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

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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, Ca2+ 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 reported2–5 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
families (three individuals) with the disease. All reported XLTT patients carried the same exon 4 GATA1 p.R216Q mutation. Similarities with the autosomal inherited disorder gray platelet syndrome (GPS) have been noted regarding deficiency of platelet α-granules, splenomegaly, and, more recently, myelofibrosis. 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. 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-Ayyun, Johns Hopkins University, e-mail, 24 October 2018), aiming to disclose similarities and differences.

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. The phenotypical aspects, including bleedings, of the four adult patients were described previously. Additionally, an 8-year old boy (at sampling) from family B, was now included for proteomics and routine investigations. The control material for proteomics consisted of platelets from five sex- and age-matched healthy volunteers.

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 GPIIIb/IIa 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.

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 hemorrhages. 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.

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). Among routine coagulation tests, APTT and PF-INR were slightly elevated in three and two adults, respectively, from family B. Plasma levels of plasminogen activator inhibitor 1 (P-FAI-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 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).
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”. 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 and one report of dense granule 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.29

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.30 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,31 47 proteins were here identified to be differentially regulated. Congruent to findings from TEM of sparse numbers of granules, 59 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 (TTL12), spectrin α chain, non-erythrocytic 1 (SPTAN1) and neulin (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 to originate from different subcellular compartments, the majority from the cytoplasm (not shown).

Pathway and network analyses
In a core analysis in IPA (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 (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,23 Online Supplementary Table S2 containing 250 proteins with FC ≥±1.2 from one GPS
patient compared to one control, revealed that nine pro-
teins, all of them known to be present in α-granules, were
downregulated in both XLTT and GPS (for XLTT with cri-
tera 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 pro-
tein, SACM1L, and six granule proteins that were down-
regulated 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 Ca2+ transporting proteins ATP2A3,
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   |       |      |
| Q5MS12     | SLICSD3 | Solute carrier family 35 member D3              | -3.36   | 0.0156   |       |      |
| P01211     | 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 |
| P017228    | CLEC2L | C-type lectin domain family 2 member L          | -2.10   | 0.0319   |       |      |
| P00775     | PBPB   | Platelet basic protein                          | -2.08   | 0.0103   | x     | Down |
| Q12191     | LRMP   | Lymphoid-restricted membrane protein             | -2.01   | 0.0312   |       |      |
| P16019     | SELP   | P-selectin                                       | -1.74   | 0.0041   | x     | Down |
| P09480     | SPARC  | Secreted protein acidic and rich in cysteine    | -1.74   | 0.0242   | x     |      |
| Q13201     | MMRN1  | Multimerin-1                                    | -1.69   | 0.0220   | x     | Down |
| Q13567     | IQGAP2 | Ras GTPase-activating-like protein IQGAP2       | -1.67   | 0.0009   | x     |      |
| P04275     | VWF    | von Willebrand factor                            | -1.67   | 0.0092   | x     |      |
| Q8WX7F     | ATL1   | Atlantin-1                                       | -1.63   | 0.0202   | x     |      |
| Q9UB8      | CD84   | SLAM family member 5                             | -1.62   | 0.0110   | x     |      |
| Q9NRW1     | RAB6B  | Ras-related protein Rab-6B                      | -1.60   | 0.0243   | x     |      |
| Q16463     | DNB1   | Drebir                                            | -1.59   | 0.0364   | x     |      |
| P19677     | APP    | Amyloid beta A protein                           | -1.58   | 0.0194   | x     | Down |
| Q8WKE9     | STON2  | Stomin-2                                         | -1.57   | 0.0259   | x     |      |
| P77389     | MAOB   | Amin oxidase [flavin-containing] B                | -1.57   | 0.0088   | x     |      |
| Q8WMAI     | TMEM40 | Transmembrane protein 40                        | -1.55   | 0.0259   | x     |      |
| Q8TD25     | MICAL1 | Protein-methionine sulfoxide oxidase             | -1.48   | 0.0074   | x     |      |
| Q5WC8      | HACD4  | Very-long-chain (3R)-3-hydroxyacyl-[acyl-carrier protein] dehydrotase 4 | -1.45   | 0.0335   | x     |      |
| Q5SQ84     | LYG66F | Lymphocyte antigen 6 complex locus protein G6f  | -1.45   | 0.0103   | x     |      |
| O95219     | SNX4   | Sorting nexin-4                                  | -1.45   | 0.0006   |       |      |
| O43283     | AKR72A | Allofaxin B1 aldehyde reductase member 2         | -1.44   | 0.0333   | x     |      |
| Q7LUL1     | MOB1B  | MOB kinase activator 1B                          | -1.43   | 0.0333   | x     |      |
| P31114     | CORO1A | Coronin-1A                                       | -1.43   | 0.0497   | x     |      |
| P00390     | GSR    | Glutathione reductase, mitochondrial             | -1.41   | 0.0220   |       |      |
| P47757     | 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 |
| Q4917      | YWHAH  | 14-3-3 protein eta                               | -1.37   | 0.0061   | x     | Up   |
| P43304     | GPD2   | Glycerol-3-phosphate dehydrogenase, mitochondrial| -1.35   | 0.0074   | x     |      |
| P51655     | HSD17B4| Peroxosomal multifunctional enzyme type 2       | -1.32   | 0.0220   | x     | Down |
| Q9NR12     | PDLIM7 | PDZ and LIM domain protein 7                     | -1.31   | 0.0383   | x     |      |
| Q4KM92     | ANO6   | Anoctamin-6                                      | -1.31   | 0.0043   | x     | Down |
| Q93084     | ATP2A3 | Sarcoplasmic/endoplasmic reticulum calcium ATPase 3 | -1.30   | 0.0103   | x     | Up   |
| Q75038     | WDR1   | WD repeat-containing protein 1                   | -1.30   | 0.0331   | x     | Up   |
| Q14165     | MLEC   | Malectin                                         | -1.29   | 0.0413   | x     | Up   |
| P23219     | PTTGS1 | Prostaglandin GH synthase                        | -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     | Down |
| Q9CC89     | UBE2O  | Ubiquitin-conjugating enzyme E2 O               | -1.23   | 0.0219   | x     |      |
| Q16581     | UGP2   | UTP-glucose-1-phosphate uridylyltransferase     | -1.23   | 0.0241   | x     |      |

*Proteins potentially associated with platelet granules, as published before.** *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.†(Online Supplementary File 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    | RPS5A  | 40S ribosomal protein S5a                         | 2.59    | 0.0092   |       |      |
| P42947    | RPL1A  | Ribose-5-phosphate isomerase                      | 2.17    | 0.0262   |       |      |
| Q14166    | TTB1L2 | 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    | MTUN   | Maturin                                           | 1.79    | 0.0022   |       |      |
| Q6830     | PRDX1  | Peroxiredoxin-1                                  | 1.70    | 0.0355   | 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   |       |      |
| P4727     | RAD23B | UV excision repair protein RAD23 homolog B        | 1.51    | 0.0076   | x     |      |
| P34949    | MPI    | Mannose-6-phosphate isomerase                     | 1.48    | 0.0227   |       |      |
| Q691K3    | 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   |       |      |
| Q570N5    | FNP1L  | Formin-binding protein 1-like                     | 1.41    | 0.0373   |       |      |
| Q0UX71    | PLXDC2 | Plexin domain-containing protein 2                | 1.41    | 0.0103   |       |      |
| P05023    | ATP1A1 | Sodium/potassium-transporting ATPase subunit alpha-1 | 1.35    | 0.0028   |       |      |
| Q09733    | NAP1L4 | Nucleosome assembly protein 1-like 4              | 1.35    | 0.0333   | x     |      |
| P28070    | PSMB4  | Proteasome subunit beta type-4                    | 1.35    | 0.0468   |       |      |
| P9302     | 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   |       |      |
| P43666    | 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    | 0.0487   |       |      |
| Q9NM75    | SACM1L | Phosphatidylinositol phosphatase SAC1             | 1.29    | 0.0330   | x     | Up   |
| Q15257    | PTPA   | Serine/threonine-protein phosphatase 2A activator | 1.27    | 0.0202   |       |      |
| P43527    | PASN   | Fatty acid synthase                               | 1.26    | 0.0103   |       |      |
| P08107    | HSPAR1/HSP1B | Heat shock 70 kDa protein 1A/1B | 1.26    | 0.0220   | x     |      |
| Q0Q3C3    | RTN4   | Reticulin-4                                       | 1.25    | 0.0312   |       |      |
| P25789    | PSMA4  | Proteasome subunit alpha type-4                   | 1.21    | 0.0333   |       |      |
| P61201    | COP52  | COP9 signalosome complex subunit 2               | 1.21    | 0.0317   |       |      |

*Proteins potentially associated with platelet granules, as published before. ††Protein levels elevated or reduced, here defined as fold change (FC) ≥1.2, in a published gray platelet syndrome (GPS) platelet organelle fraction proteome. †(Online SupplementaryTable 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), 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 (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; Supplementary Figure S5B). VIPAS39, a protein that regulates platelet granule biogenesis, had predicted inhibited activity in both XLTT and GPS (Figure 3; Supplementary Figure S5A and B).
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

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**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, TGFB1 and TGFB2 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, 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, 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 (GPIIbα) 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. 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). 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.
Hemostatic platelet functions are largely mediated by soluble factors released from membrane-bound storage organelles including α-granules, dense granules and lysosomes. 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. 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 life-span of platelets and regulated by external and internal signaling. 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. 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). Some dysregulations of the platelet proteome could be due to absorption/endocytosis of proteins from plasma into circulating platelets, thus reflecting plasma concentrations. 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...

Discussion

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 receptor activation. 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.
amounts of proteasomal proteins have recently been reported also in GFI1B mutated macrothrombocytopenia.\textsuperscript{31}

Some blood analyses may aid in the differential diagnosis of XLTT patients. For example, the high S-TPO noted in XLTT noted here differs from the reported normal or mildly elevated level in ITP.\textsuperscript{35} Increased P-TPO levels were found in mice with the hypomorphic Gata1\textsuperscript{mut} mutation.\textsuperscript{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,\textsuperscript{33} and the TPO-MPL axis has crucial effects on platelet production and life span.\textsuperscript{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. F-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.\textsuperscript{37} Interestingly, a partially cleaved form of F5 (comprising approximately 20% of the total F5 in blood) resides in the \(\alpha\)-granules of platelets, in complex with the protein multimerin (MMPRN1).\textsuperscript{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)\textsuperscript{39} might be of interest for future evaluations regarding bleeding diathesis.\textsuperscript{34}

The platelet QMS revealed significant (FC \(\geq\) ±1.2, q<0.05) reductions of 39 proteins associated with granules. These included SERPINE1/P-AI-1, vWF, SELP/P-selectin and PTGS1/COX1 which are all important for hemostasis (Table 2). Latent transforming growth factor \(\beta\)-binding protein 1 (LTBP1), the fourth most downregulated protein and found in \(\alpha\)-granules, has not previously been associated with bleeding diathesis, but its interactions with transforming growth factor \(\beta\) (TGF\(\beta\)), found in platelets in high concentrations, might be of significance for BM fibrosis development. This has also been discussed for GPS.\textsuperscript{30} Notably, TGF\(\beta\)1 was identified as a predicted inhibited upstream regulator in XLTT, but appeared somehow activated in GPS (Figure 5), with possible implications for the respective myelofibrosis developments.\textsuperscript{39} In XLTT, both the present study (Figure 3 and LTBP1) and our former IHC investigation on BM expression of CTGF and VEGF showed low TGF\(\beta\) stimulated protein expression 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.\textsuperscript{40} XLTT BM fibrosis is characterized by increased angiogenesis.\textsuperscript{41} Though the exact role of THBS1 in hemostasis is unclear, it may interact with coagulation factor 13/F13A1\textsuperscript{42} which was also found in reduced amounts in the XLTT and GPS platelet proteomes.

The identification of several jointly downregulated \(\alpha\)-granule proteins in XLTT and GPS is congruent with ultrastructural similarities regarding deficiencies of \(\alpha\)-granules. However, NBEAL2 mutated GPS platelets have shown normal morphology and numbers of dense bodies/\(\alpha\)-granules\textsuperscript{43,44} whereas our XLTT results suggested ultrastructural and functional dense granule deficiency, consistent with an earlier ultrastructural study.\textsuperscript{10} Possible proteome correlates to a suggested functional difference between XLTT and GPS regarding dense granule release included contraregulations of the three Ca\textsuperscript{2+} transporting ATPases ATP2A3, ATP2A2\textsuperscript{45} and ATP2C1,\textsuperscript{46} 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).\textsuperscript{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).\textsuperscript{41} 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.\textsuperscript{45}

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).\textsuperscript{47}

A recent investigation based on patients with GPS (NBEAL2 mutations) and the GATA1 mutations p.D218D and p.D218Y suggested that GATA1 enhances NBEAL2 expression via interaction with the GATA1 co-activator friend of GATA1 (FOG1),\textsuperscript{48} possibly explaining the \(\alpha\)-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.\textsuperscript{49,50} 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.\textsuperscript{45} We observed a release of \(\alpha\)-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 \(\alpha\)- 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 \(\alpha\)-granules indeed caused the low F-selectin exposure in XLTT platelets. In addition, as fibrinogen is normally stored in platelet \(\alpha\)-granules and released upon activation to aid aggregation,\textsuperscript{39} 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.\textsuperscript{39} The TEM picture in combination with low levels of proteins affecting dense granule biogenesis (SLC35D3)\textsuperscript{51} and function (ATP2A3, ATP2A2\textsuperscript{45} and ATP2C1)\textsuperscript{46} 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-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
MA and JP included patients; DB, CK, CS and JB worked with proteomics; DB and MA performed bioinformatics analyses; SR performed flow cytometry and analyzed the results; JP, AGE and MA performed and evaluated immunohistochemistry; KH performed electron microscopy; DB, MÅ, SR and JP wrote the manuscript, which was revised and approved by all authors.

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References
1. Thompson AR, Wood WG, Stamatoyanopoulou G. X-linked syndrome of platelet dysfunction, thrombocytopenia, and imbalanced globin chain synthesis with hemolysis. Blood. 1977;50(2):303-316.
2. Baldini CL, Pecci A, Loffredo G, et al. Effects of the R216Q mutation of GATA-1 on erythropoiesis and megakaryocytopoiesis. Thromb Haemost. 2004;91(1):129-140.
3. Hughan SC, Senis Y, Best D, et al. Selective impairment of platelet activation to collagen in the absence of GATA1. Blood. 2005;105(11):4569-4574.
4. Tubuleni VN, Levine JE, Campagna DR, et al. X-linked gray platelet syndrome due to a GATA1 Arg216Gln mutation. Blood. 2007;109(8):3297-3300.
5. Baldini CL, De Canio E, Saveo A. Why the disorder induced by GATA1 Arg216Gln mutation should be called "X-linked thrombocytopenia with thalassemia" rather than "X-linked gray platelet syndrome". Blood. 2007;110(7):2771-2771, author reply 2771.
6. Åström M, Hahn-Strömberg V, Zetterberg E, Örberg J, Åström M. [X-linked thrombocytopenia with thalassemia in two families in Sweden. Consider hereditary causes of thrombocytopenia and bone marrow fibrosis]. Läkartidningen. 2010;107(54-55):1474-1477.
7. Danielsson S, Verup M, Olsson L, Falmblad J, Åström M. [X-linked thrombocytopenia with thalassemia in two families in Sweden. Consider hereditary causes of thrombocytopenia and bone marrow fibrosis]. Läkartidningen. 2010;107(54-55):1786-1796.
8. Ciovacco WA, Raskind WH, Kacena MA. NBEAL2 is mutated in gray platelet syndrome due to a novel frameshift mutation at the NBEAL2 gene. Blood. 2008;111(10):4369-4376.
9. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, et al. Characterization of the platelet granule proteome: evidence of the presence of MHC1 in alpha-granules. J Proteomics. 2014;101:130-140.
10. Meng R, Wang Y, Yao Y, et al. SLC35D3 delivery from megakaryocyte early endosomes is required for platelet dense granule biogenesis and is differentially defective in Hermansky-Pudlak syndrome models. Blood. 2012;120(2):404-414.
11. Maynard DM, Heijnen G, Cai W, Gunay-Aygun M. The alpha-granule proteome: novel proteins in normal and ghost granules in gray platelet syndrome. J Thromb Haemost. 2010;8(3):1786-1796.
12. Connolly-Andersen AM, Sundberg E, Ahlm C, et al. Increased thrombopoiesis and megakaryocytic proliferation in a patient with the X-linked thrombocytopenia due to a missense mutation in GATA1. Blood. 2005;105(11):4369-4376.
13. Danielsson S, Merup M, Olsson L, Falmblad J, Åström M. [X-linked thrombocytopenia with thalassemia in two families in Sweden. Consider hereditary causes of thrombocytopenia and bone marrow fibrosis]. Läkartidningen. 2010;107(54-55):1474-1477.
14. Boknäs N, Ramström S, Fåvälö L, Lindahl TL. Flow cytometry-based platelet function testing is predictive of symptom burden in a cohort of bleeders. Platelets. 2018;29(5):512-519.
15. Connolly-Andersen AM, Sundberg E, Ahlm C, et al. Increased thrombopoiesis and platelet activation in Hantavirus-infected patients. J Infect Dis. 2015;212(7):1061-1069.
16. Data were analyzed through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, CA, USA). https://www.qiagen.com/ingenuity.
17. Boknäs N, Ramström S, Fåvälö L, Lindahl TL. Flow cytometry-based platelet function testing is predictive of symptom burden in a cohort of bleeders. Platelets. 2018;29(5):512-519.
18. Ollinger K. Thrombin-induced lysosomal damage-α-granule dysfunction in gray platelet syndrome. J Hematol. 2010;158(5):799-800.
19. Zufferey A, Schvartz D, Nolli S, Reny JL, Sanchez JC, Fontana F. Characterization of the platelet granule proteome: evidence of the presence of MHC1 in alpha-granules. J Proteomics. 2014;101:130-140.
20. Meng R, Wang Y, Yao Y, et al. SLC35D3 delivery from megakaryocyte early endosomes is required for platelet dense granule biogenesis and is differentially defective in Hermansky-Pudlak syndrome models. Blood. 2012;120(2):404-414.
21. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2019;47(D1):D607-D613.
22. Norden AT, Norden P. Should we really test the platelet alpha-granules in platelets classified as gray platelet syndrome? Am J Hematol. 2016;91(7):714-715.
23. Specht E, Kaemmerer D, Sanger J, Wirtz RM, Schulz S, Lupp A. Comparison of immunoreactive score, HER2/neu score and H score for the immunohistochemical evaluation of somatostatin receptors in bronchopulmonary neuroendocrine neoplasms. Histopathology. 2015;67(3):365-377.
24. Santos MT, Valles J, Lago A, et al. Residual platelet thrombocytopenia A2 and prothrombotic effects of erythrocytes are important determinants of aspirin resistance in patients with vascular disease. J Thromb Haemost. 2006;4(6):615-621.
25. Frelinger AL, 3rd, Grace RE, Gerrits AJ, et al. Platelet function tests, independent of platelet count, are associated with bleeding severity in ITP. Blood. 2015;126(7):875-879.
26. Boknäs N, Macowan AS, Stögergen AL, Ramström S. Platelet function testing at low platelet counts: when can you trust your analysis? Res Pract Thromb Haemost. 2019;3(2):285-290.
27. Stögergen AL, Svensson Holm AC, Ramström S, Lindström EG, Grenégard M, Ollinger K. Thrombin-induced lysosomal exocytosis in eosinophils is dependent on secondary activation by ADF and regulated by endothelial-derived substances. Platelets. 2016;27(1):86-92.
28. Heijnen H, van der Stuijs P. Platelet secretory behaviour: as diverse as the granules... or
29. Weyrich AS, Lindemann S, Tolley ND, et al. Change in protein phenotype without a nucleus: translational control in platelets. Semin Thromb Hemost. 2004;30(4):491-498.

30. Yu C, Niakan KK, Matsushita M, Stamatoyanopoulos G, Orkin SH, Raskind WH. X-linked thrombocytopenia with thalassemia from a mutation in the amino finger of GATA-1 affecting DNA binding rather than FOG-1 interaction. Blood. 2002;100(6):2040-2045.

31. van Oorschot R, Hansen M, Koornneef JM, et al. Molecular mechanisms of bleeding disorderassociated GFI1B(Q287*) mutation and its affected pathways in megakaryocytes and platelets. Haematologica. 2019;104(7):1460-1472.

32. Palmblad J. To give and take - life of a platelet. Blood. 2009;113(12):2617.

33. Makar RS, Zhukov OS, Sahud MA, Kuter DJ. Thrombopoietin levels in patients with disorders of platelet production: diagnostic potential and utility in predicting response to TPO receptor agonists. Am J Hematol. 2015;88(12):1041-1044.

34. Zingariello M, Sancillo L, Martelli F, et al. The thrombopoietin/MPL axis is activated in the Gata1(low) mouse model of myelofibrosis and is associated with a defective RPS14 signature. Blood Cancer J. 2017;7(6):e572.

35. Sunohara M, Morikawa S, Fuse A, Sato I. GATA-dependent regulation of TPO-induced c-mpl gene expression during megakaryopoiesis. Ökajimas Folia Anat Jpn. 2014;90(4):101-106.

36. Saur SJ, Sanghrajka V, Geddis AE, Kaushansky K, Hitchcock IS. Ubiquitination and degradation of the thrombopoietin receptor c-Mpl. Blood. 2010;115(6):1254-1263.

37. Dahlbäck B. Pro- and anticoagulant properties of factor V in pathogenesis of thrombosis and bleeding disorders. Int J Lab Hematol. 2016;38(Suppl 1):S4-11.

38. Jeimy SB, Fuller N, Tasneem S, et al. Multimerin 1 binds factor V and activated factor V with high affinity and inhibits thrombin generation. Thromb Haemost. 2008;100(6):1058-1067.

39. Malara A, Abbonante V, Zingariello M, Migliaccio A, Baldini A. Megakaryocyte contribution to bone marrow fibrosis: many arrows in the quiver. Meditter J Hematol Infect Dis. 2018;10(1):e2018068.

40. Huang T, Sun L, Yuan X, Qiu H. Thrombospondin-1 is a multifaceted player in tumor progression. Oncotarget. 2017;8(48):84546-84558.

41. Dardik R, Solomon A, Loscalzo J, et al. Novel proangiogenic effect of factor XIII associated with suppression of thrombospondin 1 expression. Arterioscler Thromb Vasc Biol. 2008;28(8):1472-1477.

42. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet α-granules. Nat Genet. 2011;43(8):732-734.

43. Bottega R, Pecchi A, De Candia E, et al. Correlation between platelet phenotype and NBEAL2 genotype in patients with congenital thrombocytopenia and α-granule deficiency. Haematologica. 2015;99(6):868-874.

44. Feng M, Elab Z, Borgel D, et al. NAADP/SECA3-dependent Ca2+ stores pathway specifically controls early autocrine ADF secretion potentiating platelet activation. Circ Res. 2020;127(7):e166-e183.

45. Unsworth AJ, Bomnik I, Pinto-Fernandez A, et al. Human platelet protein ubiquitylation and changes following GPVI activation. Thromb Haemost. 2019;119(1):104-116.

46. Jakubowski M, Debski J, Szahidewicz-Krupska E, et al. Platelet carbonic anhydrase II, a forgotten enzyme, may be responsible for aspirin resistance. Oxid Med Cell Longev. 2017;2017:3132068.

47. Bourquin JP, Subramanian A, Langebrake C, et al. Identification of distinct molecular phenotypes in acute megakaryoblastic leukemia by gene expression profiling. Proc Natl Acad Sci U S A. 2006;103(59):3539-3544.

48. Wigaarts A, Wittevrongel C, Thys C, et al. The transcription factor GATA1 regulates NBEAL2 expression through a long-distance enhancer. Haematologica. 2017;102(4):695-706.

49. Larroca LM, Heller FG, Podda G, et al. Megakaryocytic emperipolesis and platelet function abnormalities in five patients with gray platelet syndrome. Platelets. 2015;26(8):751-757.