5 kIU/L)	0.37	0.55	0.52	0.86	0.70
P-Factor 8 (0.5-1.8 kIU/L	1.21	0.90	1.89	1.19	0.88
P-PAI-1 (<15 kIU/L)	< 2	< 2	< 2	3.5	< 2
P-VWF (0.5-1.5 kIU/L)	0.78	0.86	0.89	0.96	1.98 (ref 0.5-2.0)
S-TPO (14-75 pg/mL)	156	121	99	ND	ND

*Patients were from two unrelated families, where I, II, III and IV belong to family B and V belongs to family A.[6] †Range from several sampling occasions. The maximum platelet count of patient V (101×10⁹/L) was sampled at an infectious episode. P: plasma; S: serum; MPV: mean platelet volume; APTT: activated partial thromboplastin time; PK-INR: prothrombin complex – international normalized ratio; PAI-1: plasminogen activator inhibitor 1; VWF: Von Willebrand Factor; TPO: thrombopoietin; ND: not determined.

We investigated whether or not the thrombocytopenia could be explained by defective regulation of thrombopoietin (TPO) turnover. Serum TPO levels were higher in all three sampled XLTT males (range, 99–156 pg/mL), and in a female carrier (95 pg/mL), compared to age- and sex-matched controls (n=10) showing a mean value of 47 pg/mL (range, 14–75) pg/mL (P<0.001) (Table 1; *Online Supplementary Figure S3*)

Transmission electron microscopy

TEM of platelets from three males with XLTT, representing both families, showed the presence of abnormally large platelets and deficiencies in the numbers and contents of α-granules compared to controls investigated in parallel (Figure 1A to I). Empty looking vacuoles were abundant, probably representing "ghost α-granules".[12] The dense tubular system and open canalicular system were well represented. Dense granules were not observed in XLTT but were found in platelets from healthy controls, although whole mounts were not used. Thus, we corroborated previous reports of α-granule[2,4,10] and one report of dense granule[10] deficiencies in XLTT, with probable significance for the bleeding diathesis. Overall, the ultrastructural alterations in XLTT platelets were largely similar to those described in an earlier patient.[10]

Platelet proteomics results

In order to explore potential alterations in the platelet proteome, we used QMS. In isolated platelets from the five XLTT patients and five age-matched male healthy controls, >3,100 proteins were identified, similar to previous reports.[17] Out of these, >2,200 proteins could be quantified in both patients and controls and further analyzed by statistical comparison of groups (*Online Supplementary Methods*). Eighty-three proteins were shown to be significantly altered (fold change [FC] ≥±1.2, q<0.05); 46 showing reduced and 37 elevated levels (Tables 2 and 3).

From two previously reported datasets of >800 proteins predicted to be of granule origin in healthy individuals,[18,19] 47 proteins were here identified to be differentially regulated. Congruent to findings from TEM of sparse numbers of granules, 39 of 47 (83%) predicted granule proteins identified by QMS with FC ≥±1.2 and q<0.05 were downregulated in XLTT (Table 2). SLC35D3, a protein involved in the biogenesis of platelet dense granules,[20] was downregulated in all XLTT patients, mean 3.4-times compared to healthy controls. Similar to the finding in plasma (Table 1), SERPINE1/PAI-1 (stored in α-granules) showed significantly reduced levels in XLTT platelets. In accordance with the slightly hemolytic phenotype, haptoglobin (HP) was four-times downregulated (Table 2).

In contrast, the antioxidative enzyme carbonic anhydrase 2 (CA2) was almost three-times more abundant in XLTT compared to healthy controls. Also the seventh among the most upregulated significant proteins in XLTT platelets, peroxiredoxin 1 (PRDX1), has antioxidant effects. Some other top upregulated proteins including tubulin-tyrosine ligase-like protein 12 (TTLL12), spectrin α chain, non-erythrocytic 1 (SPTAN1) and nexilin (NEXN) were cytoskeletal components (Table 3). Notably, the protein level of NBEAL2 (mutated in GPS) was not significantly altered in XLTT compared to control platelets (FC =1.05, q=0.24). The 83 significantly altered proteins were predicted in IPA[16] to originate from different subcellular compartments, the majority from the cytoplasm (*not shown*).

Pathway and network analyses

In a core analysis in IPA[16] (13/05/2019) of the 83 platelet proteins with FC ≥±1.2 and q<0.05 compared to the controls, coagulation system was the most significant pathway, with reductions of the α-granule proteins F13A1, SERPINE1/PAI-1 and von Willebrand factor (VWF). The second most significant pathway was protein ubiquitination, with five upregulated proteins (HSPA1A/HSPA1B, PSMA4, PSMB2, PSMB4, PSMC4) and one downregulated (UBE2O). Protein–protein interaction network analysis using STRING[21] (28/07/2020) suggested two clusters with altered granule and vesicle domain proteins, and one cluster with altered proteasomal proteins (Figure 2).

X-linked thrombocytopenia with thalassemia *versus* gray platelet syndrome

Comparison with a published GPS platelet α-granule fraction sub-proteome,[12] *Online Supplementary Table S2* containing 230 proteins with FC ≥±1.2 from one GPS

patient compared to one control, revealed that nine proteins, all of them known to be present in α-granules, were downregulated in both XLTT and GPS (for XLTT with criteria FC ≥±1.2 and q<0.05): LTBP1, PPBP, THBS1, SELP/P-selectin, MMRN1, APP, F13A1, HSD17B4 and ANO6. In addition, there was one jointly upregulated granule protein, SACM1L, and six granule proteins that were downregulated in XLTT but upregulated in GPS: FHL1, YWHAH, ATP2A3, WDR1, MLEC and ATP2A2 (Tables 2 and 3). Using equal criteria as for the GPS study, among 729 XLTT platelet proteins with FC ≥±1.2 regardless of statistical significance, six were found to be commonly upregulated and 30 commonly downregulated whereas 24 were contraregulated in XLTT in comparison to GPS (*Online Supplementary Figure S4*; *Online Supplementary Table S1*). The three Ca^{2+} transporting proteins ATP2A3,

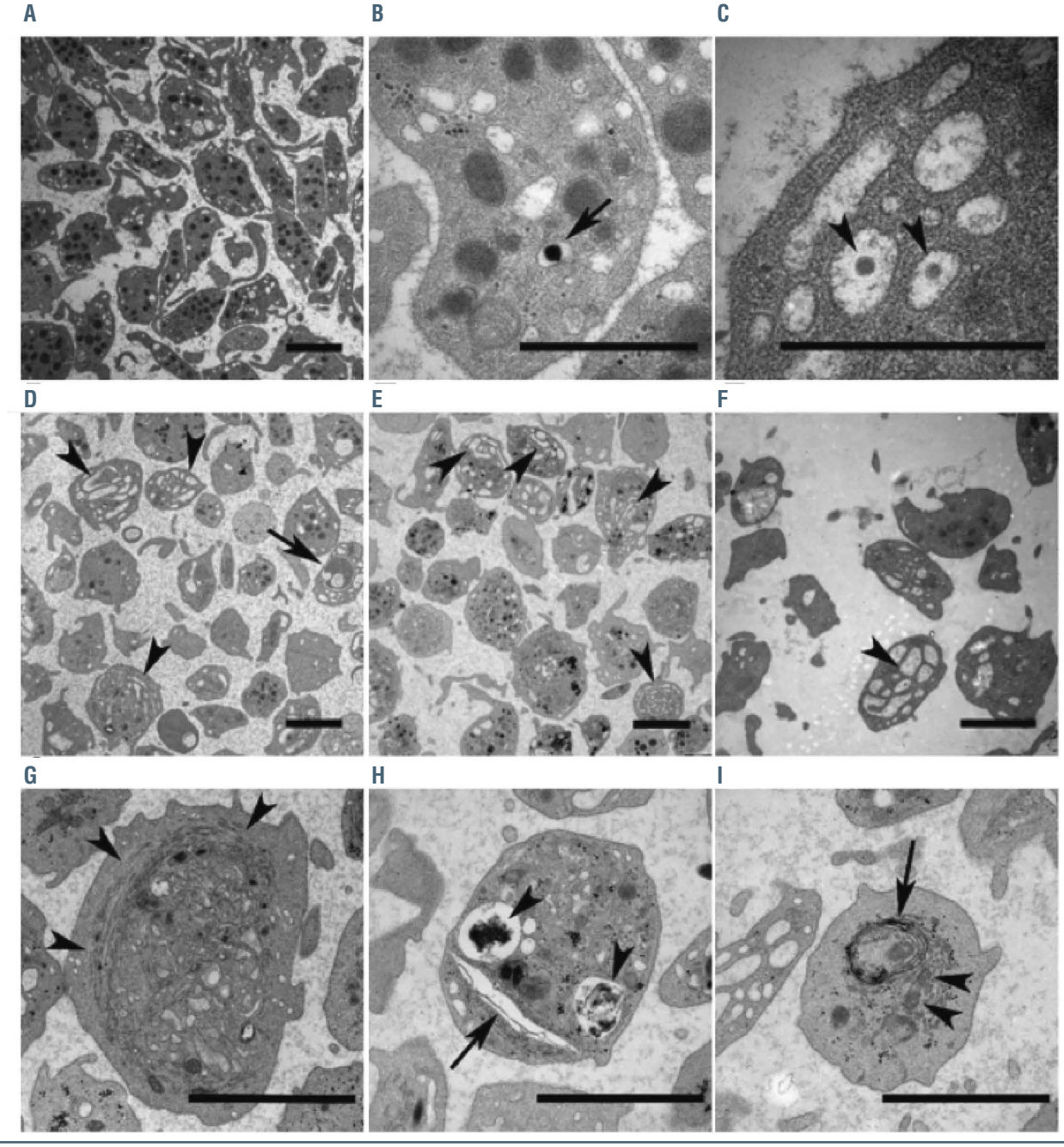

Figure 1. Occurrence of giant platelets and granule deficiency in X-linked thrombocytopenia with thalassemia. Transmission electron microscopy (TEM) graphs of platelets (A and B) from a healthy control and (C to I) from three X-linked thrombocytopenia with thalassemia (XLTT) patients. Bars in (A and D–I) =2 μm, in (B and C) =1 μm. (A) Platelets from controls show a normal morphology with few mitochondria, some open canalicular system components and several rounded α-granules. (B) A few dense granules (arrow) were also observed in the controls. (C) Detail of a platelet from XLTT patient I (Table 1) shows reduced amounts of α-granules and mostly empty vacuoles, some containing small remnants (arrowheads) of α-granules. (D) Overview of platelets from XLTT patient V (Table 1) showing reduced amounts of α-granules and some platelets with increased dilated open canicular system (arrowheads). A platelet containing a secondary platelet was also observed (arrow). (E) Overview of platelets from XLTT patient II (Table 1) showing reduced amounts of α-granules and platelets with increased dilated open canicular system (arrowheads). (F) Overview of platelets from XLTT patient I, showing reduced amounts of α-granules and increased dilated open canicular system (arrowhead). (G) An agranular macrothrombocyte from XLTT patient II, with microtubuli in the periphery (arrowheads), vacuoles and some mitochondria. (H) An agranular macrothrombocyte from XLTT patient II, with a tubular inclusion (arrow) and some vacuoles containing electron dense cell debris (arrowheads). (I) An agranular macrothrombocyte from XLTT patient II, containing some mitochondria (arrowheads) and elements of the dense tubular system (arrow).

Table 2. **Significantly downregulated proteins in X-linked thrombocytopenia with thalassemia platelets and overlap in gray platelet syndrome.**

Uniprot ID	IPA ID	Protein name	XLTT FC	q-values	Gran*	GPS†
P00738	HP	Haptoglobin	-4.03	0.0319		
Q5M8T2	SLC35D3	Solute carrier family 35 member D3	-3.36	0.0156		
P05121	SERPINE1	Plasminogen activator inhibitor 1	-2.17	0.0041	x	
Q14766	LTBP1	Latent-transforming growth factor beta-binding protein 1	-2.11	0.0028	x	Down
P0C7M8	CLEC2L	C-type lectin domain family 2 member L	-2.10	0.0319		
P02775	PPBP	Platelet basic protein	-2.08	0.0103	x	Down
Q12912	LRMP	Lymphoid-restricted membrane protein	-2.01	0.0312		
Q16799	RTN1	Reticulon-1	-2.00	0.0202		
P07996	THBS1	Thrombospondin-1	-1.78	0.0088	x	Down
Q13642	FHL1	Four and a half LIM domains protein 1	-1.77	0.0285	x	Up
P16109	SELP	P-selectin	-1.74	0.0041	x	Down
P09486	SPARC	Secreted protein acidic and rich in cystein	-1.74	0.0242	x	
Q13201	MMRN1	Multimerin-1	-1.69	0.0220	x	Down
Q13576	IQGAP2	Ras GTPase-activating-like protein IQGAP2	-1.67	0.0009	x	
P04275	VWF	von Willebrand factor	-1.67	0.0092	x	
Q8WXF7	ATL1	Atlastin-1	-1.63	0.0202	x	
Q9UIB8	CD84	SLAM family member 5	-1.62	0.0110	x	
Q9NRW1	RAB6B	Ras-related protein Rab-6B	-1.60	0.0243	x	
Q16643	DBN1	Drebrin	-1.59	0.0364	x	
P05067	APP	Amyloid beta A4 protein	-1.58	0.0194	x	Down
Q8WXE9	STON2	Stonin-2	-1.57	0.0259	x	
P27338	MAOB	Amine oxidase [flavin-containing] B	-1.57	0.0088	x	
Q8WWA1	TMEM40	Transmembrane protein 40	-1.55	0.0259	x	
Q8TDZ2	MICAL1	Protein-methionine sulfoxide oxidase	-1.48	0.0074	x	
Q5VWC8	HACD4	Very-long-chain (3R)-3-hydroxyacyl-[acyl-carrier protein] dehydratase 4	-1.45	0.0335	x	
Q5SQ64	LY6G6F	Lymphocyte antigen 6 complex locus protein G6f	-1.45	0.0103	x	
O95219	SNX4	Sorting nexin-4	-1.45	0.0006		
O43488	AKR7A2	Aflatoxin B1 aldehyde reductase member 2	-1.44	0.0333	x	
Q7L9L4	MOB1B	MOB kinase activator 1B	-1.43	0.0333	x	
P31146	CORO1A	Coronin-1A	-1.43	0.0497	x	
P00390	GSR	Glutathione reductase, mitochondrial	-1.41	0.0220		
P47755	CAPZA2	F-actin-capping protein subunit alpha-2	-1.40	0.0223	x	
P00488	F13A1	Coagulation factor XIII A chain	-1.39	0.0220	x	Down
Q04917	YWHAH	14-3-3 protein eta	-1.37	0.0061	x	Up
P43304	GPD2	Glycerol-3-phosphate dehydrogenase, mitochondrial	-1.35	0.0074	x	
P51659	HSD17B4	Peroxisomal multifunctional enzyme type 2	-1.32	0.0220	x	Down
Q9NR12	PDLIM7	PDZ and LIM domain protein 7	-1.31	0.0393	x	
Q4KMQ2	ANO6	Anoctamin-6	-1.31	0.0043	x	Down
Q93084	ATP2A3	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3	-1.30	0.0103	x	Up
O75083	WDR1	WD repeat-containing protein 1	-1.30	0.0331	x	Up
Q14165	MLEC	Malectin	-1.29	0.0413	x	Up
P23219	PTGS1	Prostaglandin G/H synthase 1	-1.28	0.0460	x	
Q96AX2	RAB37	Ras-related protein Rab-37	-1.24	0.0471	x	
P16615	ATP2A2	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	-1.24	0.0333	x	Up
Q9C0C9	UBE2O	Ubiquitin-conjugating enzyme E2 O	-1.23	0.0219	x	
Q16851	UGP2	UTP--glucose-1-phosphate uridylyltransferase	-1.23	0.0241	x	

*Proteins potentially associated with platelet granules, as published before.[17,18] †Protein levels elevated or reduced, here defined as fold change [FC] ≥±1.2, in a published gray platelet syndrome (GPS) platelet α-granule fraction proteome.[12] *(Online Supplementary Table S2)* XLTT: X-linked thrombocytopenia with thalassemia.

Table 3. Significantly upregulated proteins in X-linked thrombocytopenia with thalassemia platelets and overlap in gray platelet syndrome.

Uniprot ID	IPA ID	Protein name	XLTT FC	q-values	Gran*	GPS†
P00918	CA2	Carbonic anhydrase 2	2.91	0.0006	x	
P61247	RPS3A	40S ribosomal protein S3a	2.59	0.0092		
P49247	RPIA	Ribose-5-phosphate isomerase	2.17	0.0202		
Q14166	TTLL12	Tubulin--tyrosine ligase-like protein 12	1.94	0.0156	x	
P51692	STAT5B	Signal transducer and activator of transcription 5B	1.86	0.0202		
Q8N3F0	MTURN	Maturin	1.79	0.0022		
Q06830	PRDX1	Peroxiredoxin-1	1.70	0.0335	x	
Q13813	SPTAN1	Spectrin alpha chain, non-erythrocytic 1	1.68	0.0082		
Q0ZGT2	NEXN	Nexilin	1.60	0.0317	x	
Q99447	PCYT2	Ethanolamine-phosphate cytidylyltransferase	1.58	0.0061		
Q9UK76	JPT1	Hematological and neurological expressed 1 protein	1.55	0.0202		
P04080	CSTB	Cystatin-B	1.54	0.0202		
P54727	RAD23B	UV excision repair protein RAD23 homolog B	1.51	0.0076	x	
P34949	MPI	Mannose-6-phosphate isomerase	1.48	0.0227		
Q6YHK3	CD109	CD109 antigen	1.47	0.0092	x	
P46783	RPS10	40S ribosomal protein S10	1.46	0.0223		
P48147	PREP	Prolyl endopeptidase	1.41	0.0103		
Q5T0N5	FNBP1L	Formin-binding protein 1-like	1.41	0.0373		
Q6UX71	PLXDC2	Plexin domain-containing protein 2	1.41	0.0103		
P05023	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	1.35	0.0028		
Q99733	NAP1L4	Nucleosome assembly protein 1-like 4	1.35	0.0333	x	
P28070	PSMB4	Proteasome subunit beta type-4	1.35	0.0468		
P98082	DAB2	Disabled homolog 2	1.34	0.0487		
P49721	PSMB2	Proteasome subunit beta type-2	1.33	0.0227		
Q14392	LRRC32	Leucine-rich repeat-containing protein 32	1.33	0.0317	x	
P30085	CMPK1	UMP-CMP kinase	1.32	0.0220		
P26640	VARS	Valine--tRNA ligase	1.31	0.0010		
P43686	PSMC4	26S protease regulatory subunit 6B	1.31	0.0357		
P07814	EPRS	Bifunctional glutamate/proline--tRNA ligase	1.29	0.0471		
P50454	SERPINH1	Serpin H1	1.29	0.0497		
Q9NTJ5	SACM1L	Phosphatidylinositide phosphatase SAC1	1.29	0.0330	x	Up
Q15257	PTPA	Serine/threonine-protein phosphatase 2A activator	1.27	0.0202		
P49327	FASN	Fatty acid synthase	1.26	0.0103		
P08107	HSPA1A/HSPA1B	Heat shock 70 kDa protein 1A/1B	1.26	0.0220	x	
Q9NQC3	RTN4	Reticulon-4	1.25	0.0312		
P25789	PSMA4	Proteasome subunit alpha type-4	1.21	0.0333		
P61201	COPS2	COP9 signalosome complex subunit 2	1.21	0.0317		

*Proteins potentially associated with platelet granules, as published before.[17,18] †Protein levels elevated or reduced, here defined as fold change [FC] ≥±1.2, in a published gray platelet syndrome (GPS) platelet α-granule fraction proteome.[12] (*Online Supplementary Table S2*) XLTT: X-linked thrombocytopenia with thalassemia.

ATP2A2 and ATP2C1 were downregulated in XLTT but upregulated in GPS. One of the jointly upregulated proteins was PRDX2 (FC =2.56, *P*=0.03 but q=0.15 in XLTT, FC =1.35 in GPS),[12] *Online Supplementary Table S2* with similar antioxidant functions as PRDX1 (that was only found in XLTT).

Upstream regulators, predicted from the dataset using IPA, were then compared between the XLTT and GPS datasets. All proteins with FC ≥±1.2 (compared to the respective controls regardless of statistical significance) were included. The upstream regulators with predicted inhibition and activation, respectively (Z-score ≥±2.0; *P*<0.05), in XLTT and GPS are presented in Figure 3. RPTOR independent companion of mTOR complex 2 (aka RICTOR) showed the strongest predicted altered activity in XLTT, with its inhibition predicted mainly by elevated expression of downstream proteasome proteins (*Online Supplementary Figure S5A*) participating in the protein ubiquitination pathway. In GPS, the X-box binding protein 1 (XBP1, which responds to unfolded protein increases) had the highest absolute Z-score (activated with Z-score 4.02) among upstream regulators (Figure 3; *Online Supplementary Figure S5B*). VIPAS39, a protein that regulates platelet granule biogenesis,[22] had predicted inhibited activity in both XLTT and GPS (Figure 3; *Online Supplementary Figure S5A* and *B*).

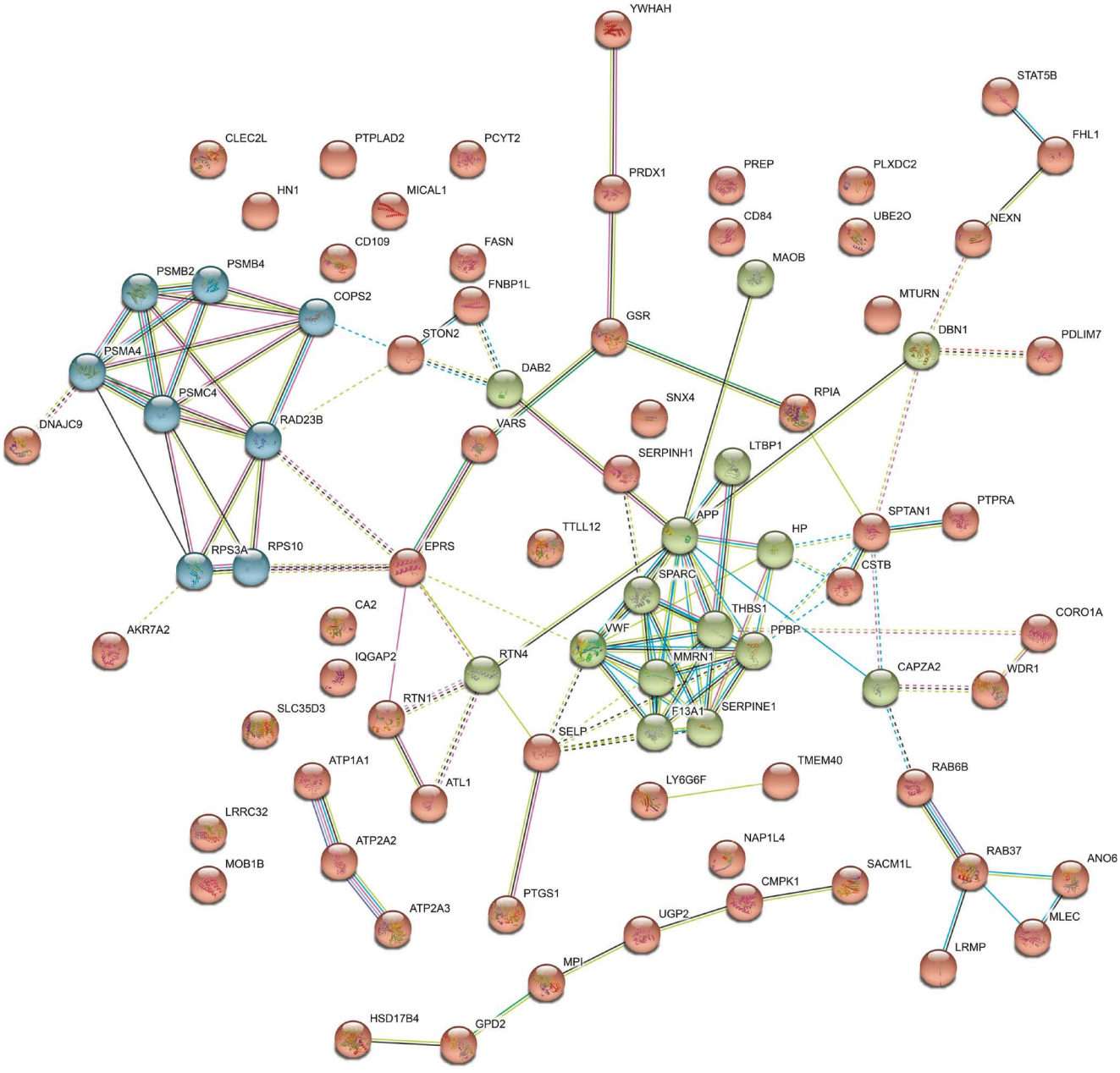

Figure 2. Analysis of possible protein–protein interaction network using STRING tool. The list of 83 differentially expressed proteins in X-linked thrombocytopenia with thalassemia (XLTT) platelets was subjected to STRING analysis, which found significantly more associations between proteins than would have occurred by chance (*P*=7.85e-12). Three clusters detected by k-means algorithm were colored as follows: cluster 1, 60 proteins, red (those of the 83 proteins of Table 2 and 3 not included in cluster 2 and 3); cluster 2, 15 proteins, green; cluster 3, eight proteins, cyan. The top three gene ontology (GO) enrichment terms regarding Biological Process given in STRING were for cluster 1: regulated exocytosis, vesicle-mediated transport, and secretion; for cluster 2: platelet degranulation, regulated exocytosis, and vesicle-mediated transport; for cluster 3: protein modification by small protein removal, cellular macromolecular catabolic process, and protein deubiquitination.

Validation of proteomics data

Immunohistochemistry

We assumed that protein alterations in the platelet proteome could reflect regulations taking place in precursor megakaryocytes in the BM, and performed IHC on two downregulated proteins (see the *Online Supplementary Methods*). The established semi-quantitative H-score ("histo-score") method was used for evaluation of megakaryocyte staining intensities. The H-score is obtained by the formula: three-times the percentage of strongly staining cells + twice the percentage of moderately staining cells + the percentage of weakly staining cells, giving a range of 0-300.[23]

Prostaglandin G/H synthase 1 (PTGS1/COX1, FC =-1.28) plays a role in production of the autocrine platelet activator thromboxane A2, important for hemostasis.[24] Although megakaryocytes from both controls and XLTT stained for the presence of PTGS1 in the perinuclear region and cytoplasm, there was an almost 50% reduction in median megakaryocyte H-score for cytoplasmic staining intensity in XLTT compared to controls (P=0.012) (Figure 4A). The perinuclear PTGS1 staining

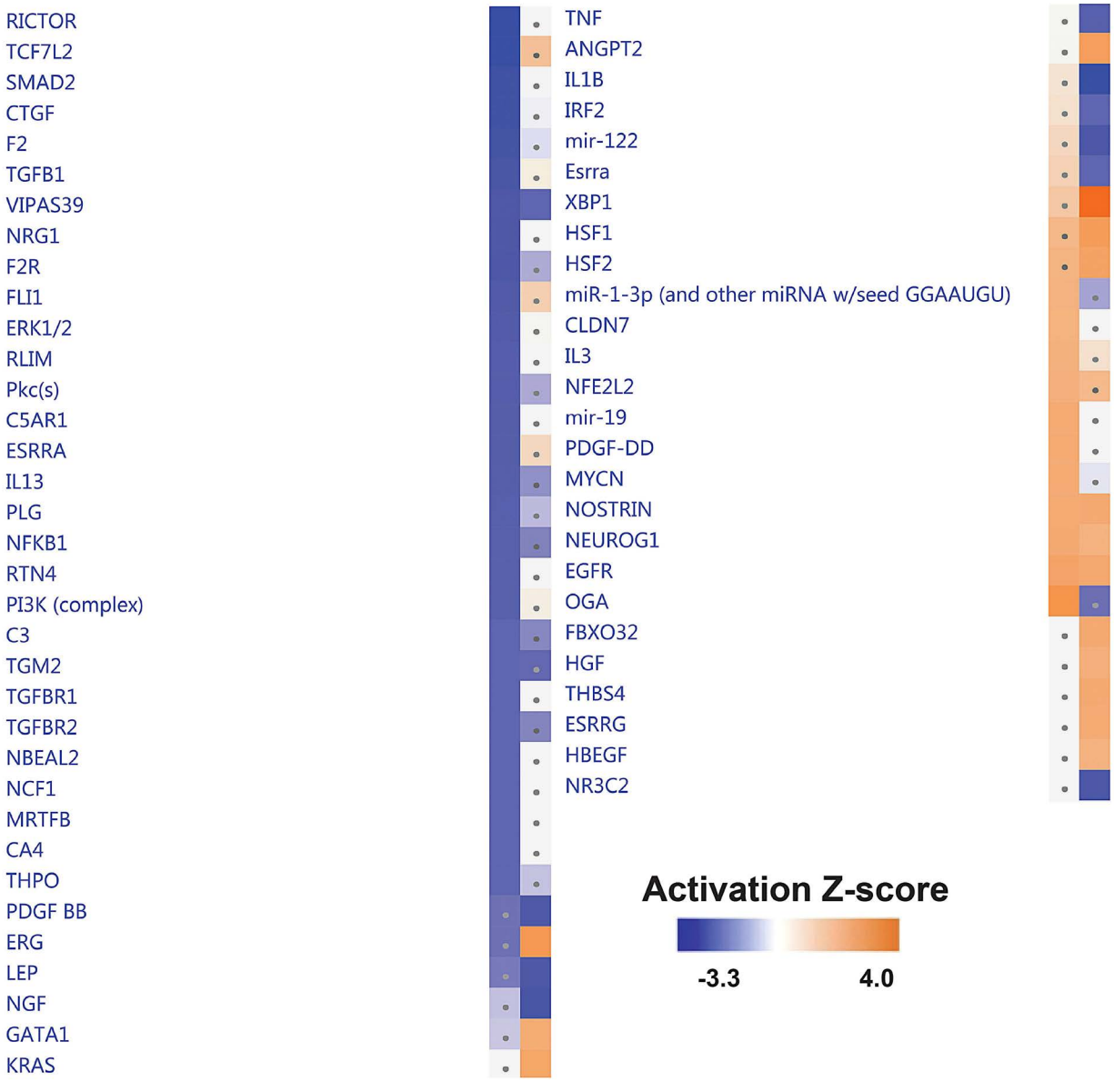

Figure 3. Analysis of upstream regulators in X-linked thrombocytopenia with thalassemia and gray platelet syndrome. Comparison of Ingenuity Pathway Analysis (IPA) core analyses of X-linked thrombocytopenia with thalassemia (XLTT) and gray platelet syndrome (GPS), where fold change (FC) ≥±1.2 compared to controls was used as the only criterion for inclusion of dysregulated proteins. The upstream regulators are sorted by ascending Z-scores in XLTT. The results are filtered to show genes, mRNA and proteins with upstream regulator Benjamini-Hochberg adjusted P<0.05 and absolute Z-score ≥±2. However, when present as a result of comparison with the other group, predicted regulators with Z-score <±2 are marked by dots in the heatmap. Several known/suggested fibrosis regulators including RICTOR, SMAD2, CTGF, TGFB1, FLI1, ERK1/2, PI3K, TGFBR1 and TGFBR2 were predicted to be inhibited in XLTT, and less so or activated in GPS. Contrarily, EGFR and PDGF-DD (also involved in fibrosis) were predicted to be activated in XLTT. NBEAL2 (mutated in GPS) showed predicted inhibited activity in XLTT. GATA1 (mutated in XLTT) was predicted to be activated in GPS. IL1, OSM, VIPAS39, RICTOR, HIF1A and F2 were significant predicted inhibited upstream regulators (Z-score ≤ -2) in XLTT when only the 83 proteins with FC ≥±1.2 and q<0.05 were included for the underlying core analysis; no upstream regulator was then predicted to be activated with Z-score ≥2 (*not shown*).

was slightly but not significantly reduced in XLTT (numerical data *not shown*).

When BM biopsies from controls and XLTT patients were stained for solute carrier family 35 member D3 (SLC35D3), the second most downregulated protein (FC =-3.40), important for dense granule formation,[20] we found a greater than 50% reduction in median megakaryocyte cytoplasmic H-score in XLTT compared to controls (P=0.006) (Figure 4B).

Platelet function testing by flow cytometry

As flow cytometry can be used for platelet function testing even at low platelet counts,[15,25,26] this method was chosen for testing platelet activation responses *ex vivo*. Blood samples from XLTT patients II and V from Table 1 were analyzed by flow cytometry for evaluation of platelet function (Figure 5). The common surface markers CD41 (GPIIb) and CD42b (GPIbα) were used to identify the platelets. These levels were not quantified, but no marked differences were noticed between healthy donors and patients (*not shown*). Platelets from patient II showed a markedly low activation response to ADP, thrombin receptor, PAR4-activating peptide (AP) and CRP-XL (cross-linked collagen-related peptide, activates collagen receptor GPVI), both considering the binding of fibrinogen to its receptor and the exposure of the α-granule protein P-selectin upon activation (Figure 5A and B). Only the response to PAR1-AP (platelet thrombin receptor PAR1-activating peptide, also known as TRAP) was close to normal. Patient V also showed a low activation response, especially to PAR4-AP and CRP-XL.

For both patients, the decrease of activation in the presence of apyrase was very low (Figure 5C and D), even for the agonists where the primary response was closer to normal. This indicates that the release and contribution to activation by ADP from platelet dense granules was low.[14,15] Control experiments were performed with blood from a normal donor diluted to the same platelet count as patient V. In these control experiments, apyrase decreased the platelet activation response considerably for all agonists, showing that released ADP normally can contribute to platelet activation even at these low platelet counts (*not shown*). No pronounced abnormalities in the capacity for induction of pro-coagulant platelet features (exposure of phosphatidylserine on the cell surface, detected as annexin V binding) upon strong stimulation by a combination of CRP-XL and PAR-activating peptides were observed for any of the patients (*Online Supplementary Table S2*), although patient V showed results in the lower part of the reference range for normal donors.

The platelets from the two patients showed a capacity to expose the lysosomal protein LAMP1 on their surface upon activation, indicating the presence of lysosomes (*Online Supplementary Table S2*).[27] As for P-selectin and fibrinogen receptor activation, the LAMP1 exposure for patient V was relatively low as compared to results in normal donors. This could suggest the presence of fewer than normal lysosomes in XLTT platelets, but no conclusions should be drawn as the platelets showed a generally low activation potential, leading to lower potential of lysosome granule release even if normal numbers of lysosomes were present.

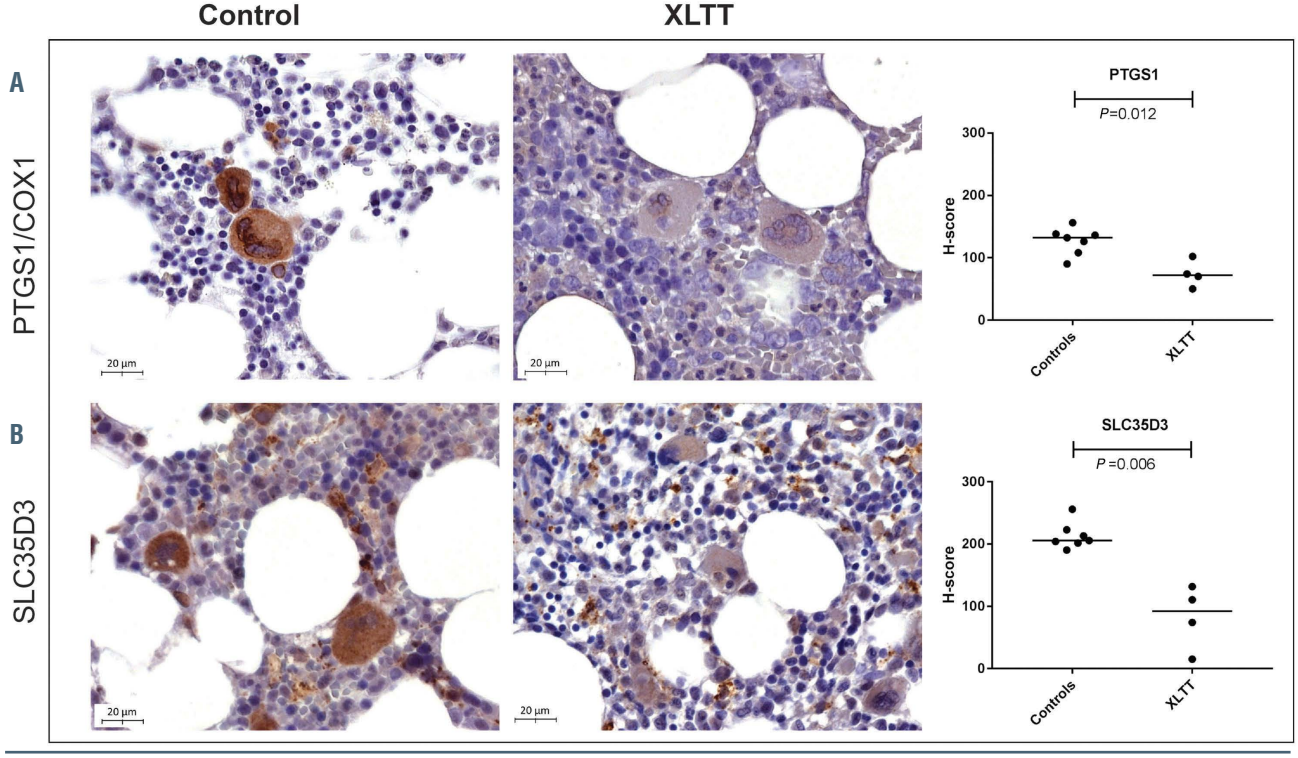

Figure 4. PTGS1/COX1 and SLC35D3 megakaryocyte staining in X-linked thrombocytopenia with thalassemia and controls. (A) Representative immunohistochemical staining of PTGS1/COX1 in bone marrow (BM) megakaryocytes from healthy control (left) and X-linked thrombocytopenia with thalassemia (XLTT) patient (center). Original magnification 60x. The graph (right) shows H-score distribution between controls (n=7) and XLTT (n=4) where the lines represent median H-scores (132 in controls, 72 in XLTT). (B) Representative immunohistochemical staining of SLC35D3 in BM megakaryocytes from healthy control (left) and XLTT patient (center). Original magnification 60x. The graph (right) shows H-score distribution between controls (n=7) and XLTT (n=4) where the lines represent median H-scores (206 in controls, 92 in XLTT).

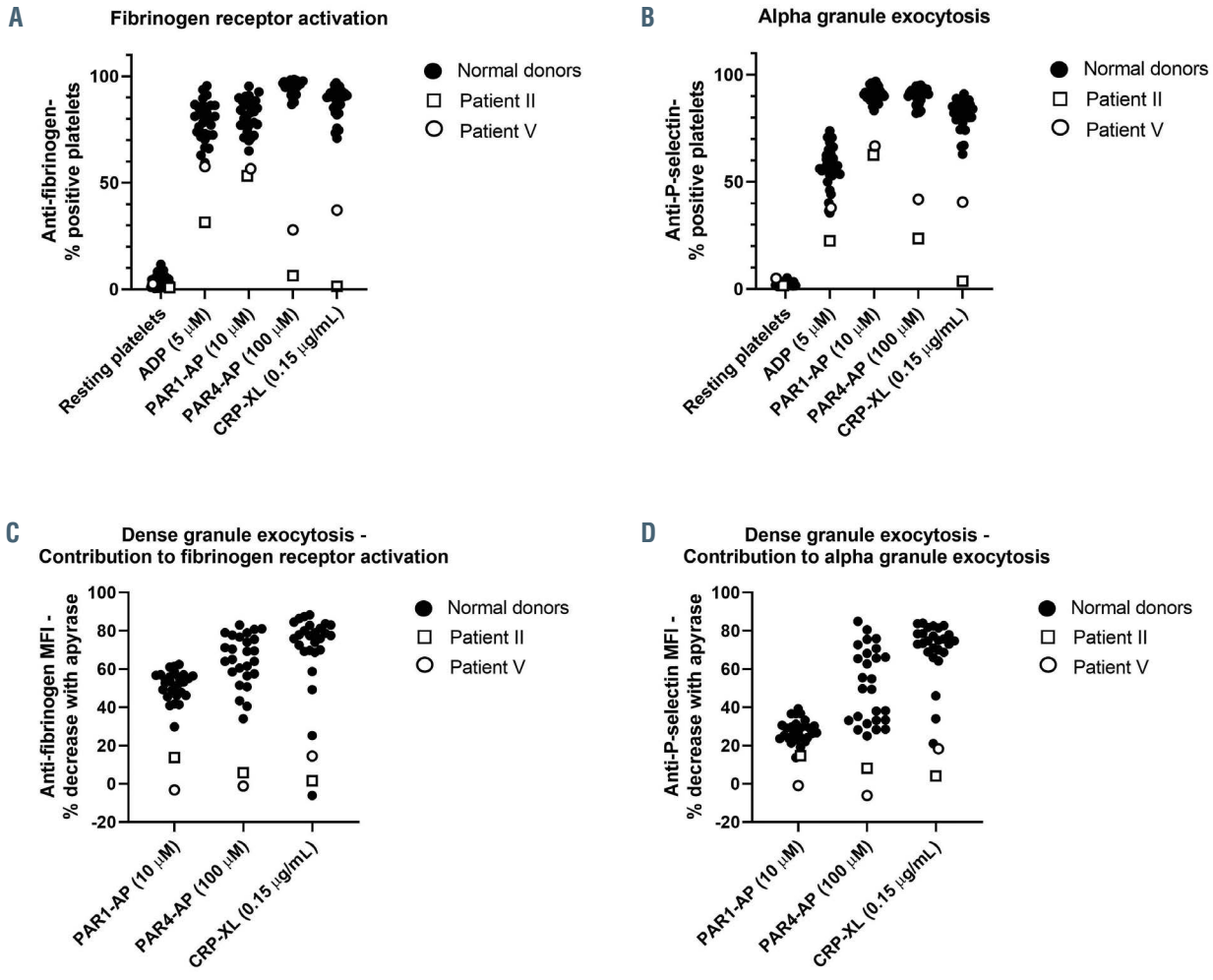

Figure 5. Examination of platelet activation by flow cytometry. Platelet activation responses were measured as binding of an antibody towards fibrinogen and binding of an antibody towards the α-granule protein P-selectin in response to exposure to platelet agonists specifically activating receptors for ADP, thrombin receptor PAR1 (PAR1-AP), PAR4 (PAR4-AP) and collagen receptor GPVI (CRP-XL). The scatter plots show results for all normal donors (n=26-30) as black circles, while results for patient II are displayed as open squares and for patient V as open circles. (A) Fibrinogen receptor activation. Percentage of platelets binding fibrinogen upon activation. (B) α-granule exocytosis. Percentage of platelets exposing P-selectin upon activation. (C) Dense granule exocytosis: contribution to fibrinogen binding. Percentage decrease in median fluorescence intensity (MFI) for the anti-fibrinogen antibody in the presence of apyrase to degrade ADP released from dense granules. (D) Dense granule exocytosis: contribution to α-granule exocytosis. Percentage decrease in MFI for the anti-P-selectin antibody in the presence of apyrase to degrade ADP released from dense granules.

Discussion

Hemostatic platelet functions are largely mediated by soluble factors released from membrane-bound storage organelles including α-granules, dense granules and lysosomes.[28] In the present study of XLTT patients, platelet TEM suggested diminished numbers of both dense- and α-granules (Figure 1), in congruence with earlier studies.[2,4,10] For an improved understanding of the molecular mechanisms behind the bleeding diathesis in XLTT, we compared the platelet proteome of five patients with five matched controls. In addition to findings of granule content deficiencies, altered protein ubiquitination was thereby suggested in XLTT.

Exploration of the cellular effects of the XLTT causing *GATA1* mutation in platelets is hampered by their lack of nuclear DNA and transcriptional regulation. Protein translation, however, is continuous throughout the lifespan of platelets and regulated by external and internal signaling.[29] Several causes of platelet protein alterations might exist. Some might be the result of *GATA1* mutation p.R216Q induced transcriptional changes, in similarity to the transcriptional dysregulation of the erythropoiesis in XLTT due to defect GATA1, leading to low expression of β-globin chains and thereby the β-thalassemia-like trait.[30] Other mechanisms could include altered trafficking of vesicles/granules from megakaryocytes to proplatelets, and changes in vesicle/granule release *in vivo* (including such occurring after platelet/megakaryocyte activation).[31] Some dysregulations of the platelet proteome could be due to absorption/endocytosis of proteins from plasma into circulating platelets, thus reflecting plasma concentrations.[32] One example of the latter is haptoglobin (FC =-4.03, q=0.03), which was generally low in XLTT plasma, probably largely due to the continuous thalassemia-like hemolysis. Notably, the altered protein ubiquitination pathway in our data (mostly elevated levels of proteasomal proteins) could imply protein degradation as an important cause and/or effect of granule deficiencies. Increased

amounts of proteasomal proteins have recently been reported also in *GFI1B* mutated macrothrombocytopenia.[31]

Some blood analyses may aid in the differential diagnosis of XLTT patients. For example, the high S-TPO in XLTT noted here differs from the reported normal or mildly elevated level in ITP.[33] Increased P-TPO levels were found in mice with the hypomorphic *Gata1*^{low} mutation.[34] If the high S-TPO level reflects deficient uptake of TPO by the TPO-receptor MPL in platelets and megakaryocytes of the thrombocytopenic XLTT patients should be further evaluated. *MPL* is transcriptionally regulated by *GATA1*,[35] and the TPO-MPL axis has crucial effects on platelet production and life span.[36] The MPL protein seemed possibly downregulated (FC =-1.84, q=0.06) in XLTT platelets.

P-PAI-1 (aka SERPINE1, an inhibitor of fibrinolysis) was low in all XLTT patients, which could contribute to bleeding. P-F5 values were below normal in three of the patients, and in the lower normal range in two. Deficiency of F5 has previously been associated with a modest bleeding diathesis.[37] Interestingly, a partially cleaved form of F5 (comprising approximately 20% of the total F5 in blood) resides in the α-granules of platelets, in complex with the protein multimerin (MMRN1).[38] Multimerin was downregulated in our proteomics assay (FC =-1.68, q=0.02) whereas the platelet F5 level change was not statistically significant. Multimerin deficiency (found also in the platelet proteome of GPS)[12] might be of interest for future evaluations regarding bleeding diathesis.[38]

The platelet QMS revealed significant (FC ≥±1.2, q<0.05) reductions of 39 proteins associated with granules. These included SERPINE1/PAI-1, vWF, SELP/P-selectin and PTGS1/COX1 which are all important for hemostasis (Table 2). Latent transforming growth factor β-binding protein 1 (LTBP1), the fourth most downregulated protein and found in α-granules, has not previously been associated with bleeding diathesis, but its interactions with transforming growth factor β (TGFB), found in platelets in high concentrations, might be of significance for BM fibrosis development. This has also been discussed for GPS.[12] Notably, TGFB1 was identified as a predicted inhibited upstream regulator in XLTT, but appeared somewhat activated in GPS (Figure 3), with possible implications for the respective myelofibrosis developments.[39] In XLTT, both the present study (Figure 3 and LTBP1) and our former IHC investigation on BM expression of CTGF and VEGF[6] showed low TGFB stimulated protein expressions in XLTT megakaryocytes/platelets.

Thrombospondin-1 (THBS1) was downregulated in XLTT platelets, and downregulated also in GPS. THBS1 is a matricellular glycoprotein first discovered in activated platelets. It interacts with a number of ligands and is of significance for, inter alia, inhibition of angiogenesis:[40] XLTT BM fibrosis is characterized by increased angiogenesis.[6] Though the exact role of THBS1 in hemostasis is unclear, it may interact with coagulation factor 13/F13A1[41] which was also found in reduced amounts in the XLTT and GPS platelet proteomes.

The identification of several jointly downregulated α-granule proteins in XLTT and GPS is congruent with ultrastructural similarities regarding deficiencies of α-granules. However, *NBEAL2* mutated GPS platelets have shown normal morphology and numbers of dense bodies/granules,[42,43] whereas our XLTT results suggested ultrastructural and functional dense granule deficiency, consistent with an earlier ultrastructural study.[10] Possible proteomic correlates to a suggested functional difference between XLTT and GPS regarding dense granule release included contraregulations of the three Ca^{2+} transporting ATPases ATP2A3, ATP2A2[44] and ATP2C1,[45] downregulated in XLTT (although q=0.057 for ATP2C1) but upregulated in GPS (*Online Supplementary Table S1*). Deficient dense granule ADP release could be a consequence in XLTT (Figure 5C and D).[44,45]

The most upregulated protein of our study, CA2 (FC =2.91, q=0.0006), was one of 26 dysregulated proteins included in the "response to stress" gene ontology found significantly enriched in STRING analysis (*not shown*).[21] CA2 participates in several biological processes, including regulations of ion transport and cytosol acidity. CA2 was recently found to predict aspirin resistance in platelet aggregation tests with arachidonic acid.[46] Elevated expression of *CA2* mRNA was found in Down syndrome-associated acute megakaryoblastic leukemia (AMKL-DS), which harbors *GATA1* exon 2 mutations, compared to other AMKL (NCBI GEO2 Accession: GSE4119).[47]

A recent investigation based on patients with GPS (*NBEAL2* mutations) and the *GATA1* mutations p.D218G and p.D218Y suggested that GATA1 enhances *NBEAL2* expression via interaction with the GATA1 co-activator friend of GATA1 (FOG1),[48] possibly explaining the α-granule deficiency in the *GATA1* mutated patients. However, GATA1 interaction with FOG1 should not be affected in XLTT due to the different mutation localization of p.R216Q which does not alter the FOG1 binding site.[8,30] In addition, NBEAL2 protein expression was not altered in XLTT platelets in our study. Thus, the mechanisms for the platelet defects in XLTT must be investigated and evaluated on their own terms.

Flow cytometry is the only method available for reliable studies of platelet functional responses at low platelet counts. Both investigated XLTT patients' platelets showed low reactivity to several platelet agonists (Figure 5), as has similarly been described in GPS.[49] We observed a release of α-granules and lysosomes, but to a lower extent compared to normal donors. If this was just a consequence of the low primary reactivity or due to lower numbers or contents of the granules is difficult to ascertain with this method. However, the TEM and proteomics data also strongly suggested a decrease in both α- and dense granules in XLTT platelets, and activation was low even at very high agonist concentrations (*not shown*). Taken together, this suggests that a lower amount of dense- and α-granules indeed caused the low P-selectin exposure in XLTT platelets. In addition, as fibrinogen is normally stored in platelet α-granules and released upon activation to aid aggregation,[29] reduced levels of platelet fibrinogen in XLTT (non-significant by QMS) might contribute to the low fibrinogen binding observed upon platelet activation. For both patients, the dense granule ADP release seemed very deficient, indicating reduced dense granule contents and/or a dense granule release defect.[25] The TEM picture in combination with low levels of proteins affecting dense granule biogenesis (SLC35D3)[20] and function (ATP2A3, ATP2A2[44] and ATP2C1)[45] could support both mechanisms.

Although investigation of a rare disease such as XLTT implies limited statistical power, 83 dysregulated proteins (FC ≥±1.2, q<0.05) were identified in our proteomics study. Combining the proteomic results analyzed by IPA and STRING with flow cytometry and information from electron microscopy and IHC, several pieces of evidence pointed to dense- and α-granule deficiencies as contributors to platelet functional defects. Impaired dense granule biogenesis and function might differentiate XLTT from GPS, but further studies in additional families are needed. Novel findings suggesting altered protein ubiquitination and degradation should be investigated further in relation to the pathogenesis of platelet granule disorders.

Disclosure

No conflicts of interest to disclose.

Contribution

MÅ and JP included patients; DB, CK, CS and JB worked with proteomics; DB and MÅ performed bioinformatics analyses; SR performed flow cytometry and analyzed the results; JP, AGE and MÅ performed and evaluated immunohistochemistry; KH performed electron microscopy; DB, MÅ, SR and JP co-wrote the manuscript, which was revised and approved by all authors.

Acknowledgements

The authors would like to thank all participating individuals with XLTT as well as the healthy volunteers. Doctor Anders Fritzberg at the Department of Pediatrics, Falun Hospital, Sweden, included the pediatric patient. Professor Tomas Lindahl at the Department of Clinical Chemistry, and bioinformatics scientist Rada Ellegard at the Department of Clinical Genetics, both at Linköping University, Sweden, carried out the TruSight panel genetic investigation. Inger Vedin (PhD) at the Departments of Medicine and Hematology, Karolinska Institute, Karolinska University Hospital Huddinge, Sweden, performed TPO measurements and immunohistochemistry. Doctor Ian Jones at the Department of Laboratory Medicine, Falun Hospital, Sweden, revised the language.

Funding

Örebro County Council Research Committee, grant numbers OLL-158661, OLL-164431, OLL-239301, OLL-255251, OLL-268261 and OLL-347141.

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