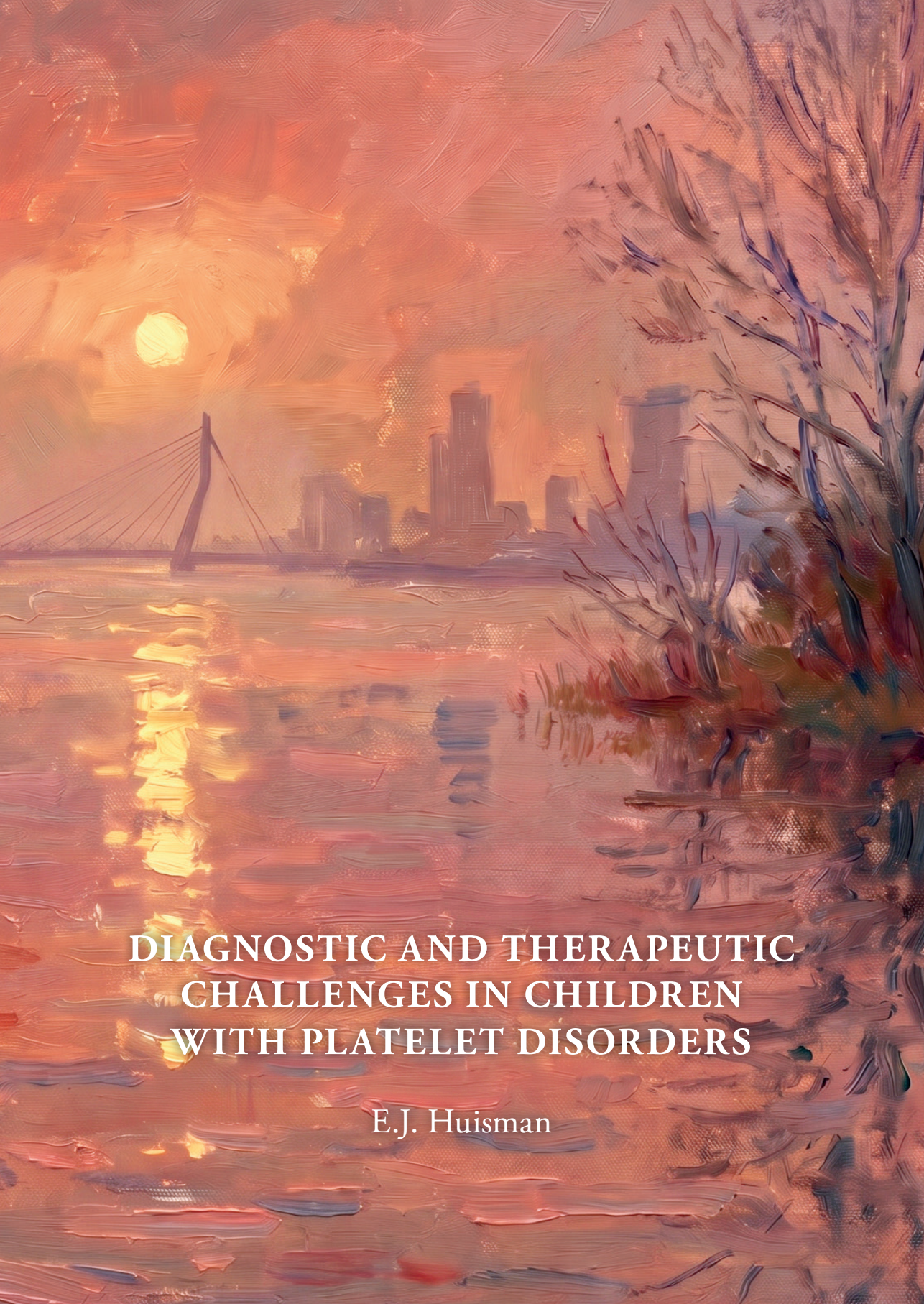


DIAGNOSTIC AND THERAPEUTIC CHALLENGES IN CHILDREN WITH PLATELET DISORDERS

E.J. Huisman

PROPOSITIONS

1. Some bleeding disorders are more equal than others (this thesis)

2. Molecular genetic analysis should be part of the overall workup of patients suspect of a platelet disorder, as results impact classification, counseling and treatment (this thesis)

3. Platelet disorders affect female adolescents more than males, as heavy menstrual bleeding physiologically occurs every month (this thesis)

4. Some clues are in the unexpected, as persistent or abundant expression of CD34, CD36 or CD44 on peripheral platelets points to respectively *GFI1B, FLI1* or *ETV6*-related platelets disorders (this thesis)

5. Minor risks associated with alloimmunization having potential for considerable impact, warrant a precautionary approach until we have fully addressed existing knowledge gaps (this thesis)

6. Early investment in children's healthcare is a future proof investment (adapted from Heckman's curve; Heckman, 2008)

7. Individualizing transfusion medicine is crucial to improve outcomes in different disease-related and patient-related conditions (Curley et al , NEJM 2019)

8. Investigating one single blood cell, gene or protein risks an oversimplification of the body's complex pathways (Gunnink-Fustolo et al, Nature 2025)

9. Real-world data bridges knowledge gaps when (randomized) clinical trials are not feasible in rare diseases (Wilson et al, Lancet 2024)

10. Acquiring knowledge is only rarely expanding what is known, but more often reveals what is not (adapted from Copernicus, 15th century)

11. You know that your doctoral thesis has become a long journey when you realize that you started without reading glasses

Diagnostic and Therapeutic Challenges in Children with Platelet Disorders

Elisabeth Janneke Huisman

Cover design Stefanie van den Herik | Herikmedia.nl; gebaseerd op Monet's Sunset over the Seine, en de zonsondergang over Rotterdam

Printed copies of this thesis were kindly supported by Sanquin

ISBN 9789465344805

Layout and printing by ProefschriftMaken | proefschriftmaken.nl

Diagnostische en therapeutische uitdagingen in kinderen met bloedplaatjesafwijkingen

Diagnostic and Therapeutic Challenges in Children with Platelet Disorders

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr.ir. Jantine Schuit

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

10 september 2026 om 10.30 uur

door

Elisabeth Janneke Huisman
geboren te Sliedrecht.

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotoren	Prof.dr. M.H. Cnossen
	Prof.dr. M. de Haas
Beoordelingscommissie	Prof.dr. F.W.G. Leebeek
	Prof.dr. R.E.G. Schutgens
	Prof.dr. T. Kleefstra

Little by little, a little becomes a lot

Tanzanian proverb

TABLE OF CONTENTS

INTRODUCTION		**9**
Chapter 1	General introduction & outline of the thesis	9
PART I	**Unmet needs in clinical management and diagnosis**	**29**
Chapter 2	Knowledge gaps in health-related quality of life research performed in children with bleeding disorders – A scoping review	31
Chapter 3	Bleeding symptoms, laboratory and molecular genetic results in platelet disorders	59
Chapter 4	Proteomic landscapes of inherited platelet disorders with different etiologies	91
Chapter 5	Interstitial 11q deletions and terminal 11q duplications cause a bleeding tendency due to platelet dysfunction that is similar to 11q deletions causing Jacobsen syndrome	121
Chapter 6	Patients with chromosome 11q deletions are characterized by inborn errors of immunity involving both B- and T-lymphocytes	147
Chapter 7	Heterozygous nonsense *FLI1* p.Met100* variant results in thrombocytopenia and increased erythroid gene expression	171
Chapter 8	*ETV6/TEL* knockout leads to impaired megakaryoid gene regulation, which is phenocopied by the ETS-domain mutant ETV6-R369W via dominant negative actions.	217
PART II	**Therapeutic dilemmas**	**253**
Chapter 9	Implementing HLA and HPA-matched platelet transfusions for patients with Glanzmann and Bernard-Soulier syndrome as standardized care? A Dutch survey and recommendations	255

Chapter 10 Are thrombocytopenia and platelet transfusions associated with major bleeding in preterm neonates? A systematic review 281

Chapter 11 Desmopressin response in children with platelet disorders may depend on the presence of a genetic cause when response is measured with Von Willebrand activity and Platelet Function Analyzer 323

GENERAL DISCUSSION AND SUMMARY **335**

Chapter 12 General discussion and conclusions 337

Chapter 13 Summary/Samenvatting 359

APPENDICES **377**

Authors and affiliations 379
List of publications 387
List of abbreviations 393
PhD portfolio 400
About the author 404
Dankwoord 407

Litle things are indeed little,
but to be faithful in little things is a great thing

Mother Theresa (1910-1997)

1 GENERAL INTRODUCTION & OUTLINE OF THE THESIS

GENERAL INTRODUCTION

The discovery of platelets

In 1882, Giulio Bizzozero (1846-1901) discovered that the discoid corpuscles that he named "blutt plättchen", were not the disintegrated leftovers of granulocytes but a unique, third form of blood cell, we now call thrombocytes or platelets.(1-3) Bizzozero was able to see these platelets by using a microscope, a relatively novel tool that caused a small revolution since its use to investigate blood was suggested by Petrus Borellus in 1653. He published an article in which he described that "a number of things may be discovered in the blood of patients with this microscope".(4) He was right and today, we still study the fundaments of megakaryopoiesis and the role of platelets in the cellular processes of hemostasis and thrombosis with old and novel technology.

Platelet production

Platelets are small (1 till 3 µm in diameter), discoid shaped, enucleated cell fragments. Platelets are produced by megakaryocytes.(5, 6) These relatively large cells (50 till 150 µm) are situated in the bone marrow. The bone marrow is the soft tissue inside the medullary cavity of bones consisting of an extensive labyrinth of sinusoidal vessels and it houses the pluripotent hematopoietic stem cells (HSC) from which all blood cells are formed through a complex maturation and differentiation process. Megakaryocytes are formed from megakaryocyte-erythroid progenitors (MEP) which are the precursors of both the erythroid and megakaryocytic blood cell lineages.

Platelet production follows a sophisticated two-step production process in these megakaryocyte-erythroid progenitors. The first phase leads to proplatelet formation through polyploidization, enucleation and maturation for approximately five days. This polyploidy is achieved by a proliferation phase followed by several rounds of endomitosis, which is a form of DNA replication without cell division, resulting in an accumulation of DNA from 4N up to 128N per multilobular nucleus.(5, 7, 8) See Figure 1.

Figure 1 Endomitosis of megakaryocytes

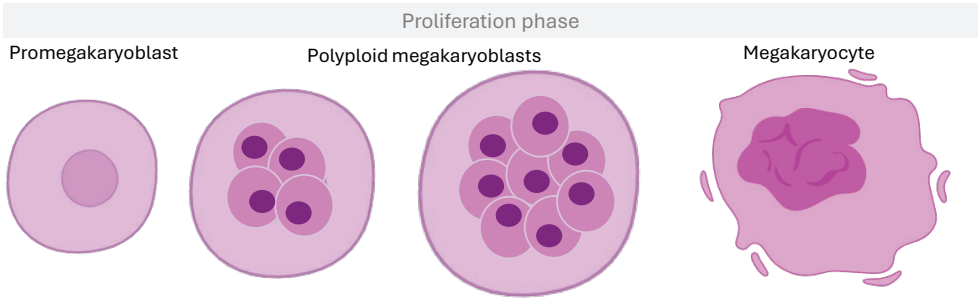


Figure created in BioRender© and Microsoft Powerpoint

Hereafter, enucleation and maturation occur by expanding the cytoplasm through an increase of cytoskeletal proteins, platelet specific granules, and membrane elongation by activation of the Golgi network. When matured, megakaryocytes start to form protrusions, called proplatelets. Beta1-tubulin and actin are essential to form and modulate these proplatelets to form the proplatelet-tip, which extends into the sinusoidal blood vessels of the bone marrow looping back at the end of each process.(5, 7) Examples of genes that are known to regulate megakaryocyte maturation are: *RHOA, MYL9*, *MYH9*, *MYH10, RUNX1, GEF-H1, ECT2, GFI1B, FLI1, GATA-1, and ETV6*. Examples of genes that are specifically involved in proplatelet formation are *TUBB1, ACTN1, WAS* and *FLNA* (5, 9)

The second phase leads to release of platelets through organelle and granule delivery via microtubules to the preplatelet tip, followed by preplatelet assembly through the formation of bead-like swellings.(5) These coiled structures already have the characteristic platelet shape of a mature platelet, and after a process of looping and coiling, platelets are released into the blood stream. Examples of genes involved in preplatelet formation are *NFE2, TUBB1* and *RAB27B*.(8) This second phase is relatively rapid and completed within hours.(5, 10)

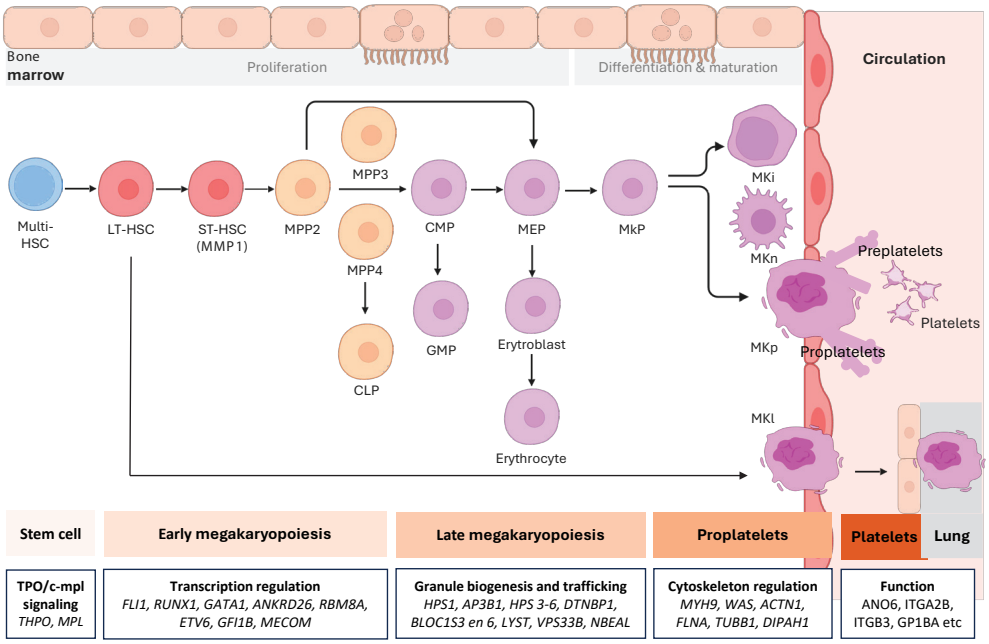
Each megakaryocyte releases approximately 500-2000 platelets over its lifetime, leading, altogether, to a total platelet production of 1 x 10^{11} platelets per day. In the peripheral blood circulation, human platelets survive seven to ten days, resulting in a platelet count of 150-400 x 10^{9}/L in the peripheral blood.(5) See Figure 2. The production process of megakaryocytes is regulated by a feed-back mechanism with thrombopoietin (TPO) as one of the driving factors acting through the c-Mpl receptor on megakaryocytes. TPO is regulated by *THPO* and produced in the liver.(5, 12)

Platelet functioning

Platelets play an important role in vascular integrity and hemostasis, but are also involved in wound healing, angiogenesis, immunity and inflammation. When activated, platelets bind to endothelium and subsequent aggregation of platelets is important for the formation of a hemostatic clot. In this process, two phases are recognized, the primary and secondary hemostasis; followed by controlled clot degradation through fibrinolysis.(13)

During the primary hemostasis, blood vessels contract in response to endothelial damage, von Willebrand factor (VWF) is released and platelets are activated forming podocytes. Subsequently, platelets adhere to the subendothelium by platelet membrane receptors called integrins or glycoproteins (GP). After binding with GPIb from the GPIb/V/IX-complex and von Willebrand factor, the activated platelet, enforces its connections by binding to collagen with integrin α2ß1 (formerly known as: GPIa/IIa) and GPVI.(13) This binding leads to acceleration of platelet activation and acceleration of the secondary hemostasis by initiation of various concomitant activating pathways (also See Figure 3):

Figure 2 Megakaryopoiesis and platelet formation and examples of genes involved



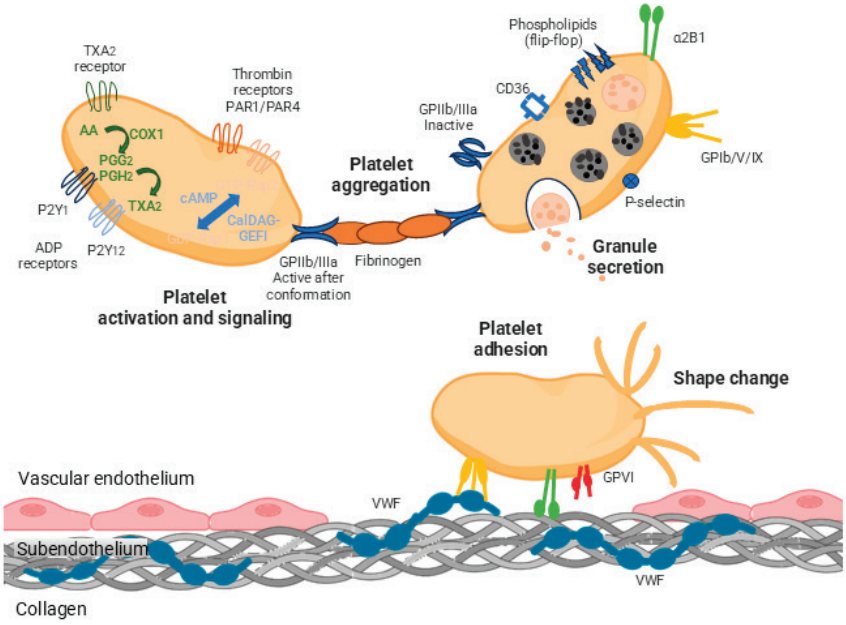
Legend CLP = common lymphoid progenitor, CMP = common myeloid progenitor, Ery = erythrocyte, GMP = granulocyte-monocyte progenitor, HSC = hematopoietic stem cell, MKi = megakaryocytes involved in immunity, MEP = megakaryocyte-erythrocyte progenitor, MkP = megakaryocyte progenitor, MMP = multipotent progenitors, MKl = lung megakaryocytes, MKn = niche-supporting megakaryocyte, pMK = platelet producing megakaryocyte, ST-HSC = short-term hematopoietic stem cell (or multipotent progenitor 1). Figure created in BioRender© and Microsoft Powerpoint, adapted from Lentaigne et al 2019, and Du et al, 2025.(7, 11)

- An increased calcium efflux and intracellular calcium release in platelets under the genetic regulation of *PLCB, PLCG2* and *CalDAG-GEFI* will occur.
- In the meantime, zymogen *Factor VII* (FVII) is activated by the release of tissue factor from the endothelium of the damaged vascular wall. Through multiple steps, this activation leads to the formation of thrombin, an important endpoint of the secondary hemostasis.
- The presence of thrombin also induces movement of negatively charged phospholipids from the inner to the outer leaflet of the membrane. This so-called flip-flop mechanism provides a negatively charged surface that is essential for binding to vitamin K dependent coagulation factors which in return further stimulated thrombin formation.(14)
- It also leads to degranulation causing an increased production of thromboxane (*TBXAS1*) through the enzyme's phospholipase A2, cyclo-oxygenase 1 and thromboxane-A-synthesis. This subsequently activates thromboxane-receptor (encoded by *TBXA2R*)

and integrin αIIbß3 (formerly known as: GPIIb/IIIa, encoded by *ITGA2B* and *ITGB3*). (13, 15, 16)

- Integrin αIIbß3 undergoes a conformational change by a process called inside-out signaling. In inactive platelets, integrin αIIbß3 has low affinity to fibrinogen, but upon activation and changing it confirmation it becomes a high affinity receptor for fibrinogen. If fibrinogen is bound, this leads to outside-in signaling resulting in further platelet activation and aggregation.(17)
- Platelet aggregation occurs upon degranulation of the intracellular platelet granules. These granules contain important coagulation proteins (such as von Willebrand factor, FV, FXIII and fibrinogen), but also P-selectin, platelet factor 4, other growth factors and fibronectin. P-selectin, platelet factor 4 and various growth factors play an important role in wound healing and inflammation.(16, 18)
- During this process, platelets change their shapes and develop small filipodia.(19)

Figure 3 Platelet activation, aggregation, adhesion and shape change



Legend AA = arachidonic acid, ADP = adenosine diphosphate, COX = cyclo-oxygenase, GDP = guanosine diphosphate, GP = glycoprotein, GTP = guanosine triphosphate, PGG2 = prostaglandin G2, PGH2 = prostaglandin H2, TXA2 = thromboxane 2. Figure created in BioRender© and Microsoft Powerpoint, adapted from Du et al, 2025 and Paul et al, 1999.(7, 19)

Platelet disorders

Taking the complexity of platelet production and functioning into account, a serious disruption in these processes or mutations of involved genes may result in a shortage or loss of function or even both. All patients with aberrant platelet functions are diagnosed with a so-called platelet disorder. However, since many types of disruption can lead to platelet shortage or loss of function, the group of patients with platelets disorders (PD) is extremely heterogeneous.(18) The spectrum is even more diverse when PD is classified by their molecular genetic cause; because there is not always a direct translation of a genotype into a phenotype. Molecular genetics has also changed the concept that PD are a rare disease with an estimated incidence of 1:1,000,000, as the increasing knowledge and use of genetic testing reveal more PD subgroups, leading to current incidence estimations of 1:10,000.(18, 20)

Despite new insights based on molecular genetic testing, a classification for PD is still lacking. This is relevant, as PD can occur as an isolated entity or as part of an underlying syndrome affecting multiple organs and tissues. For example, a gene defect that leads to an inherited PD may also cause white blood cells to dysfunction, such as *GATA1* or *AP3B1*. The latter gene, which causes type 2 Hermansky-Pudlak syndrome, also causes albinism and ophthalmological defects due to nystagmus. Other well-known examples are the *MYH9*-related disorders, which are characterized by cataract and kidney problems. A genetic diagnosis is important to monitor the patient, plan additional screening and to install protective measures such as avoidance of drug toxicity when organ dysfunction is expected. (18) Surveillance is especially essential in patients with a genetic mutation that encodes for transcription factors, such as *RUNX1, ANKRD26* or *ETV6*, of which dysregulation of their expression may lead to hematological malignancies. Therefore, if mutations in these genes are found in patients with thrombocytopenia, a lifelong monitoring for occurrence of hematological malignancies is necessary, since these occur in 10-30% of patients.(21, 22) Therefore, a classification of PD in relation to syndromes as well as predisposition to hematological malignancies seems warranted. Also, counseling and follow-up can be individualized.

This individualization of counseling and follow-up also depends on the expected or actual bleeding tendency. Bleeding phenotype, however, varies between patients and is not always related to platelet count nor to type of functional defect.(18, 20) From some inherited PD it is known that bleeding tendencies are high, such as Glanzmann Thrombasthenia or Bernard-Soulier syndrome. But this association is not known in all patients with PD. Modifying factors involving disease-related factors and additional coagulation factor deficiencies, as well as other intrinsic and extrinsic factors may also influence bleeding tendency and risk. Examples are physical activity and behavior, sex, age, co-medication or the occurrence of medical procedures such as dental interventions or surgery. Due to these factors, follow-up is difficult to individualize upfront.

Treatment options

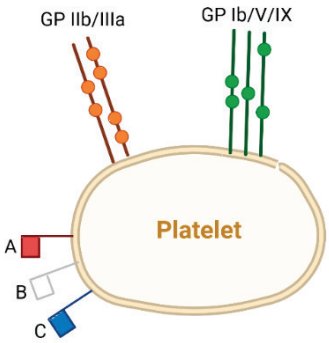
Treatment in children with PD is indicated when active bleeding occurs (therapeutic intervention) or to prevent bleeding (prophylactic treatment). Choice of treatment is based on the prevention or treatment of the bleeding, bleeding type, severity, and location. It can consist of a single drug, but often a combination of drugs and interventions is used. Current treatment options for PD are mainly non-specific and include supportive measures such as local pressure of coagulation, and medication such as tranexamic acid, desmopressin, and platelet transfusions to increase the number of functional platelets. Tranexamic acid and desmopressin enhance hemostasis by targeting other parts of the coagulation. Tranexamic acid, for example, inhibits fibrinolysis. It is especially effective to treat or prevent mucocutaneous bleeding.(23) Desmopressin (or DDAVP) leads to an increase in Von Willebrand Factor and Factor VIII (FVIII) by mobilization of Von Willebrand Factor from the vascular endothelium. In children, use of desmopressin is limited in children <2 years due to potential adverse events.

Specific treatment options enhancing platelet production have not yet been registered for PD patients, but a recent phase II study have shown positive and hopeful results in patients with *WAS* and *MYH9*-related PD for the thrombopoietin (TPO) agonists romiplostim and eltrombopag.(24, 25) Both TPO agonists stimulate megakaryopoiesis and thereby, partially, correcting the severe thrombocytopenia. The last specific treatment option is the use of recombinant activated FVII (rFVIIa); as is registered for treatment in Glanzmann Thrombasthenia patients.(26-28) Sporadically, hematopoietic stem cell transplantations are performed. Indications are most often based on predisposition for malignant disease as is known to occur in *RUNX1, GATA2 or ETV6*-related PD. Indications are only rarely the bleeding tendency alone, as this clinical benefit does not often outweigh the potential short-term and long-term side-effects. To date, no gene therapies are available in Europe for PD.

More specific for platelet transfusions, in the Netherlands there are guidelines for blood transfusions leading to a high quality and safety standard. When a donor donates ~500 mL of whole blood, it is processed, leucoreduced, and separated into plasma, red blood cells and platelets. A finalized platelet transfusion unit containing ≥250 x 10^9/L of platelets and is produced from four to five whole blood donations or from a single platelet apheresis procedure. In patients with PD, platelet transfusions are indicated for both thrombocytopenia and thrombocytopathy. The administration of transfusions is, however, not without debate. One of the risks is alloimmunization, as platelets carry various structures that are necessary for their function and immune recognition, such as human leucocyte and platelet antigens (HLA class I and HPA); unfortunately, these structures are polymorphic and can elicit alloimmune responses in a patient leading to transfusion reactions or platelet refractoriness. In PD patients with Glanzmann Thrombasthenia and to a lesser extent those with Bernard-Soulier syndrome, the potential risk on development of

either HPA antibodies or antibodies directed against glycoprotein-related antibodies (isoantibodies) may be even higher as those patients respectively lack the platelet-specific GPIIb/IIIa receptor complex (also named integrin αIIbß3 or fibrinogen receptor) or the GPIb/V/IX glycoprotein receptor complex (VWF receptor). Absence of these glycoprotein complexes will also result in the absence of these HPA antigens on platelets, see Figure 4.

Figure 4 Platelet containing HLA Class I, glycoprotein complex IIb/IIIa and Ib/V/IX as well as HPA antigen expression on glycoproteins



Legend Red-white-blue flags represent class I HLA-A, B and C proteins; Orange and green structures represent the platelet glycoprotein (GP) IIb/IIIa, also named integrin αIIbß3, and glycoprotein receptor complex Ib/V/IX. Figure created in BioRender© and Microsoft Powerpoint.

Multiple risk factors influence the risk of developing HLA and/or HPA antibodies, such as transfusion frequency, HLA or HPA mismatch between donor and recipient, immunogenicity of an HLA or HPA antigen, which in turn depends on the protein structure and the immune response of the recipient, and lastly the expression of any of the HPA-expressing integrin chains as part of other type of structures by other tissues. For example, GPIIIa (beta3) is not only expressed as GPIIb/IIIa complex on platelets, but also as alfaVbeta3 glycoprotein (vitronectin receptor) by many types of white blood cells and endothelial cells.

Overview of diagnostic tests

Here, we provide a general overview of diagnostic tests to confirm and specify suspected PD, as applied in children.

Platelet count, indices and function

The first and easily accessible test to measure platelet count, immature platelet fraction (IPF) and mean platelet volume (MPV) is an automatic cell counter that identifies platelets by fluorescence flowcytometry (PLT-F).(29) After perforating the platelet membranes with the lysing reagent, the fluorescence marker labels the residual RNA inside the platelet.

Using the forward scattered light reflecting size and the fluorescence signal reflecting labelled nucleic acid content, the platelets are separated from red and white blood cells and displayed in a scattergram. Results are quantified as normal, decreased or increased for normal reference ranges.(30)

IPF is also quantified in the scattergram, in which IPF is the percentage (%) of immature platelets containing still relatively high levels of RNA.(31) This is visualized by an increased fluorescent signal, and a stronger forward scatter signal due to the larger size of the platelets. Results are quantified as normal (1-5%, median 3.2), decreased (≤1%) or increased (≥5%).(30) A disadvantage of cell count and IPF is that results do not discriminate between an inherited or acquired thrombocytopenia.

Additionally, MPV or the diameter of the platelet is measured in a small blood volume. Results are categorized as normal (median 10.5 fL), too small (MPV <8 fL) or too large (MPV ≥12 fL), resulting in a diagnosis of microplatelets or macroplatelets.(30) A disadvantage of large MPVs is that these also do not discriminate between inherited and a acquired thrombocytopenia. However, some inherited PD may be associated with a small MPVs, e.g. *WAS*-related PD, or large MPVs, e.g. Bernard-Soulier syndrome, *NBEAL* or *TUBB1*-related PD.(16, 32) The advantage of these three techniques in children, is the very low blood volume needed for all three analyses of less than 100 µL.

The examination of a blood smear by microscopy will show respectively platelet morphology as well as red and white blood cell morphology.(33) Results are descriptive but can be semi-quantified in a range from 0 (absent) to 4 (present in all examined samples). In children, an advantage is the low blood volume needed of 0.5 mL. Also, a pseudo-thrombocytopenia, resulting from platelet aggregation not related to PD, can be identified. (33) A disadvantage is the need for specific expertise as well as the subjectivity of results. However, some inherited PDs are associated with specific morphological features, such as Grey Platelet Syndrome (either *NBEAL* or *GFI1B*-related) in which platelets are lighter and grey colored. In addition, other blood cell lines may demonstrate typical features such as Döhle-like inclusions in the MYH9-RDs which are blue-gray, oval, basophilic, neutrophil inclusions located in the peripheral cytoplasm. However, Döhle-like inclusions can also be observed during infections or G-CSF use.(33, 34)

Furthermore, platelet function screening and testing is of great importance. Screening can be done by the Platelet Function Analyzer (PFA 100 or 200®, Siemens healthcare diagnostics). This instrument monitors platelet interaction on a collagen-ADP (PFA-ADP) or a collagen-epinephrin (PFA-EPI) coated membrane in citrated blood and under controlled flow conditions. The platelet and Von Willebrand activity are measured by the closure time of the membrane aperture, as the growing plug progressively blocks the blood flow through the aperture.(35) Blood volume demands are relatively moderate (1 – 1.8 mL),

making it applicable in children. A disadvantage is the low sensitivity for PD, as normal PFA results do not rule out a PD.(36, 37)

The golden standard for platelet function testing is light transmission aggregometry (LTA). Platelet aggregation testing measures the ability of various platelet agonists to induce *in vitro* activation and platelet-to-platelet activation in platelet rich plasma (PRP), which is stirred in a cuvette, and placed between a light source and a photocell. Patient's platelets are inactively suspended in the PRP, leading to a cloudy suspension that scatters and dims the light source. Thereafter, in different incubations, a set of platelet agonists is added activating the platelets, inducing shape change and *in vitro* platelet aggregation. This results in a decreased absorption of light which is subsequently detected by the photocell and visualized in a graphic.(20, 38-40) The results are quantified per agonist as normal (≥50% for weak agonists and ≥70% for strong agonists), weak (<50% respectively <70%), reversible (after a good amplitude, the curve did not hold), biphasic or as no response.(40) Although LTA is the golden standard for platelet function testing, its disadvantage for children is significant as a large whole blood volume is required (minimum of 20 mL) which can only be acquired by venipuncture. Although international guidelines advise a minimum standard containing the following agonists: ADP, collagen, ristocetin in two concentrations, arachidonic acid and TRAP, overall standardized practices are lacking making comparison of results between laboratories difficult.(41), Additionally, epinephrin and a thromboxane A2 analogue like U46619 can be added to the analysis.(40) Importantly for PD diagnosis, the requirement of a PRP ≥150 x 10^9/L restricts its application to PD presenting with low platelet numbers. Multiple (pre and post) analytical conditions may also influence results, for example medication or food. Repeated measurements show varying test results within one individual.(42) And finally, normal LTA results do not rule out a PD, especially so called storage pool diseases with δ granule defects are not always identified by LTA.(43)

A parameter that can be informative in the diagnosis of PD is the plasma level of thrombopoietin (TPO). In PD with defects in megakaryopoiesis or with platelet instability, TPO levels are increased. In the Netherlands, TPO levels are quantified as normal (≤33 U/mL) or increased. For pediatric use, an advantage is the relatively low blood volume needed (0.5 mL). Disadvantages are that the result is not specific and only discriminating against normal, increased and very high levels. In the latter situation results point more to a failure in megakaryocyte production than a PD. Lastly, a disadvantage is the availability of testing, as it is only be performed in one reference laboratory.

If a PD is suspected, one can also determine the expression of certain platelet membrane structures by flow cytometry. By labelling the structure of interest with a specific fluorescent-labeled monoclonal antibody, the presence of the structure can be visualized by various lasers used in the flowcytometry.(44) The platelet surface markers CD41, CD61

and CD42a, CD42b are generally investigated, as they correspond respectively with integrin αIIbß3 and GPIb/V/IX, absent in respectively Glanzmann Thrombasthenia or Bernard-Soulier syndrome.(44) Sometimes mepacrine labelling is added as a marker for storage pool defects.(45, 46) Results are the percentage (%) of the normal reference range and quantified as type I (<5%), type II (5-20%) and type III (≥20% up to normal expression). For application in children, an advantage is the relative low blood volume needed regardless of platelet count (1 mL). Disadvantages are the low availability of the test and the fact that expressed but dysfunctional integrins (type III) will not be recognized.(28)

DNA analysis of genes involved in platelet production or function

In platelet production and function, many genes are involved. The possibilities to investigate the genes involved in platelet production and function have significantly changed the field of PD diagnostics and research during the last 10 years.(11) It has become clear that there are many genes that can give rise to PD through different mechanisms, and still more genes involved in platelet production are being identified. Abnormal results of genetic analysis will be first classified based on the nature of this variant, involving single nucleotide variants, insertions, deletions, or duplications. Subsequently, they are classified based on the significance and pathogenicity of the detected aberration. Current guidelines identify five classes: class 1 being (certainly) benign, class 2 likely benign, class 3 unknown, named Variant of Unknown Significance (VUS), class 4 likely pathogenic, and class 5 pathogenic.(47)

Molecular genetic analysis consists of different techniques varying from single gene analysis through Sanger sequencing or MLPA to predefined targeted panels analyzed with next-generation (tNGS) sequencing, whole-exome sequencing with a predefined filter of genes of interest or whole exome analysis of all exomes (WES) and (targeted) whole genome sequencing ((t)WGS). Recently, standard copy number variation analysis (CNV) is added, or an additional SNP array can be performed. Novel techniques as RNA-analysis are being more widely applied. Furthermore, population databases improve by using genome-wide association studies (GWAS). For routine DNA analysis, a reasonable blood volume of 2-5 mL of whole blood is sufficient, if leucocyte counts are normal. Also, alternative DNA sampling isolated from mucosal swabs is possible. However, some genetic tests and RNA analysis require specific blood containers (PAX) or even skin biopsies for fibroblast cultures, which limit the use of tests. The advantage in PD diagnostics is the fact that it specifies the diagnosis of an inherited PD, and in some, recommended follow-up, screening and treatment. With the technical advancements of predefined panels, multiple genes of interest can be sequenced in one run, making costs more acceptable. Relative disadvantages are the need for optimal patient and parental counseling of unexpected findings, as well as potential impact of a genetic diagnosis on patient and other family members. Most of PD do not have a monogenetic etiology, and genetic variants or

polymorphisms are often categorized as VUS. Segregation analysis and additional laboratory tests are then needed, but capacity and research options may be limited.

Research tools to measure platelet production or function

Flowcytometry and platelet function tests are combined in so called whole blood flow cytometric function tests. The basis of this test is flow cytometry. The additional step is that activating agonists as used in LTA are added. The resulting platelet activation is measured by the new occurring presence of certain platelet surface markers, such as P-selectin (CD62P).(48-50). An advantage for use in children is the moderate blood volume required (5 mL). Currently, use is restricted, as this test is not yet commercially available and CE-marked, neither is it validated in children. Another disadvantage is that platelet activation can only be tested by measurable changes on platelet surface markers. Lastly, a practical limitation is that the test must be performed on fresh blood samples to ensure stability and integrity of antibody labeling, making transport over longer distances to a specialized laboratory difficult.

Mass-spectrometry (MS) is a technique that can be used to evaluate the presence of certain proteins in a cell fraction or body fluid. As PD are the result of a dysfunction or dysregulation in the platelet production or function, abnormalities in signaling pathways are usually involved. These pathways are coordinated by a variety of genes that, when translated and transcribed correctly, result in functional proteins. When pathogenic genetic variants impact correct production of certain gene products, it may be possible to detect this in the platelet proteome by proteomic-based MS. This technique identifies and quantifies proteins in a fraction. The platelet proteome can be visualized without and with isotope labelling making it suitable for specific indications, as well as using various stimulating agonists making testing of the effect of platelet activation on the proteome visible.(51) The advantage of MS lays in the ability to perform testing in 2-5 mL (depending on platelet count), analyzing 4,000-6,000 platelet proteins in one run. A limitation of the technology is that only quantification of a protein does not correlate with its function. Therefore, bioinformatics is necessary to define the set of proteins of which the presence may be impacted by a disturbed function of a given platelet protein. The knowledge to do these in-depth analysis is still growing.(52) Moreover, pre-analytic conditions need to be defined and used for all samples; it is important to isolate platelets within a few hours after drawing the blood sample. Also, MS demands specific, not widely available instruments, supercomputers, as well as highly qualified staff to perform and analyze the abundance of data.

Electron microscopy (EM) is a technique in which a beam of electrons and their wave-like characteristics are used to magnify an object, in our situation, platelets. After being accelerated through a vacuum by positive electrical potential, the beam is confined and focused through a magnetic lens. By doing so an image is magnified up to 1 x 10^6 times.

(53) A major advantage of EM is the possibility to see and quantify structures within the platelet, such as number and size of granules, lysosomes, mitochondria or Golgi structures. A limitation of the use of this powerful technique is that fixation of the platelets needs to be done within 24 hours after blood sampling. Also, the test is costly and not widely available, and it demands qualified staff for performing as well as analyzing the results.(54)

Finally, induced pluripotent stem cells (iPSC) are a type of pluripotent stem cells that can be generated from peripherally derived somatic cells, such as nucleated leucocytes. Hence, these end-differentiated nucleated cells are then in vitro cultured and backwards differentiated to become a stem cell, which can be differentiated into a megakaryocyte. By doing this in patients with inherited platelet disorders, this creates a unique opportunity to investigate the fundamentals of the disease mechanism in a laboratory setting. (55)

For a detailed overview of all techniques available, we refer to Larsen et al.(56) Tests such as the lumiaggregometry, multiplate assay, viscoelastic tests or VerifyNow® were not available for the study and are therefore not described.

OUTLINE OF THE THESIS

In this thesis, we studied clinical and diagnostic challenges and therapeutic dilemmas observed in children with inherited platelet disorders. We aimed to gain a deeper understanding of the clinical phenotype and underlying disease mechanisms of PD; to advance knowledge of available and experimental diagnostics and to provide evidence to support in therapeutic choices.

In this thesis, we present the subjects studied in **Chapter 1,** followed by a scoping review on health-related QoL (HRQoL) in children with PD in **Chapter 2**. In **Chapter 3**, we describe an observational cohort (DoReMi study) of almost 100 children with various presentations of PD, their clinical symptoms, and laboratory test results. We propose a PD classification based on genetic and functional test results. In **Chapter 4**, we present the outcome of mass-spectrometry (MS) analysis to investigate its potential role in PD diagnostics. In **Chapter 5,** we investigate the bleeding symptoms and platelet function in a cohort of patients with terminal 11q deletion syndrome (known as Jacobsen syndrome) but also with other 11q disorders. Additionally, in **Chapter 6,** we investigate in the same cohort of patients with 11q disorders the hypothesis that both platelets and granulocytes granule defects can be found. Additionally we investigated T cell function tests, as B cell dysfunction resulting in hypogammaglobulinemia is common in these patients, but T cells have not yet been examined. To gain more insight on the pathophysiology of in-

herited PD we investigate the effect of two novel pathogenic mutations by analyzing the effect on megakaryopoiesis in IPSCs. **Chapter 7** describes the effect of a novel frame shift mutation in *FLI1*, and **Chapter 8** of a novel single variant in *ETV6*.

We also investigated the use and indications for HLA and/or HPA matched platelet transfusions for PD patients diagnosed with Glanzmann Thrombasthenia or Bernard-Soulier syndrome in **Chapter 9.** In **Chapter 10**, we performed a systematic review to investigate if prophylactic platelet transfusions are protective against major bleeding in premature neonates. In **Chapter 11,** we analyzed the data of 51 children with PD that underwent desmopressin response testing. These results are of importance as patients with PD have varying desmopressin responses. We conclude with a discussion (**Chapter 12**) and summary/samenvatting in **Chapter 13**.

REFERENCES

1. Mazzarello P, Calligaro AL, Calligaro A. Giulio bizzozero: A pioneer of cell biology (vol 2, pg 776, 2001). Nat Rev Mol Cell Bio. 2001;2(11):848-.
2. Ribatti D, Crivellato E. Giulio Bizzozero and the discovery of platelets. Leukemia Res. 2007;31(10):1339-41.
3. Sivagnanam K. Remembering the Discovery of Platelets: Max Schultze (1865) and Giulio Bizzozero (1882). Indian J Vascular En. 2020;7(1):78-9.
4. Hajdu SI. A note from history: The first use of the microscope in medicine. Ann Clin Lab Sci. 2002;32(3):309-10.
5. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. J Cell Biol. 2013;201(6):785-96.
6. Zhou JX, Wang HT, He J, Xu CL, Chen XY, Yang H, et al. Decoding Human Megakaryocyte Development. Exp Hematol. 2021;100:S113-S.
7. Du CH, Chen J, Wang JP. New insights into the generation and function of megakaryocytes in health and disease. Haematologica. 2025;110(7):1500-12.
8. Tijssen MR, Ghevaert C. Transcription factors in late megakaryopoiesis and related platelet disorders. J Thromb Haemost. 2013;11(4):593-604.
9. Wang HT, He J, Xu CL, Chen XY, Yang H, Shi SJ, et al. Decoding Human Megakaryocyte Development. Cell Stem Cell. 2021;28(3):535-549.
10. Italiano JE, Jr., Patel-Hett S, Hartwig JH. Mechanics of proplatelet elaboration. J Thromb Haemost. 2007;5 Suppl 1:18-23.
11. Lentaigne C, Freson K, Laffan MA, Turro E, Ouwehand WH, Consortium B-B, the ThromboGenomics C. Inherited platelet disorders: toward DNA-based diagnosis. Blood. 2016;127(23):2814-23.
12. Varghese LN, Defour JP, Pecquet C, Constantinescu SN. The Thrombopoietin Receptor: Structural Basis of Traffic and Activation by Ligand, Mutations, Agonists, and Mutated Calreticulin. Front Endocrinol. 2017;8.
13. Clemetson KJ. Platelets and Primary Haemostasis. Thrombosis Research. 2012;129(3):220-4.
14. Hemker HC, van Rijn JL, Rosing J, van Dieijen G, Bevers EM, Zwaal RF. Platelet membrane involvement in blood coagulation. Blood Cells. 1983;9(2):303-17.
15. Scridon A. Platelets and Their Role in Hemostasis and Thrombosis-From Physiology to Pathophysiology and Therapeutic Implications. International Journal of Molecular Sciences. 2022;23(21).
16. Israels SJ, Kahr WH, Blanchette VS, Luban NL, Rivard GE, Rand ML. Platelet disorders in children: A diagnostic approach. Pediatr Blood Cancer. 2011;56(6):975-83.
17. Armstrong PC, Peter K. GPIIb/IIIa inhibitors: From bench to bedside and back to bench again. Thromb Haemostasis. 2012;107(5):808-14.
18. Palma-Barqueros V, Revilla N, Sanchez A, Zamora Canovas A, Rodriguez-Alen A, Marin-Quilez A, et al. Inherited Platelet Disorders: An Updated Overview. Int J Mol Sci. 2021;22(9).
19. Paul BZS, Daniel JL, Kunapuli SP. Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways: Role of P160 rock in platelet shape change. Thromb Haemostasis. 1999:439-.
20. Bourguignon A, Tasneem S, Hayward CP. Screening and diagnosis of inherited platelet disorders. Crit Rev Cl Lab Sci. 2022;59(6):405-44.
21. Illango J, Nair AS, Gor R, Fernando RW, Malik M, Siddiqui NA, Hamid P. A Systematic Review of the Role of Runt-Related Transcription Factor 1 (RUNX1) in the Pathogenesis of Hemato-

logical Malignancies in Patients With Inherited Bone Marrow Failure Syndromes. Cureus J Med Science. 2022;14(5).
22. Di Paola J, Porter CC. ETV6-related thrombocytopenia and leukemia predisposition. Blood. 2019;134(8):663-7.
23. Chauncey JM, Patel P. Tranexamic Acid. 2025.
24. Zaninetti C, Gresele P, Bertomoro A, Klersy C, De Candia E, Veneri D, et al. Eltrombopag for the treatment of inherited thrombocytopenias: a phase II clinical trial. Haematologica. 2020;105(3):820-8.
25. Bastida JM, Gonzalez-Porras JR, Rivera J, Lozano ML. Role of Thrombopoietin Receptor Agonists in Inherited Thrombocytopenia. International Journal of Molecular Sciences. 2021;22(9).
26. https://www.ema.europa.eu/en/medicines/human/EPAR/novoseven
27. Lisman T, Adelmeijer J, Cauwenberghs S, Van Pampus ECM, Heemskerk JWM, De Groott PG. Recombinant factor VIIa enhances platelet adhesion and activation under flow conditions at normal and reduced platelet count. Journal of Thrombosis and Haemostasis. 2005;3(4):742-51.
28. Mathews N, Rivard GE, Bonnefoy A. Glanzmann Thrombasthenia: Perspectives from Clinical Practice on Accurate Diagnosis and Optimal Treatment Strategies. Journal of Blood Medicine. 2021;12:449-63.
29. https://www.sysmex-europe.com/academy/knowledge-centre/technologies/fluorescence-platelet-count-application-plt-f/
30. Pereira KN, de Carvalho JAM, Paniz C. Reference intervals of the platelet indexes in a healthy population in Santa Maria, Rio Grande do Sul, Brazil. Hematol Transf Cell. 2019;41(2):187-9.
31. https://www.sysmex.co.uk/education/knowledge-centre/sysmex-parameters/immature-platelet-fraction-ipf
32. Israels SJ. Diagnostic evaluation of platelet function disorders in neonates and children: an update. Semin Thromb Hemost. 2009;35(2):181-8.
33. Robier C. Platelet morphology. J Lab Med. 2020;44(5):231-9.
34. Iqbal NT, Li W. Pay attention to neutrophil inclusions in pediatric patients with thrombocytopenia. Blood. 2019;134(11):907.
35. https://www.platelet-research.org/techniques-available/pfa-100-and-pfa-200/
36. Sladky JL, Klima J, Gróoms L, Kerlin BA, O'Brien SH. The PFA-100 Does Not Predict Delta-Granule Platelet Storage Pool Deficiencies. Blood. 2010;116(21):1043-.
37. Hayward CPM, Harrison P, Cattaneo M, Ortel TL, Rao AK, Soc CI. Platelet function analyzer (PFA)- closure time in the evaluation of platelet disorders and platelet function:: reply to a rebuttal. Journal of Thrombosis and Haemostasis. 2006;4(6):1432-.
38. Hayward CP, Pai M, Liu Y, Moffat KA, Seecharan J, Webert KE, et al. Diagnostic utility of light transmission platelet aggregometry: results from a prospective study of individuals referred for bleeding disorder assessments. J Thromb Haemost. 2009;7(4):676-8439.
39. Alessi MC, Sie P, Payrastre B. Strengths and Weaknesses of Light Transmission Aggregometry in Diagnosing Hereditary Platelet Function Disorders. J Clin Med. 2020;9(3).
40. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. Journal of Thrombosis and Haemostasis. 2013;11(6):1183-9.42. Yaw HP, van den Helm S, Linden M, Monagle P, Ignjatovic V. Whole blood flow cytometry protocol for the assessment of platelet phenotype, function, and cellular interactions. Platelets. 2021;32(6):786-93.

41. Munnix ICA, Van Oerle R, Verhezen P, Kuijper P, Hackeng CM, Hopman-Kerkhoff HIJ, et al. Harmonizing light transmission aggregometry in the Netherlands by implementation of the SSC-ISTH guideline. Platelets. 2021;32(4):516-23.
42. Alessi MC, Coxon C, Ibrahim-Kosta M, Bacci M, Voisin S, Rivera J, et al. Multicenter evaluation of light transmission platelet aggregation reagents: communication from the ISTH SSC Subcommittee on Platelet Physiology. Journal of Thrombosis and Haemostasis. 2023;21(9):2596-610.
43. Yoxtheimer L, Smith MR, Gunning WT. Platelet aggregation assays do not reliably diagnose platelet storage pool deficiency. Journal of Thrombosis and Haemostasis. 2015;13:47-.
44. Linden MD, Frelinger AIII, Barnard MR, Przyklenk K, Furman MI, Michelson AD. Application of flow cytometry to platelet disorders. Seminars in Thrombosis and Hemostasis. 2004;30(5):501-11.
45. https://practical-haemostasis.com/Platelets/platelet_function_testing_nucleotide_assays.html
46. van Asten I, Blaauwgeers M, Granneman L, Heijnen HFG, Kruip MJHA, Beckers EAM, et al. Flow cytometric mepacrine fluorescence can be used for the exclusion of platelet dense granule deficiency. Journal of Thrombosis and Haemostasis. 2020;18(3):706-13.
47. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-24.
48. Kicken CH, Roest M, Henskens YMC, de Laat B, Huskens D. Application of an optimized flow cytometry-based quantification of Platelet Activation (PACT): Monitoring platelet activation in platelet concentrates. Plos One. 2017;12(2).
49. van Asten I, Schutgens REG, Baaij M, Zandstra J, Roest M, Pasterkamp G, et al. Validation of flow cytometric analysis of platelet function in patients with a suspected platelet function defect. J Thromb Haemost. 2018;16(4):689-98.
50. Yaw HP, van den Helm S, Linden M, Monagle P, Ignjatovic V. Whole blood flow cytometry protocol for the assessment of platelet phenotype, function, and cellular interactions. Platelets. 2021;32(6):786-93.
51. Huang JN, Zhang PY, Solari FA, Sickmann A, Garcia A, Jurk K, Heemskerk JWM. Molecular Proteomics and Signalling of Human Platelets in Health and Disease. International Journal of Molecular Sciences. 2021;22(18).
52. Zivkovic M, Shamorkina TM, Blaauwgeers MW, Post H, Heck AJR, Schutgens REG, et al. Proteomic analysis indicates lower abundance of platelet α-granule proteins in Glanzmann thrombasthenia. Journal of Thrombosis and Haemostasis. 2025;23(7):2284-96.
53. Brunet JG, Iyer JK, Badin MS, Graf L, Moffat KA, Timleck M, et al. Electron microscopy examination of platelet whole mount preparations to quantitate platelet dense granule
54. https://www.news-medical.net/life-sciences/Advantages-and-Disadvantages-of-Electron-Microscopy.aspx
55. Hansen M, von Lindern M, van den Akker E, Varga E. Human-induced pluripotent stem cell-derived blood products: state of the art and future directions. Febs Lett. 2019;593(23):3288-303.
56. Larsen JB, Hvas AM, Hojbjerg JA. Platelet Function Testing: Update and Future Directions. Seminars in Thrombosis and Hemostasis. 2023;49(06):600-8.

Human behavior flows from three main sources: desire, emotion, and knowledge

Plato (428 – 348 BC)

PART I

UNMET NEEDS IN CLINICAL MANAGEMENT & DIAGNOSIS

It's the strength of emotion that guides us

Claude Monet (1840 - 1926 AD)

2 Knowledge gaps in health-related quality of life research performed in children with bleeding disorders – A scoping review

E.J. Huisman
C.M. Mussert
G. Bai
H. Raat
M.H. Cnossen

Haemophilia 2024;30(2):295-305

ABSTRACT

Introduction

Bleeding disorders (BDs) may influence health-related quality of life (HRQoL) in children and caregivers. Measuring HRQoL gives insight into domains requiring support and provides an opportunity to evaluate the effects of novel therapies.

Aim

To gain insight in the current body of literature on HRQoL in children with BDs in order to identify knowledge gaps for research and further development of this field.

Methods

Scoping review.

Results

We included 53 articles, describing studies mainly performed in Europe and North-America (60.4%) and mostly within the last ten years. Only 32% studies included children <4 years. Almost all studies (47/53, 88.7%) were performed in boys with haemophilia, pooling haemophilia A and B (n=21) and different disease severities (n=20). Thirteen different generic and five disease-specific HRQoL-questionnaires were applied; all questionnaires were validated for haemophilia specifically. Six (11.3%) combined generic and disease-specific questionnaires. Self-reports were most frequently applied (40/53, 75.5%), sometimes combined with proxy and/or parent-reports (17/53, 32.1%). Eleven studies used a reference group (20.8%). Statistical analyses mostly consisted of mean and SD (77.4%).

Conclusion

HRQoL-research is mainly performed in school-aged boys with haemophilia, treated in developed countries. Pitfalls encountered are the pooling of various BDs, subtypes and severities, as well as the application of multiple generic questionnaires prohibiting comparison of results. More attention is needed for broader study populations including other BDs such as young children, feminine bleeding issues and platelet disorders, as well as the use of HRQoL as an effect-measurement tool for medical interventions, and more thorough statistical analysis.

Key words

Blood coagulation disorders [MESH], Child [MESH], haemophilia [MESH], quality of life [MESH], review [MESH], research design [MESH]

INTRODUCTION

Bleeding disorders (BDs) lead to an increased bleeding tendency due to a quantitative or qualitative defect in either platelets or coagulation factors. Due to heterogeneity in pathophysiology and severity, inter-individual bleeding phenotype varies greatly between affected individuals. Typical bleeding manifestations of BDs affecting the primary hemostasis are extensive bruising and mucosal bleeding such as epistaxis heavy menstrual bleeding and surgical bleeding.(1) When the secondary hemostasis is affected, bleeding also present as muscle and joint bleeds. Severe bleeding such as intracranial or gastrointestinal bleeding is less frequently encountered but devastating.(2) Children with BDs also endure lifestyle interventions, medical appointments, venipunctures, and treatment. This may cause anxiety, depression, atypical behavior, or isolation due to less participation in social and physical activities.(3) When the level of individual disease control, societal participation and general life expectations is affected by the BD it may negatively influence quality of life.(4)

Quality of life (QoL) is defined by the World Health Organization as 'an individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns'.(5, 6) Health-Related QoL (HRQoL) specifically focusses on the effects of health, illness and treatment on an individual's life and excludes all non-health-related aspects of QoL.(6, 7) Over the past years, HRQoL-assessment is increasingly acknowledged as a marker of health outcome in children with chronic diseases as it visualizes disease burden.(8) It provides insights into the need for treatment or interventions, in return, giving physicians patient-tailored goals to improve medical care.(9) To measure HRQoL, two categories of questionnaires are used: generic and disease-specific questionnaires. Generic questionnaires are broadly applicable and facilitate comparisons between groups with different (chronic) diseases or healthy children from a general population sample. Disease-specific questionnaires focus on the influence of the specific symptoms and characteristics of a certain disease on the patients, being validated for this specific condition.(9) In children, both the child and the caregiver rate the impact of the disease on the HRQoL of the child. These different points of view are relevant and could be assessed simultaneously. For this, three subcategories of HRQoL measurement questionnaires are distinguished: firstly age-appropriate self-reports measuring the child's HRQoL as reported by the child itself. Secondly, proxy-reports, evaluating the child's HRQoL as reported by the parents or caregivers. Thirdly, parent reports, measuring how the child's disease impacts parenthood and family life.(10, 11) The aim of this study was to gain insight in the steadily increasing body of literature on HRQoL in children with all types of BDs in order to identify knowledge gaps for research and further development of this field. For that purpose, we performed a systematic review of current literature, and details of published studies were summarized. Subsequently, the unmet needs observed were addressed and presented as a scope for future research.

MATERIAL AND METHODS

Study selection

We used the methodological framework for scoping reviews as described by Arksey and O'Malley (2005)(12) and recommendations by Levac et al. (2010)(13) and Pham et al. (2014)(14). The following stages were as follows: 1| identify the research question, 2| identify relevant articles, 3| select articles, 4| chart the data, and 5| collate, summarize and report the results. Not applicable was stage 6 (consult of consumers and stakeholders). (12-14)

Eligibility criteria

Articles were eligible if HRQoL results were published of children (<18 years old) with a BD, defined as either an inherited platelet disorder (IPD) or deficiency or abnormal function of coagulation factors. When results of children and adults were combined, articles were solely included if the results for the paediatric population were presented and analyzed separately. Articles had to be written in Dutch or English. Exclusion criteria were no HRQoL questionnaire results available, no full text available, conference abstracts, methodology of validation or cross-cultural adaptation of HRQoL-questionnaires; acquired BDs and articles presenting only aggregated results from children and adults.

Search strategy

We performed a systematic search in the following electronic databases: EMBASE, MEDLINE Ovid and Cochrane CENTRAL on April the 29th of 2019 and updated June 1st 2021 with the help of an experienced biomedical librarian and Cochrane information specialist (WB). A search for grey literature was not conducted. The search-query consisted of a broad range of key words related to BDs, (HR)QoL and children (supplement 1*)*. The query was adjusted to the requirements of specific databases. No publication date limitations were applied. All records were imported into an EndNote^{R} library for bibliographic management and duplicates were removed. Records were screened on title and abstract by two independent researchers (CM/EH). Only articles meeting the inclusion criteria were included after reading the full text. Of the included articles, references were checked for missed articles.

Data collection

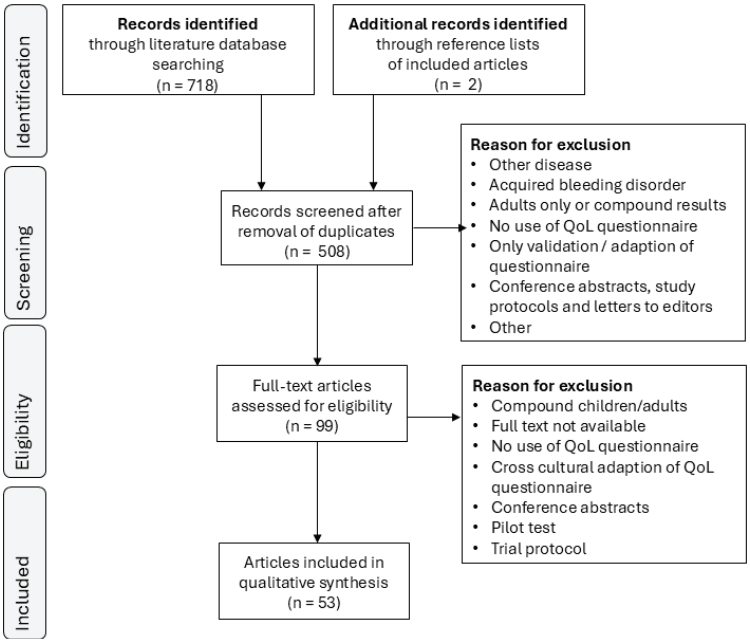
A pre-set data format was developed prior to extracting data of the final set of included articles in an Excel (Microsoft® 2016) spreadsheet, including author(s); year of publication; journal of publication; country of origin; type of BD; disease severity and whether the HRQoL results were reported separately or pooled according to severity, age-appropriate and validated; number of included patients and caregivers; age; application of a reference group; type of generic and/or disease-specific HRQoL questionnaire, and lastly the

used descriptive statistics for the HRQoL-results. Tables were made in Word (Microsoft® 2018), illustrations were designed using Pages (Apple Inc®, 2007).

RESULTS

The search yielded 718 records and using references two additional articles were found. After removal of duplicates, 508 records were screened on title/abstract. After the first screening, 99 articles were reviewed in full text independently; after discussion and agreement by two independent reviewers on incongruent articles, 53 articles were included in this scoping review.(15-65) These final articles were subdivided into two different subgroups: 21 articles described studies that measured the effect of an intervention, such as treatment and/or sporting activities,(47-54, 57-58, 60-61, 65-68) and 32 articles described studies that measured HRQoL at a single time point.(15-16, 20-22, 24-29, 31-37, 39-40, 69) An overview of the search and screening process for this scoping review is illustrated in a PRISMA Flow Diagram (Figure 1).

Figure 1 PRISMA 2009 Flow Diagram



Abbreviations n = number, QoL = quality of life

General characteristics of included articles

General characteristics of the included articles are demonstrated in Table 1. All articles were published after the year 2000 with the majority published after 2010 (86.8%). In summary, HRQoL-research in children with BDs has been performed in a variety of countries around the world, but the majority of studies were performed in Europe (21/53, 39.6%) and Northern America (11/53; 21%). The others were performed in Asia (10/53, 18.9%), the Middle East (4/53, 7.5%) and Australia (2/53, 3.8%, details in supplement 2). Five papers described the results of HRQoL-studies performed by an international cooperation. HRQoL was measured at a single time-point in 32 articles (32/52, 61.9%). In 19 articles (17/53, 32.1%) the change in HRQoL was analyzed in children with haemophilia by measuring a within-patient change as a measurement for the impact of interventions such as education,(49, 51, 57, 67) sports or yoga,(48, 50, 52, 59, 60, 66) or around the introduction of novel factor replacement therapies.(54, 56, 59, 61, 62, 64) In 4 articles (4/21, 19.0%), an inter-patient difference was investigated, comparing HRQoL in a group with and without an intracranial or frequent bleeding with HRQoL in on demand treatment versus prophylaxis groups.(47, 53, 58, 68) In two articles (2/21, 9.5%), non-haemophilia patients were pooled with haemophilia patients in the final analysis (details in supplement 2).(50, 60)

Table 1 Characteristics of included publications

General characteristics	Number (n)	Percentage (%)
Subgroups	(n=53)	
QoL at single time point	32	60,4
QoL before and after intervention	21	39,6
Publication year		
2000 – 2004	4	7,5
2005 – 2009	3	5,7
2010 – 2014	16	30,2
2015 – 2019	21	39,6
2020 – 2021	9	17,0
Country		
Europe	19	35,8
Northern America	13	24,5
Asia	10	18,9
Middle East	4	7,5
Oceania	2	3,8
Mixed	5	9,4

Abbreviation QoL = quality of life

General characteristics of included patients

An overview of the general patient characteristics is presented in Table 2. Importantly, study populations ranged from 13 to 339 children, but most included a maximum of 149 children (44/53, 86.3%). Fifty articles mentioned the minimal age of the included children

(50/53, 94.3%). Sixteen included children <4 years of age (16/50, 32.0%), with five articles including children from the age of <1 year (5/16, 31.3%). The other 34 articles included patients ≥4 years of age (34/50, 68.0%). The vast majority of included articles studied HRQoL in children with haemophilia (47/53, 88.7%), of which 36 pooled haemophilia A en B patients (36/47, 76.6). When children with haemophilia A and B were reported separately, 16 articles addressed haemophilia A (16/21, 76.2%) and 5 haemophilia B (5/21, 23.8). Articles that published data of children with other BDs than haemophilia, were sparse (3/53, 5.7%): one article reported on children with Von Willebrand Disease (VWD),(21) and one on children with IPD.(37) Three articles included patients with various BDs other than haemophilia and one article pooled haemophilia with VWD.(32, 35, 50, 60) These four articles pooled the results in single analysis. For a visualization of the main body of research. See Figure 2, details in Supplement 2.

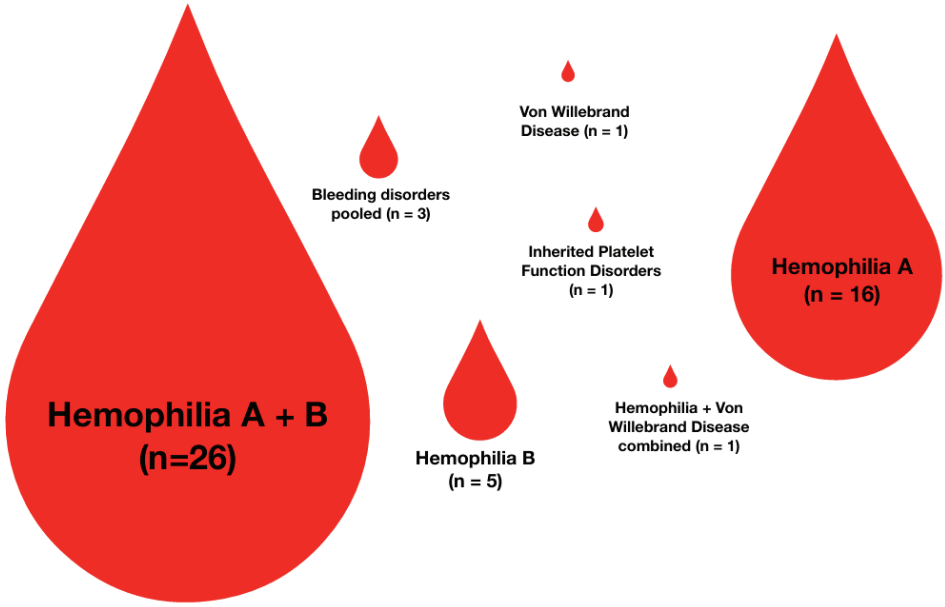
Table 2 Characteristics of included patients

Patient characteristics	Number (n)	Percentage (%)
Number of patients in included publications†	(n = 51)	
<50	23	45,1
50-99	13	25,5
100-149‡	8	15,7
150-199‡	4	7,8
200-299	1	2,0
300-399‡	2	3,9
Youngest age of included children	(n = 50)§	
< 4 years	16	32,0
3 4 years	34	68,0
Type of bleeding disorder	(n = 53)	
Haemophilia A + B pooled	26	49,1
Haemophilia A only	16	30.2
Haemophilia B only	5	9,4
Bleeding disorders pooled	3	5,7
Inherited Platelet Disorders	1	1,9
Von Willebrand Disease (VWD)	1	1,9
Haemophilia A + B and VWD pooled	1	1,9
Severity of bleeding disorder stated	(n = 44)	(83,0)
Multiple severity types pooled	30	68,2
Results pooled¶	20	
Results separated	10	31,8
Severely affected patients only	14	
Severity of bleeding disorder omitted	(n = 9)	(17,0)

Abbreviations QoL = Quality of life, RBDs = Rare Bleedings Disorders, VWD = Von Willebrand Disease

Legend † Number of included patients that filled in the QoL questionnaire; ‡ Three articles reported the number of children on behalf of which caregivers had filled in the questionnaire; § Three articles did not mention the minimal age of the included children; ¶ All articles that included different grades of disease severity but pooled the QoL results.

Figure 2 Visualisation of the main body of research that has been performed in the field of QoL in pediatric patients with different bleeding disorders



Legend Figure to scale

Severity of bleeding in relation to HRQoL-research

Besides the fact that different BDs may influence HRQoL differently, the severity of disease will of course affect HRQoL as well. For example, haemophilia is divided into mild, moderate and severe haemophilia, based on the lowest documented FVIII or FIX activity level.(70) VWD is subdivided into type 1, type 2 and type 3. Type 1 and 3 are defined as quantitative defects, and type 2 is defined as qualitative defects.(71) Other BDs are either heterogeneous or homogenous, with the residual activity of the specific coagulation factor sometimes associated with bleeding phenotype. Inherited platelet disorders are divided into multiple subgroups based on platelet number, receptor defects, flowcytometric results, or are clustered by results of DNA-analysis.(72) In the context of disease severity, we found that 44 articles specifically mentioned the severity of BDs. In 14/44 (31.8%) articles, patients with similar disease severity were included. In the other 30 articles, various disease severities were pooled at inclusion (68.2%), but in 10/30 (33.3%) results were depicted per severity. This made it possible to correlate HRQoL-results with a specific disease and/or disease severity in 24/44 (54.5%; details in Supplement 2).

HRQoL-questionnaires

Twenty-seven studies used 13 different generic questionnaires due to the requirement of age-appropriate questionnaires, but also to the large variation on generic questionnaires. Use of multiple questionnaires was observed in one study. The generic questionnaires

PedsQoL and KINDL, and the utility measurement tool EQ-5D were most often used (17/27, 63.0%; Table 3). Thirty-five studies used a disease-specific questionnaire, all developed for haemophilia (n=35). Five different disease-specific questionnaires were used in patients with haemophilia. The HaemoQoL (26/35, 74.3%) and CHO-KLAT (5/35, 14.3%) were used most often, followed by the Adapted InhibQoL (2/35, 5.7%), QUAL-HEMO (1/35, 2.9%) and Hemo-Sat (1/35, 2.9%). These haemophilia-questionnaires were also used once in VWD patients (n=1, (50)) and once in a pooled patient group with rare BDs (n=1, (35)) In one article, HRQoL was assessed in children with IPD by a disease-specific questionnaire developed for acquired immune thrombocytopenia (ITP).(37) (Table 3). All HRQoL questionnaires were age-appropriate for the included patients (Table 4).

Six studies combined a generic and disease-specific questionnaire (11.3%).(16, 33, 46, 52, 56, 68) To compare HRQoL results, 11 studies used a reference group (20.8%), of which 10 studies compared with a healthy reference group,(21, 26, 31-33, 42, 47, 50, 60, 66) and three (also) compared the results with HRQoL obtained in children with another chronic disease.(16, 31, 32) Two HRQoL studies in children with haemophilia used a control group that also suffered from haemophilia but did not experience an intracranial hemorrhage or was not on prophylaxis.(47, 58)

Table 3 Overview of generic and disease-specific HRQoL-questionnaires

Characteristics of questionnaires	Number (n)	Percentage (%)
Generic QoL questionnaires	(n = 27)	
EQ-5D, subtypes VAS, Y-3L and 5L	6	22,2
PedsQL	6	22,2
KINDL	5	18,5
CHQ	3	11,1
SF-36	3	11,1
CarerQoL	1	3,7
CHIP-CE	1	3,7
GAD-7	1	3,7
ITQOL	1	3,7
KIDSCREEN	1	3,7
PHQ-9	1	3,7
Quality of My Life questionnaire	1	3,7
TAPQOL	1	3,7
Disease-specific questionnaires - Haemophillia[†]	(n = 35)	
Haemo-QoL	26	74,3
CHO-KLAT	5	14,3
Adapted Inhib QoL	2	5,7
QUAL-HEMO	1	2,9
Hemo-Sat	1	2,9
Disease-specific questionnaires - ITP[‡]	(n=1)	
Kids ITP Tool	1	

Table 3 Overview of generic and disease-specific HRQoL-questionnaires (*continued*)

Characteristics of questionnaires	Number (n)	Percentage (%)
Reference group	(n = 53)	
No reference group	42	79,2
Healthy reference only	8	15,1
Healthy reference + other disease reference	2	3,8
Other disease reference only	1	1,9

Abbreviations CarerQoL = Care Related Quality of Life; CHIP-CE = Child Health and Illness Profile - Child Edition; CHO-KLAT = Canadian hemophilia outcome-Kids Life Assessment Tool; CHQ = Child Health Questionnaire; D= Domains; EQ = Euro Quality of Life, GAD = Generalized Anxiety Disorder; Haemo-QoL = Haemophilia Quality of Life; Hemo-Sat = Health-related quality of life and satisfaction; ITQOL = Infant and Toddler Quality of Life; Kids ITP Tools = Kids immune mediated thrombocytopenia tool; L = Levels; QoL = Quality of Life; PedsQL = Pediatric Quality of Life Inventory; PHQ = Patient Health Questionnaire; QUAL-HEMO = Hemophilia age group-specific quality of life questionnaire; SF = Short Form Health Survey; TAPQOL = TNO-AZL Preschool Children Quality of Life; VAS = Visual Analogous Scale; Y = Young;

Legend † Disease specific questionnaire validated in haemophilia patients, ‡ Disease specific questionnaire validated in immune mediated thrombocytopenia (ITP)

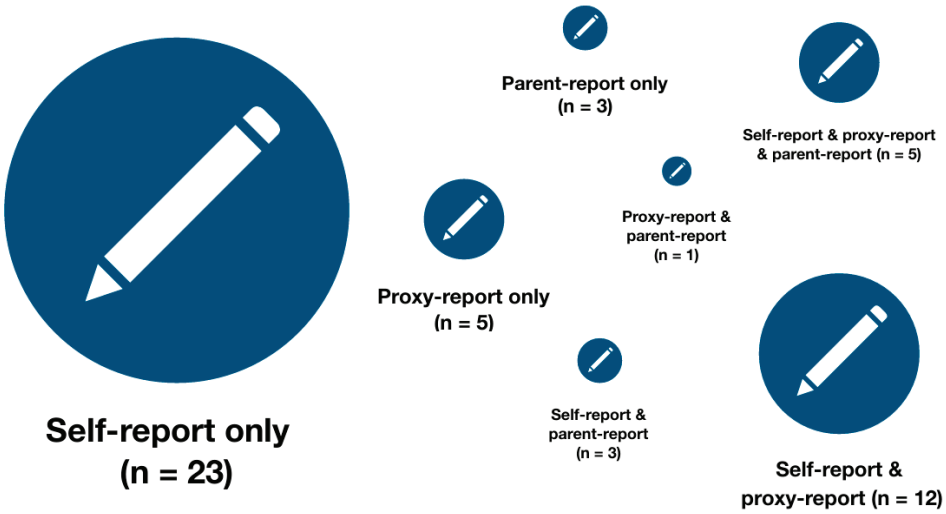
Table 4 Overview of used HRQoL questionnaires per age group

Brand Name	Full name	Age groups
CarerQoL	Care Related Quality of Life	Parents only (proxy)
CHIP-CE	Child Health and Illness Profile - Child Edition	6 till 11 years
CHO-KLAT	Canadian hemophilia outcome-Kids Life Assessment Tool	4 till 18years
CHQ	Child Health Questionnaire	5 till 18 years
EQ-5D	EuroQoL 5 Domains	≥18 years
EQ-5D VAS	Visual Analogue Scale	Not specified
EQ-5D 3L	5 Domains 3 Levels	≥18 years + parents
EQ-5D 5L	5 Domains 5 Levels	≥18 years + parents
EQ-5D-Y	5 Domains – Young	I: 4 till 7, II: 8 till 18 years
GAD-7	Generalized Anxiety Disorder-7	>8 years
Haemo-QoL	Haemophilia Quality of Life	I: 4 till 7 years, II: 8 till 12 years, III: 13-16 years
Hemo-Sat	Health-related quality of life and satisfaction	<17 years
ITQOL	Infant and Toddler Quality of Life	≥2 months till <6 years
Kids ITP Tools	Kids immune mediated thrombocytopenia tool	2 till 18 years
PedsQL	Pediatric Quality of Life Inventory	2 till 18 years
PHQ-9	Patient Health Questionnaire-9	≥11 years
QUAL-HEMO	Hemophilia age group-specific quality of life questionnaire	Child, adolescent and parents, age not specified
SF-36	36-item Short Form Health Survey	≥14 years
TAPQOL	TNO-AZL Preschool Children Quality of Life	≥9 months till <6 years

Lastly, most questionnaires standardly consist of different versions: self-report, proxy-report and/or parent-report. All possible combinations were found in the included studies (Figure 3). Five studies analyzed the child's HRQoL by surveying the child, the parents' view on the child (proxy) and the parental HRQoL.(16, 21, 48, 62, 73) The majority (23/53, 43.4%) solely evaluated the HRQoL by the answers given by the child itself. Twelve studies added a proxy-report (12/53, 22.6%).(23, 24,30 34, 39, 40, 54, 58-60, 67, 68) Three studies assessed only the impact of the child's disorder on the parental HRQoL by using a parent-report (17.0%).(28, 57, 69)

One study did not mention which versions were used.(47)

Figure 3 Visual impression of the distribution of used QoL questionnaire versions in 52/53 included articles of the scoping review



Legend Figure to scale

Descriptive statistics

The mean and standard deviation (SD) were the descriptive statistics that were used most often. They were used in 88.7% and 77.4% respectively of the included articles. Thereafter, a range (24.5%), median (24.5%) and interquartile range (IQR, 13.2%) were frequently used as descriptive statistics. The effect size was used in five articles (9.4%), followed by the difference from baseline and p-values (both 7.5%), the minimally clinically important difference (MCID) or documentation of the outliers (both 5.7%), standard error (3.8%; Supplement 2).

DISCUSSION

Summary of knowledge gaps

From the body of evidence summarized in our scoping review, we have extracted the following knowledge gaps:

- *Future HRQoL research should focus on all types of BDs, both sexes, all ages and continents*

There is hardly any research on the effect of treatment in patients with BDs other than haemophilia. Although more rare, prophylaxis with factor concentrates is also given to patients with other factor deficiencies than haemophilia, as well as on demand treatment using blood product transfusions or desmopressin.(74, 75) And only in children with haemophilia HRQoL research has been done by measuring a within-patient change around the introduction of novel interventions or therapies. The benefit of currently used (on demand) treatment options, transfusions and desmopressin in all BDs is missing. Especially HRQoL-research in children with VWD, IPD and rare factor deficiencies is missing. As bleeding patterns differ between various BDs, HRQoL questionnaires for use in VWD and IPD should focus on skin, mucosal, and perioperative bleeding and the addition of a symptom checklist to quantify the impact of skin or feminine bleeding issues is advisable.(76, 77) This is even more relevant, because girls are grossly underrepresented in studies as is the result of the high volume of haemophilia studies which is an X-linked disease. Also, only a minority of HRQoL-research included children <4 years of age. This results in a knowledge gap on the child's gross motor development, vaccinations and initiation of prophylaxis in severe haemophilia,(11) as well as the effect of BDs on the bidirectional influence between infants and caregivers and early disease acceptance.(78) For this purpose, we advise to use a generic questionnaire that is designed to measure HRQoL in young children between 0-4 years old, such as ITQOL.(79) Finally, the distribution of countries shows that children from Africa and South America, and to a lesser extent Asia, are underrepresented. This matters, because children in developing countries may report a reduced HRQoL in other domains than children in developed countries due to differences in (parental) education, socio-economic state and medical health care systems. We advise to use validated existing questionnaires after translation, taking different cross-cultural perspectives into account.(78, 80-82)

- *Strive for homogenous study groups or present results separately*

Disease severity influences bleeding frequency and bleeding complications. Therefore, studies should aim to present results per severity type. Final outcomes will otherwise be negatively influenced. For example, mean HRQoL may be overestimated for the severely affected children when children with a severe phenotype are underrepresented. Homo-

geneity also accounts for the use of prophylaxis in haemophilia and other rare coagulation factor deficiencies, as well as whether the patient has formed inhibitors.

- *Focus on follow-up in time, using the proper questionnaire and report-form for the desired outcome*

Instead of measuring HRQOL at a single time point, evaluation during follow-up provides clinicians with continuous information.(83) In this light, it is important to strive for a patients' perspective as early as possible. In young children a proxy-report remains essential, but parallel evaluation of the caregivers' perspective on HRQoL at an older age may still give more insight into the system around the child.(84) To value the results not only within the patient group, a healthy age-adapted reference group is relevant when generic tools are used. The choice of measurement tool also depends on the desired outcome such as evaluation of 'health profile' or generation of 'utility scores' for the purpose of economic evaluations such as Quality Adjusted Life Years (QALYs). To guide this choice, it is important to realize that there are two types of generic HRQoL questionnaires: 'profile measurement tools' such as the PedsQoL and CHQ generating health profiles. Of these, our study shows that the PedsQoL was most often used for children (6/27, 22%). The 'utility measurement tools' generate utility as HRQoL based outcome. Our review shows that the EQ-5D is most often used for utility outcomes (6/27, 22%). For the disease specific questionnaires, we conclude that for haemophilia patients, HaemoQoL is most frequently used (26/35, 74%) both the long (77 items) and the short version (16 or 35 items) which support comparisons of outcomes. CHO-KLAT was used less frequently (5/35, 14%), but it has been validated against the PedsQoL and is therefore also valuable. For other BDs no disease specific outcome measurement tools are available.

- *Tools are needed to compare or meta-analyze results obtained from various questionnaires*

In line with the former, the large number of generic and disease-specific questionnaires used in HRQoL-research is striking. As there is no universal tool to compare the various questionnaires, comparison of differences in standard deviations of comparable domains should be made possible as an alternative to meta-analysis data.(85, 86) The recently developed 'Item Response Theory' (IRT) may however be key to overcome these issues. IRT scales items and persons on a single metric, comparing the results between the groups longitudinally in time.(87) In paediatric care, this is even more relevant as longitudinal follow-up of a child into adulthood is advisable and otherwise requires various age-adjusted tools.

- *HRQoL research should provide detailed descriptive statistics*

Many studies solely used the mean and standard deviation (SD), or median and interquartile range (IQR) as the single descriptive statistics for HRQoL results. By doing so, data only gives insight into the average well-being but neglects the possible coping strengths of the upper outliers or the need for extra supporting strategies for the lower outliers. We therefore advise to provide a SD/IQR and a range as well as outliers, especially in studies pooling multiple disease severities. Finally, the minimal clinically important difference (MCID) was rarely used, but MCID is of value in studies evaluating novel or various treatment options. As it overcomes the gap, statistically significant effects are not always of clinical importance to a patient. Likewise, the effect size can quantify the effectiveness of a specific intervention.(88)

Strengths and limitations

The search query for this scoping review consisted of a broad range of keywords and did not set limitations on publication date and study type, creating an overview on HRQoL in all children with a BD. Limitations are the language restriction to English and Dutch. In addition, other bibliographic databases than PubMed, Cochrane and Embase may have yielded additional articles. Also, sometimes the parental questionnaire was added as a subpart of the proxy questionnaire. We could only identify this if this intervention was described in the methods section of included articles. If not, the parental questionnaire was not scored.

Conclusions and future perspectives

In this scoping review, we provide an overview of the available research on HRQoL in children with all types and severities of BDs. We demonstrate that HRQoL research in children with BDs is an upcoming research field with still important knowledge gaps which should be addressed. But we also demonstrated that certain areas have been studied more than others. So far, studies have been mainly performed in school-aged boys with haemophilia treated in developed countries. As a result, we used the identified knowledge gaps to suggest five recommendations for future research. Focus should be on performing HRQoL-research worldwide in other BDs than haemophilia, with attention to female gender-related bleeding problems and the utilization of more sophisticated statistical parameters to identify not only the risk groups but also the groups with better coping strategies.

Acknowledgments

Wichor Bramer (WB), librarian of the Erasmus University Medical Center, performed the library search.

Supplementary Method S1 Data Search Terms

Embase.com

('blood clotting disorder'/de OR 'blood clotting factor deficiency'/exp OR 'thrombocyte disorder'/exp OR 'bleeding disorder'/de OR (((blood-clot* OR coagulat* OR thrombocyte* OR Willebrand OR platelet* OR bleeding OR factor-VIII OR factor-IX) NEAR/3 (disorder* OR disease* OR dysfunction*)) OR thrombocytop* OR hemophili* OR haemophili*):ab,ti) AND (('quality of life'/exp AND ('questionnaire'/exp OR 'assessment of humans'/exp)) OR 'quality of life assessment'/exp OR (((Quality-of-Life OR hrql OR hrqol) NEAR/10 (assess* OR questionnaire* OR measure* OR evaluat* OR instrument* OR survey*)) OR eq5d OR eq-5d OR euroqol OR short-form-36 OR SF-36 OR SF36 OR short-form-20 OR SF-20 OR SF20 OR itqol OR it-qol OR chq OR pedsqol OR peds-qol OR pedqol OR ped-qol OR pedsql OR peds-ql OR pedql OR ped-ql OR hemoqol OR haemoqol OR hemo-qol OR haemo-qol OR kids-itp-tool*):ab,ti) AND ('juvenile'/exp OR childhood/exp OR adolescence/exp OR pediatrics/exp OR 'childhood disease'/de OR 'parent'/exp OR (child* OR juvenile* OR infan* OR adolescen* OR pediatr* OR paediatr* OR parent OR parents OR father* OR mother* OR paternal* OR maternal*):ab,ti) NOT ([Conference Abstract]/lim) AND [English]/lim *Medline Ovid* (Blood Coagulation Disorders/ OR exp Blood Coagulation Disorders, Inherited/ OR (((blood-clot* OR coagulat* OR thrombocyte* OR Willebrand OR platelet* OR bleeding OR factor-VIII OR factor-IX) ADJ3 (disorder* OR disease* OR dysfunction*)) OR thrombocytop* OR hemophili* OR haemophili*).ab,ti.) AND ((Quality of Life/ AND ("Surveys and Questionnaires"/)) OR (((Quality-of-Life OR hrql OR hrqol) ADJ10 (assess* OR questionnaire* OR measure* OR evaluat* OR instrument* OR survey*)) OR eq5d OR eq-5d OR euroqol OR short-form-36 OR SF-36 OR SF36 OR short-form-20 OR SF-20 OR SF20 OR itqol OR it-qol OR chq OR pedsqol OR peds-qol OR pedqol OR ped-qol OR pedsql OR peds-ql OR pedql OR ped-ql OR hemoqol OR haemoqol OR hemo-qol OR haemo-qol OR kids-itp-tool*).ab,ti.) AND (exp child/ OR exp infant/ OR adolescent/ OR pediatrics/ OR exp parents/ OR (child* OR juvenile* OR infan* OR adolescen* OR pediatr* OR paediatr* OR parent OR parents OR father* OR mother* OR paternal* OR maternal*).ab,ti.) NOT (news OR congres* OR abstract* OR book* OR chapter* OR dissertation abstract*).pt. AND english.la. *Cochrane CENTRAL* ((((blood-clot* OR coagulat* OR thrombocyte* OR Willebrand OR platelet* OR bleeding OR factor-VIII OR factor-IX) NEAR/3 (disorder* OR disease* OR dysfunction*)) OR thrombocytop* OR hemophili* OR haemophili*):ab,ti) AND ((((Quality-of-Life OR hrql OR hrqol) NEAR/10 (assess* OR questionnaire* OR measure* OR evaluat* OR instrument* OR survey*)) OR eq5d OR eq-5d OR euroqol OR short-form-36 OR SF-36 OR SF36 OR short-form-20 OR SF-20 OR SF20 OR itqol OR it-qol OR chq OR pedsqol OR peds-qol OR pedqol OR ped-qol OR pedsql OR peds-ql OR pedql OR ped-ql OR hemoqol OR haemoqol OR hemo-qol OR haemo-qol OR kids-itp-tool*):ab,ti) AND ((child* OR juvenile* OR infan* OR adolescen* OR pediatr* OR paediatr* OR parent OR parents OR father* OR mother* OR paternal* OR maternal*):ab,ti)

Supplementary Table S2 QoL studies at fixed time-point

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Shapiro(1)	Pediatrics (2001)	USA	126		9.6 (6-12)	HFA	Severe	NA	CHQ, PF50		Mean
Bullinger(2)	J Ped Hem Oncol (2003)	DE, IT, FR, ESP, UK, NL	339	330	10 (4-16)	HF	Unknown	Unknown	KINDL	Haemo-QoL	Mean, SD, Cronbach's alpha
Gringeri(3)	Haemophilia (2004)	FR, DE, IT, NL, ESP, UK	318		10 (4-16)	HFA, HFB	Severe	No		Haemo-QoL	Mean, SD
Douma-Van Riet(4)	Haemophilia (2009)	NL	158		12.7 (7-18.5)	HFA, HFB	Severe, moderate, mild	No		Haemo-QoL	Mean, SD
Mercan(5)	Pediatric Hematology and Oncology (2010)	TURK	39		Unknown (4-16)	HF	Severe	NA	NA	Haemo-QoL	Mean, SD, Crohnbach's alpha
De Wee(6)	J Thromb Hemost (2011)	NL	133	133	Unknown (0-15)	VWD	Type 1,2 and 3	No	ITQOL en CHQ		Mean, SD, effect size (Cohen's d),
Tantawy(7)	Pediatric Hematology and Oncology (2011)	EGYPT	60		Unknown (4-16)	HFA	Severe	NA		Haemo-QoL	Mean, SD, range
Gringeri(8)	J of Thrombosis and Haemostasis (2011)	IT	40	1	4 (1-7)	HFA	severe	NA		Haemo-QoL II and III	Mean, SD
Poon(9)	Haemophilia (2012)	USA	165		9.7 (2-17)	HFA	Severe, moderate, mild	Yes	PedsQL		Mean, SD, MCID
DeKoven(10)	Haemophilia (2013)	USA	52	56	8.8 (4-16)	HF	Unknown	Unknown		Haemo-QoL	Mean, median, SD, IQR, range
Lindvall(11)	Pediatr Blood Cancer (2014)	SW, UK, CA	76	143	Unknown (0-18)	HF	Unknown	Unknown	SF-36		Mean, SD

Supplementary Table S2 QoL studies at fixed time-point (*continued*)

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Poon(12)	J Gen Intern Med (2014)	USA	125		9.6 (2-17)	HFA	Severe, moderate, mild	Yes	PedsQL		Mean, SD
DeKoven(13)	Haemophilia (2014)	USA	0	310	8.33 (0-18)	HF	Severe, moderate, mild	Yes	CarerQoL		Mean, SD, range, median
Kodra(14)	Blood Transfus (2014)	IT	22	17	8 (2-17)	HF	Unknown	Unknown	EQ-5D-Y-3L (child) EQ-5D-5L (caregiver)		Mean, SD
Taha(15)	J Pediatr Hematol Oncol (2014)	IRAQ	45		(4-16)	HFA (40) HFB (5)	severe, moderate, mild	NA		Haemo-QoL I, II and III	Mean, SD, p-value
Oymak(16)	J Pediatr Hematol Oncol (2015)	TURK	33 HF, 19 JIA, 32 controls		9.7 (4-18)	HF	Severe, moderate	Yes	KINDL		Median, range
Neuner(17)	BioMed Research International (2016)	DE	74		11.5 (8-16)	Pooled BDs 1†	Unknown	Unknown	KINDL		Mean, SD, IQR, median, range, Crohnbach's alpha, outliers
St-Louis(18)	BMC Hematol (2016)	CA	13		15.7 (13-18)	HFA	Severe, moderate	Yes	SF-36	CHO-KLAT	Mean, SD, MID, range
Williams(19)	Journal of Pediatrics and Child Health (2016)	AUS	22	22	Unknown (4-16)	HFA	Severe, moderate, mild	No		Haemo-QoL	Median

Supplementary Table S2 QoL studies at fixed time-point (*continued*)

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Haghpanah(20)	Clinical and Applied Thrombosis/Hemostasis (2017)	IRAN	52	.	13.96 (4-18)	RBD	Severe, moderate, mild	Yes		Haemo-QoL	Mean, SD
Khair(21)	Haemohilia (2017)	UK	127		12.38 (8-17)	HFA + HFB	severe	No		Haemo-QoL	Mean, SD
Khair(22)	Haemophilia (2018)	UK	16	.	11 (6-17)	IPDs	Unknown	Unknown		Kids ITP Tools	Number
Buckner(23)	Eur J Haematol (2018)	USA	0	150	10 (0-18)	HFB	Severe, moderate, mild	No	PHQ-9, GAD-7		IQR, mean, median
Tang(24)	Haemophilia (2018)	CHINA	171	269	8.4 (4-4-18)	HF	Severe, moderate, mild	Yes		CHO-KLAT	Mean, SD, range, median + IQR, outliers
Zhang(25)	Health and Quality of Life outcomes (2019)	CHINA	42	42	5.48	HF	Severe, moderate, mild	No		CHO-KLAT	Mean, SD, range
Na Songkla(26)	Haemophilia (2020)	THAI	16		9.6 (0.5-20)	HFA	Severe, moderate	No	EQ-5D-5L		Median, p-value
Cikili-Uytun(27)	Egyptian Pediatric Association Gazette (2020)	TURK	20		10.95 (6-16)	HFA (16) HFB (8)	Severe, moderate, mild	Yes	KINDL		Pearman and Pearson correlation tests, p-values

Supplementary Table S2 QoL studies at fixed time-point (*continued*)

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Olden-burg(28)	Pediatric Blood & Cancer (2019)	CHINA, CR, DE, ESP, IT, JAP,USA, RSA	24	24	7.5 (8-11)	HFA	Unknown	Unknown		Haemo-QoL, Adapted Inhib-QoL	Mean, SD
Malec(29)	Am J Hematol (2020)	USA	138		14 (8-19)	HFA, HFB	Severe	No		CHO-KLAT, Haemo-Qol, Haem-A-Qol	Mean
Dsouza(30)	Ped Hemy/Oncol and Immunopa-thology (2020)	INDIA	107		11 (6-16)	HFA, HFB	Severe, moderate	No		Haemo-QoL	Mean, SD
Baek(31)	Plos One (2020)	KOREA	199		Unknown (8-16)	HFA, HFB	Severe, moderate	Yes	EQ-5D-3L, EQ VAS	Haemo-QoL	Mean, SD

Supplementary Table S2 QoL studies before and after an intervention or new therapy (*continued*)

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Revel-Vilk(32)	The Journal of Pediatrics (2004)	CA	16		9.2 (3-18)	HF	Severe, mild	Yes	Quality of My Life		Mean, SD
Van der Net(33)	Haemophilia (2006)	NL	13		11.04 (8-14.5)	HFA	Severe	NA		Haemo-QoL	Mean, SD, range
Du Treil(34)	Haemophilia (2007)	USA	31		Unknown (5-18)	HF	Unknown	Unknown	CHQ		Mean, SD

Supplementary Table S2 QoL studies before and after an intervention or new therapy (*continued*)

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Broderick(35)	Haemophilia (2010)	AUS	44		10.9 (6-17)	HF, VWD	Severe, moderate, mild	No		Haemo-QoL	Mean, SD
Duncan(36)	Haemophilia (2012)	USA	53		10.5 (<18)	HFA	Severe	NA	PedsQL		Mean
Khair(37)	Haemophilia (2012)	UK	84		11.52 (6-17)	HF	Severe, moderate, mild	Yes	KINDL	Haemo-QoL	Mean, SD
Neufeld(38)	Value in Health (2012)	USA	18	.	Unknown (2-18)	HF	Unknown	Unknown	EQ-5D, EQ-5D-VAS		Mean, SD
Santagostino(39)	Haemophilia (2014)	USA, Zuid-Amerika, Asia, EU§	64		Unknown (4-16)	HFA	Severe	NA		Haemo-QoL	Mean, SD, median, range, IQR, outliers
García-Dasí(40)	Haemophilia (2015)	ESP	68	64	(6-16)	HFA	Severe	NA		Haemo-QoL	Mean, SD
Beheshtipoor(41)	Int. j. community nurs. Midwifery (2015)	Iran	27		11.5 (8-16)	HF	Severe, moderate, mild	Yes		Haemo-QoL	Mean, SD
Polack(42)	Transfusion (2015)	FR	51	49	Unknown (3-18)	HFB	Severe, moderate	Yes	KIDSCREEN	QUAL-HEMO	Mean, SD
Cuesta-Barriuso(43)	Pediatric Physical Therapy (2016)	ESP	53 (HF) 51 (control)		10 (7-13)	HF	Severe, moderate, mild	Yes	CHIP-CE		Mean, SD
Chowdary(44)	Haemophilia (2016)	DNMK	15		14.3 (13-16)	HFB	Severe, moderate-severe	Yes	EQ-5D-3L	Haemo-QoL	Mean, SD, IQR, median, p-values

Supplementary Table S2 QoL studies before and after an intervention or new therapy (*continued*)

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Phadnis(45)	Haemophilia (2017)	India	159	133	Unknown (2-18)	HF	Severe, moderate, mild,	Yes	PedsQL		Mean, SE, range, effect size (Cohen's)
Wu(46)	Haemophilia (2017)	China	23	23	11.3 (4-16)	HFA	Severe	NA		CHO-KLAT	Mean, SD, IQR, median, range, effect size
Carcao(47)	Haemophilia (2017)	CAN	12	12	Unknown (8-12)	HFB	severe, moderate	Yes		Haemo-QoL	Median, IQR, median change from baseline
Limperg(48)	Haemophilia (2018)	NL	145		Unknown (0-18)	Pooled BDs 2‡	Severe, moderate, mild	No	TAPQOL, PedsQL		Median, range, mean, SD, effect size
Von Mackensen(49)	Haemophilia (2019)	AUS, AUT, CAN, CZE, DE, ESP, FR, ISR, ITA, RUS	20	23	5.9 (4-11)	HFB	Severe, moderate	Yes		Haemo-QoL, Hemo-Sat	Mean, SD, effect size (cohen's), MID
Mancuso(50)	Haemophilia (2020)	UK, USA, ESP, DE, IT, RSA, JAP, TURK, FR, CR	88		6.5 (1-15)	HFA, with inhibitors	Severe, moderate	Yes		Haemo-QoL SF II, and Adapted Inhib QoL	Mean, SD, change from baseline
Bladen(51)	Haemophilia (2020)	UK	9		9.77	HFA (7), HFB (2)	severe	No	EQ-5D-Y Vas		Change from baseline, adjusted mean and 75% CI

Supplementary Table S2 QoL studies before and after an intervention or new therapy (*continued*)

1st author	Journal (year)	country	No of patients	No of parents	Mean age (range, y)	Bleeding disorder	Disease severity	Disease severity pooled	Generic questionnaire	Disease specific questionnaire	Statistic variable
Chow-adry(52)	Haemophilia (2020)	UK	95		14.3 (1-17)	HFA	severe	NA	PedsQL <14yrs, SF36 =/> 14 yrs		Mean, SD and mean change from baseline

Abbreviations AUS = Australia, AUT = ??, CAN = Canada, CI = confidence interval, CZE = Czech Republic, BDs = Bleeding disorders, CR = Costa Rica, DE = Germany, DNMK = Denmark, ESP = Spain, HF = Haemophilia (A and B pooled or not otherwise specified), HFA = haemophilia A, HFB = haemophilia B, IPDs = Inherited platelet disorders, IQR = interquartile range, ISR = Israel, IT = Italy, JAP = Japan, NA = not applicable, NL = The Netherlands, RSA = South-Africa, RUS = Russia, SD = standard deviation, SW = Sweden, THAI = Thailand, TURK = Türkiye, UK = United Kingdom, USA = United States of America, VWD = Von Willebrand Disease,

Legend [†] pooled BDs1 = HFA, HFB, VWD type 2 + 3 and hereditary fibrinogen deficiency, F V, VII, XI deficiency. [‡]Pooled BDS2= HFA and HFB carriers with FVIII/IX <40IU/dL and VWD type 2+3, [§]countries not further specified.

REFERENCES

1. Bolton-Maggs, P.H., et al., A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. Br J Haematol, 2006. 135(5): p. 603-33.
2. Zanon, E. and S. Pasca, Intracranial haemorrhage in children and adults with haemophilia A and B: a literature review of the last 20 years. Blood Transfus, 2019. 17(5): p. 378-384.
3. Ghanizadeh, A. and P. Baligh-Jahromi, Depression, anxiety and suicidal behaviour in children and adolescents with Haemophilia. Haemophilia, 2009. 15(2): p. 528-32.
4. Grupe, D.W. and J.B. Nitschke, Uncertainty and anticipation in anxiety: an integrated neurobiological and psychological perspective. Nat Rev Neurosci, 2013. 14(7): p. 488-501.
5. Organization, W.H. WHOQOL: Measuring Quality of Life Available from: https://www.who.int/healthinfo/survey/whoqol-qualityoflife/en/.
6. Torrance, G.W., Utility approach to measuring health-related quality of life. J Chronic Dis, 1987. 40(6): p. 593-603.
7. Ferrans, C.E., et al., Conceptual model of health-related quality of life. J Nurs Scholarsh, 2005. 37(4): p. 336-42.
8. Eiser, C. and M. Jenney, Measuring quality of life. Arch Dis Child, 2007. 92(4): p. 348-50.
9. Koopman, H.M., et al., Het meten van de gezondheidsgerelateerde kwaliteit van leven van kinderen met een chronische ziekte. Tijdschrift voor kindergeneeskunde, 2003. 71(3): p. 142-148.
10. Davis, E., et al., Parent-proxy and child self-reported health-related quality of life: using qualitative methods to explain the discordance. Qual Life Res, 2007. 16(5): p. 863-71.
11. Germain, N., S. Aballea, and M. Toumi, Measuring the health-related quality of life in young children: how far have we come? J Mark Access Health Policy, 2019. 7(1): p. 1618661.
12. Arksey, H. and L. O'Malley, Scoping studies: towards a methodological framework. International journal of social research methodology, 2005. 8(1): p. 19-32.
13. Levac, D., H. Colquhoun, and K.K. O'Brien, Scoping studies: advancing the methodology. Implement Sci, 2010. 5: p. 69.
14. Pham, M.T., et al., A scoping review of scoping reviews: advancing the approach and enhancing the consistency. Res Synth Methods, 2014. 5(4): p. 371-85.
15. Shapiro, A.D., et al., Defining the impact of hemophilia: the Academic Achievement in Children with Hemophilia Study. Pediatrics, 2001. 108(6): p. E105.
16. Bullinger, M. and S. Von Mackensen, Quality of Life in Children and Families with Bleeding Disorders. J Pediatr Hematol Oncol, 2003. 25(SUPPL. 1): p. S64-S67.
17. Gringeri, A., et al., Health status and health-related quality of life of children with haemophilia from six West European countries. Haemophilia, Suppl, 2004. 10(1): p. 26-33.
18. Grossfeld, P.D., et al., The 11q terminal deletion disorder: a prospective study of 110 cases. Am J Med Genet A, 2004. 129A(1): p. 51-61.
19. Douma-van Riet, D.C.M., et al., Physical fitness in children with haemophilia and the effect of overweight. Haemophilia, 2009. 15(2): p. 519-527.
20. Mercan, A., et al., Hemophilia-specific quality of life index (Haemo-QoL and Haem-A-QoL Questionnaires) of children and adults: Result of a single center from Turkey. Pediatr Hematol Oncol, 2010. 27(6): p. 449-461.
21. De Wee, E.M., et al., Impact of von Willebrand disease on health-related quality of life in a pediatric population. J Thromb Haemost, 2011. 9(3): p. 502-509.
22. Tantawy, A.A.G., et al., Health-related quality of life in Egyptian children and adolescents with hemophilia A. Pediatr Hematol Oncol, 2011. 28(3): p. 222-229.

23. Gringeri, A., et al., A randomized clinical trial of prophylaxis in children with hemophilia A (the ESPRIT Study). J Thromb Haemost, 2011. 9(4): p. 700-10.
24. Poon, J.L., et al., Quality of life in haemophilia A: Hemophilia Utilization Group Study Va (HUGS-Va). Haemophilia, 2012. 18(5): p. 699-707.
25. Dekoven, M., et al., Health-related quality of life in haemophilia patients with inhibitors and their caregivers. Haemophilia, 2013. 19(2): p. 287-293.
26. Lindvall, K., et al., Increased burden on caregivers of having a child with haemophilia complicated by inhibitors. Pediatr Blood Cancer, 2014. 61(4): p. 706-711.
27. Poon, J.L., J.N. Doctor, and M.B. Nichol, Longitudinal changes in health-related quality of life for chronic diseases: An example in hemophilia A. J Gen Intern Med, 2014. 29(SUPPL. 3): p. S760-S766.
28. DeKoven, M., et al., Impact of haemophilia with inhibitors on caregiver burden in the United States. Haemophilia, 2014. 20(6): p. 822-830.
29. Kodra, Y., et al., The social burden and quality of life of patients with haemophilia in Italy. Blood Transfusion, 2014. 12(SUPPL. 3): p. s567-s575.
30. Taha, M.Y. and M.K. Hassan, Health-related quality of life in children and adolescents with hemophilia in Basra, Southern Iraq. J Pediatr Hematol Oncol, 2014. 36(3): p. 179-184.
31. Oymak, Y., et al., Comparing the quality of life of patients with hemophilia and juvenile idiopathic arthritis in which chronic arthropathy is a common complication. J Pediatr Hematol Oncol, 2015. 37(8): p. 600-604.
32. Neuner, B., et al., Health-Related Quality of Life in Children and Adolescents with Hereditary Bleeding Disorders and in Children and Adolescents with Stroke: Cross-Sectional Comparison to Siblings and Peers. BioMed Res Int, 2016. 2016.
33. St-Louis, J., et al., Generic and disease-specific quality of life among youth and young men with Hemophilia in Canada. BMC Hematol, 2016. 16(1).
34. Williams, V.K., et al., Parents' perception of quality of life in their sons with haemophilia. J Paediatr Child Health, 2016. 52(12): p. 1095-1098.
35. Haghpanah, S., et al., Quality of Life in Children and Adolescents with Rare Bleeding Disorders in Southern Iran. Clin Appl Thromb Hemost, 2017. 23(6): p. 652-656.
36. Khair, K., et al., Study of physical function in adolescents with haemophilia: The SO-FIT study. Haemophilia, 2017. 23(6): p. 918-925.
37. Khair, K. and M. Holland, The Kids' immune thrombocytopenia Tool is not suitable for assessing quality of life in children with platelet function disorders. Haemophilia, 2018. 24(4): p. e259-e261.
38. Buckner, T.W., et al., Reliability and validity of patient-reported outcome instruments in US adults with hemophilia B and caregivers in the B-HERO-S study. Eur J Haematol, 2018. 101(6): p. 781-790.
39. Tang, L., et al., Describing the quality of life of boys with haemophilia in China: Results of a multicentre study using the CHO-KLAT. Haemophilia, 2018. 24(1): p. 113-119.
40. Zhang, H., et al., Health-related quality of life in children with haemophilia in China: A 4-year follow-up prospective cohort study. Health Qual Life Outcomes, 2019. 17(1).
41. Songkla, P.N., P. Seksarn, and D. Sosothikul, Comparison of different prophylactic regimens for Thai children with moderate and severe haemophilia A. Haemophilia, 2020. 26(4): p. E211-E213.
42. Cikili-Uytun, M., et al., Psychiatric problems and its contributing factors in children and adolescents with hemophilia: a single centre study in a Turkish sample. Egyptian Pediatric Association Gazette, 2020. 68(1).

43. Oldenburg, J., et al., The effect of emicizumab prophylaxis on health-related outcomes in persons with haemophilia A with inhibitors: HAVEN 1 Study. Haemophilia, 2019. 25(1): p. 33-44.
44. Malec, L.M., et al., The impact of extended half-life factor concentrates on prophylaxis for severe hemophilia in the United States. Am J Hematol, 2020. 95(8): p. 960-965.
45. Dsouza, A., et al., Description of health related quality of life of children and adolescents with hemophilia from India. Pediatr Hematol Oncol Immunopathol, 2020. 19(3): p. 12-16.
46. Baek, H.J., et al., Health-related quality of life of moderate and severe haemophilia patients: Results of the haemophilia-specific quality of life index in Korea. PLoS One, 2020. 15(9): p. e0238686.
47. Revel-Vilk, S., et al., Effect of intracranial bleeds on the health and quality of life of boys with hemophilia. J Pediatr, 2004. 144(4): p. 490-495.
48. van der Net, J., et al., Physical fitness, functional ability and quality of life in children with severe haemophilia: A pilot study. Haemophilia, 2006. 12(5): p. 494-499.
49. Du Treil, S., J. Rice, and C.A. Leissinger, Quantifying adherence to treatment and its relationship to quality of life in a well-characterized haemophilia population. Haemophilia, 2007. 13(5): p. 493-501.
50. Broderick, C.R., et al., Fitness and quality of life in children with haemophilia. Haemophilia, 2010. 16(1): p. 118-123.
51. Duncan, N., et al., Treatment patterns, health-related quality of life and adherence to prophylaxis among haemophilia A patients in the United States. Haemophilia, 2012. 18(5): p. 760-765.
52. Khair, K., et al., The impact of sport on children with haemophilia. Haemophilia, 2012. 18(6): p. 898-905.
53. Neufeld, E.J., et al., Effect of acute bleeding on daily quality of life assessments in patients with congenital hemophilia with inhibitors and their families: observations from the dosing observational study in hemophilia. Value Health, 2012. 15(6): p. 916-925.
54. Santagostino, E., et al., Assessment of the impact of treatment on quality of life of patients with haemophilia A at different ages: Insights from two clinical trials on turoctocog alfa. Haemophilia, 2014. 20(4): p. 527-534.
55. Cuesta-Barriuso, R., et al., Effectiveness of an educational intervention of Physiotherapy in parents of children with haemophilia. Haemophilia, 2014. 20(6): p. 866-872.
56. Chowdary, P., et al., Improvement in health-related quality of life in patients with haemophilia B treated with nonacog beta pegol, a new extended half-life recombinant FIX product. Haemophilia, 2016. 22(4): p. e267-e274.
57. Phadnis, S. and A. Kar, The impact of a haemophilia education intervention on the knowledge and health related quality of life of parents of Indian children with haemophilia. Haemophilia, 2017. 23(1): p. 82-88.
58. Wu, R., et al., A prospective study of health-related quality of life of boys with severe haemophilia A in China: comparing on-demand to prophylaxis treatment. Haemophilia, 2017. 23(3): p. 430-436.
59. Carcao, M., et al., Insight into health-related quality of life of young children with haemophilia B treated with long-acting nonacog beta pegol recombinant factor IX. Haemophilia, 2017. 23(3): p. E222-E224.
60. Limperg, P.F., et al., Male gender, school attendance and sports participation are positively associated with health-related quality of life in children and adolescents with congenital bleeding disorders. Haemophilia, 2018. 24(3): p. 395-404.

61. von Mackensen, S., et al., Health-related quality of life in paediatric haemophilia B patients treated with rIX-FP. Haemophilia, 2019. 25(1): p. 45-53.
62. Mancuso, M.E., et al., Health-related quality of life and caregiver burden of emicizumab in children with haemophilia A and factor VIII inhibitors-Results from the HAVEN 2 study. Haemophilia, 2020. 26(6): p. 1009-1018.
63. Bladen, M., et al., Results of feasibility and safety of randomised controlled trial of a musculoskeletal exercise intervention versus usual care for children with haemophilia. Haemophilia, 2020. 26(5): p. e223-e225.
64. Chowdary, P., et al., Long-term safety and efficacy results from the phase 3b, open-label, multicentre Continuation study of rurioctocog alfa pegol for prophylaxis in previously treated patients with severe haemophilia A. Haemophilia, 2020. 26(4): p. e168-e178.
65. Beheshtipoor, N., et al., The effect of yoga on the quality of life in the children and adolescents with haemophilia. Int. j. community nurs. midwifery, 2015. 3(2): p. 150-155.
66. Cuesta-Barriuso, R., et al., Sporting Activities and Quality of Life in Children With Hemophilia: An Observational Study. Pediatr Phys Ther, 2016. 28(4): p. 453-459.
67. García-Dasí, M., et al., Adherence to prophylaxis and quality of life in children and adolescents with severe haemophilia A. Haemophilia, 2015. 21(4): p. 458-464.
68. Polack, B., et al., EQOFIX: A combined economic and quality-of-life study of hemophilia B treatments in France. Transfusion, 2015. 55(7): p. 1787-1797.
69. Buckner, T.W., et al., Impact of hemophilia B on quality of life in affected men, women, and caregivers—Assessment of patient-reported outcomes in the B-HERO-S study. Eur J Haematol, 2018. 100(6): p. 592-602.
70. Blanchette, V.S., et al., Definitions in hemophilia: communication from the SSC of the ISTH. J Thromb Haemost, 2014. 12(11): p. 1935-9.
71. Sadler, J.E., et al., Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J Thromb Haemost, 2006. 4(10): p. 2103-14.
72. Lambert, M.P., What to do when you suspect an inherited platelet disorder. Hematology Am Soc Hematol Educ Program, 2011. 2011: p. 377-83.
73. Oldenburg, J., et al., Outcomes in children with hemophilia A with inhibitors: Results from a noninterventional study. Pediatr Blood Cancer, 2020. 67(10).
74. DiMichele, D.M. and W.E. Hathaway, Use of DDAVP in inherited and acquired platelet dysfunction. Am J Hematol, 1990. 33(1): p. 39-45.
75. Laffan, M.A., et al., The diagnosis and management of von Willebrand disease: a United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology. Br J Haematol, 2014. 167(4): p. 453-65.
76. Weyand, A.C. and P.D. James, Sexism in the management of bleeding disorders. Research and Practice in Thrombosis and Haemostasis, 2021. 5(1): p. 51-54.
77. Von Mackensen, S., Quality of life in women with bleeding disorders. Haemophilia, 2011. 17 Suppl 1: p. 33-37.
78. Arsiwala, T., et al., Measuring What Matters for Children: A Systematic Review of Frequently Used Pediatric Generic PRO Instruments. Therapeutic Innovation & Regulatory Science, 2021. 55(5): p. 1082-1095.
79. Raat, H., et al., Reliability and validity of the Infant and Toddler Quality of Life Questionnaire (ITQOL) in a general population and respiratory disease sample. Qual Life Res, 2007. 16(3): p. 445-60.
80. Wu, R., et al., Validation of the Chinese version of the Canadian Haemophilia Outcomes-Kids' Life Assessment Tool (the CHO-KLAT). Haemophilia, 2014. 20(6): p. 794-799.

81. Lambert, C., et al., Cross-cultural adaptation and validation of the Canadian Haemophilia Outcomes-Kids' Life Assessment Tool (CHO-KLAT) in Cote d'Ivoire (the Ivory Coast). Health Qual Life Outcomes, 2020. 18(1): p. 76.
82. Young, N.L., et al., Cross-cultural validation of the CHO-KLAT and HAEMO-QoL-A in Canadian French. Haemophilia, 2012. 18(3): p. 353-357.
83. Black, N., Patient reported outcome measures could help transform healthcare. Bmj, 2013. 346: p. f167.
84. Varni, J.W., C.A. Limbers, and T.M. Burwinkle, How young can children reliably and validly self-report their health-related quality of life?: an analysis of 8,591 children across age subgroups with the PedsQL 4.0 Generic Core Scales. Health Qual Life Outcomes, 2007. 5: p. 1.
85. Hullmann, S.E., et al., Measures of general pediatric quality of life: Child Health Questionnaire (CHQ), DISABKIDS Chronic Generic Measure (DCGM), KINDL-R, Pediatric Quality of Life Inventory (PedsQL) 4.0 Generic Core Scales, and Quality of My Life Questionnaire (QoML). Arthritis Care Res (Hoboken), 2011. 63 Suppl 11: p. S420-30.
86. Kenzik, K., et al., Head-to-head Comparisons of Four Legacy Pediatric Health-Related Quality of Life Instruments: A Study on Parent Proxy-Report (MT). Quality of Life Research, 2012. 20: p. 98-98.
87. van Hoorn, E.S., et al., Harmonizing patient-reported outcome measurements in inherited bleeding disorders with PROMIS. Haemophilia, 2023. 29(1): p. 357-361.
88. Julious, S.A. and S.J. Walters, Estimating effect sizes for health-related quality of life outcomes. Stat Methods Med Res, 2014. 23(5): p. 430-9.

It's on the strength of observation and reflection that one finds a way

Claude Monet (1840 – 1926 AD)

3 Bleeding symptoms, laboratory and molecular genetic results in a cohort of children with platelet disorders

E.J. Huisman
F. Atiq
C.S.B. Veen
L. van de Watering
M. Joosten
K. van Leeuwen
L. Porcelijn
M. Kruip
M. de Haas
M.H. Cnossen

Under review in European Journal of Hematology

ABSTRACT

Introduction

Diagnosing platelet disorders (PD) is challenging, especially in children. Therefore, we evaluated clinical and laboratory data to improve diagnostic algorithms, classification and follow-up.

Methods

Between 2017-2021, we performed a prospective observational cohort study in children (0-18 years), with a suspected or confirmed PD.

Results

Ninety-eight children with a mean age of 11.6 years were included. PD was categorized at study inclusion as *proven* in 44 (45%, known genetic cause); *probable* PD in 43 (44%, functional abnormalities or thrombocytopenia); and *possible* PD in 11 (11%, suspicion of PD). Comorbidities were present in 43 (44%).

Mean ISTH-BAT score was 6.1 (SD 4.0, range 0-17), with higher scores in post-pubertal females, and lower scores in children with moderate thrombocytopenia (7.6 versus 4.8, $p<0.05$). Epistaxis, hematomas, heavy menstrual and perioperative bleeding were reported most frequently. Treatment for bleeding was required for 46 (47%) children. Bleeding as reason for referral and older age were associated with abnormally increased bleeding scores.

Standard laboratory tests consisting of MPV, IPF and morphological analysis were not able to confirm final PD diagnosis. Platelet function tested by light transmission aggregometry mainly showed lower ADP, epinephrin and thrombin responses. Molecular genetic testing reclassified PD in two and newly identified genetic cause in 10/43 (23%) children from the *probable* PD group.

Conclusions

Bleeding tendency is increased in most children with a diagnosed or suspected PD, but not in all. Comorbidities are present in almost half of these children. To provide a basis for counseling and follow-up, we categorized patients according to probability and origin of diagnosis, and subsequently propose a classification based on molecular genetics and functional analysis, differentiating between inherited PD and functional PD.

Key words

Blood coagulation disorders [MESH], Child [MESH], Classification [MESH], Diagnostic Tests, Routine [MESH], Genetic testing [MESH], Hemorrhage [MESH], Platelet Function Tests [MESH]

INTRODUCTION

Platelets are essential in hemostasis, therefore inborn errors leading to quantitative and/or qualitative platelet defects may cause bleeding that can occur already from infancy or remain asymptomatic for longer periods. Both the pathophysiology behind the defect as well as the varying bleeding tendency make platelet disorders (PD) a heterogeneous disease entity.(1, 2) A referral for diagnostic screening is usually based on a combination of bleeding symptoms, family history and exclusion of other hemostatic disorders. Unfortunately, in children, the severity of bleeding caused by the PD may be difficult to judge, due to little exposure to hemostatic challenges when patients are still young.(3)

Available diagnostic tests include platelet count, platelet indices and platelet function. Platelet function can be screened by Platelet Function Analyzer® (PFA) and tested by functional tests, such as light transmission aggregometry (LTA).(4) Although LTA is considered the golden standard, it is well known that this test has several limitations, such as the inability to identify all forms of platelet dysfunction, as well as the necessity to obtain a relatively large volume of platelet rich plasma (PRP) from a blood sample acquired by venipuncture.(5, 6) This makes functional tests especially challenging in young children and in those with a PD with low platelet counts.(7) In contrast, genetic analysis is performed on blood leukocyte or easy-accessible oral mucosal swab derived DNA. The last decade, the advances made in molecular genetic analysis have significantly changed the diagnostic landscape for PD, as >100 genes involved in megakaryopoiesis and platelet function have been identified.(8) Unfortunately, a molecular genetic defect is not always detected in children suspected for PD, making PD diagnosis and counseling of patients complex, if based on clinical and laboratory information, including basic platelet function tests. Therefore, we performed a prospective observational study in children diagnosed with or suspect of PD to evaluate if we could improve diagnostic algorithms, classification and follow-up by individualizing clinical strategies.

METHODS

Patient population and study design

We conducted a prospective observational cohort study between January 1st, 2017 and May 31th, 2021 in children aged 0-18 years, treated at or referred to the Department of Pediatric Hematology of the Erasmus MC Sophia Children's Hospital (ABR NL55101.078.16). At inclusion, PD diagnosis was categorized as follows (9):

- '*Proven*' in the presence of a (likely) pathogenic variant or chromosomal abnormality associated with PD (8);
- *'Probable*' when either an abnormal LTA was found according to international guidelines or a congenital thrombocytopenia prohibiting the use of LTA;

- '*Possible*' when a persistently prolonged PFA was found with a history of increased bleeding and normal Von Willebrand factor activity (VWF:Act) or a medical history suggestive for PD (e.g. petechiae).

Exclusion criteria were: platelet transfusions or non-steroid anti-inflammatory drugs use in the last seven days, moderate-severe Von Willebrand disease defined as a VWF:Act <0.30 U/mL (10), and/or presence of an acquired thrombocytopenia (viral, drug or immune mediated). This study was subject to the Medical Research Involving Human Subjects Act and approved by the Medical Ethics Committee of the Erasmus MC University Medical Center Rotterdam. Patients were included after written parental and/or patient informed consent according to national law and regulations.

Data collection

Data was derived from electronic patient files and included medical history, bleeding symptoms, bleeding-related treatment and transfusions, as well as laboratory test results for cell count, hemostasis, and previous genetic diagnosis. Follow-up data was available in all children for a minimum of a year after study inclusion (Supplementary Figure S1).

Bleeding symptoms were scored using the bleeding assessment tool (BAT) of the International Society of Thrombosis and Hemostasis (ISTH) ,which has been validated for PD.(11) It scores 13 specified bleeding items. The item menstrual bleeding was analyzed in girls aged ≥12 years of age, being a pragmatic choice, as the date of menarche was not known for the majority of girls. Petechiae and pediatric-specific bleeds as umbilical stump bleeding, scalp bleedings, cephalohematoma, muscular bleeding after vaccination, conjunctival hemorrhage, post-circumcision bleeding and post-venipuncture or heel prick bleeding were included under 'other' bleedings. Each item scores on absence (score 0) or presence and severity of bleeding (scores 1-4). If a bleeding symptom occurred repeatedly, we scored the most severe episode. Prophylactic treatment was not scored as bleeding-related treatment. The ISTH-BAT score is the sum of all items per individual. The cut-off values for an abnormal bleeding score is a total score of ≥3 in children <18 years. (12)

Laboratory tests

At study inclusion, 30 mL of blood was drawn by venipuncture for the following analyses: cell count and platelet indices (Sysmex XN-9100, Sysmex, Netherlands), blood smear (manual), thrombopoietin levels (TPO Sanquin Diagnostic Services), hemostatic tests and genetic analysis (See Supplemental Figure S1). The closure time (CT) of the PFA-200 collagen-epinephrine (PFA-EPI), and if abnormal, also collagen-ADP (PFA-ADP; Siemens Healthcare, Germany) were measured. A platelet count of ≥80 x 10^9/L was considered adequate, but PFA was measured for the study regardless of platelet count. Von Willebrand factor antigen (VWF:Ag; in-house ELISA assay using polyclonal rabbit antihuman

VWF-antibodies, DakoCytomation, Denmark), VWF activity (VWF:Act; Innovance VWF Act-assay, Sysmex CS-5100) and factor VIII activity (FVIII:Act; one-stage APTT-based clotting assay, Sysmex CS-5100). Results were considered normal when they were within the p5-p95 interval of age-references, and for PFA, if CT was <150 seconds. For LTA (Chrono-Log 490 (Stago Benelux) the following agonists were used: collagen, U46619, thrombin before July 2019 and TRAP thereafter, ADP, epinephrin, ristocetin, arachidonic acid. Response was considered normal when activation waves reached ≥50% for weak agonists (ADP and epinephrin) and ≥70% for strong agonists, and responses were not reversible, contained no second wave and response to low ristocetin was <10%.(4) A PRP of 150 x 10^9/L was considered adequate, but LTA was performed for study intentions if PRP was ≥50 x 10^9/L.(13)

Molecular genetic diagnosis

An in-house–developed Ampliseq custom platelet panel (Thermo Fisher Scientific) was used to sequence the coding regions of 73 genes through targeted next generation sequencing (tNGS, Supplementary Table S1).(14) Library preparation and sequencing were performed according to manufacturer protocols (S5 system, Thermo Fisher Scientific), data analysis with Ion Reporter software (Thermo Fisher Scientific). Variants were classified according to guidelines of the American College of Medical Genetics and Genomics (ACMG).(15)

Statistical analysis

We used descriptive statistics to summarize clinical and laboratory characteristics of the study population. In case of a norma distribution, data are presented as mean and standard deviation (SD). In case of a skewed distribution, as median and interquartile range (IQR). Categorical data are presented as numbers with percentages and range of minimum and maximum value. Correlation of clinical and laboratory variables to bleeding and molecular testing was analyzed with univariate and multivariate analysis. P-values of <0.05 were considered significant. Statistical data analyses were performed using SPSS version 21.0 (IBM, Armonk, NY, USA). Figure icons were derived from Power Point (Microsoft 365).

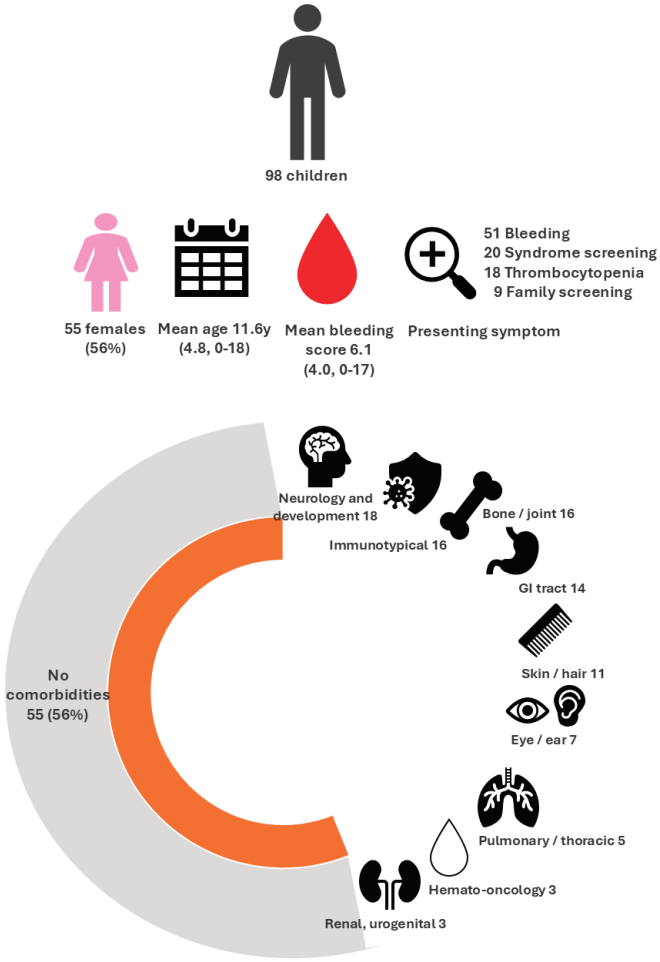
RESULTS

Patient characteristics and classification at inclusion

We included 98 children with a mean age of 11.6 years (SD 4.8, range 0-18) of whom 55 were female (56%, Figure 1). Comorbidities were present in 43 of these 98 (44%) children. Most frequently reported were developmental delays, inborn errors of immunity, or bone abnormalities (Figure 1, Supplementary Table S2). The reason for referral was atypical or excessive bleeding in the majority of cases (51/98; 52%), usually large hematomas, heavy

menstrual bleeding (HMB) and (post)surgical bleeding. In 29/98 (30%) of cases individuals were referred for hemostasis screening, either as routine screening at diagnosis of a genetic syndrome associated with a concomitant PD or due to a family history for PD.

Figure 1 Patient characteristics and bleeding scores of 98 children with platelet disorders



Abbreviations GI = gastro-intestinal, y= year(s)

Legend Mean (SD, range)

Figure 2A shows the inclusion and categorization of the study participants. Forty four of the 98 children were categorized as having *proven* PD (45%); 43 (44%) as *probable* PD of whom 34/43 (79%) had a PD based on functional abnormalities detected by LTA and nine (21%) a congenital thrombocytopenia without known genetic cause. The remaining 11 (11%) were categorized as *possible* PD based on a prolonged PFA not otherwise explained in 6/11 (55%, mean PFA-CT of 230.4 seconds, range 171 till >300) or a medical history suspect for PD in five (45%).

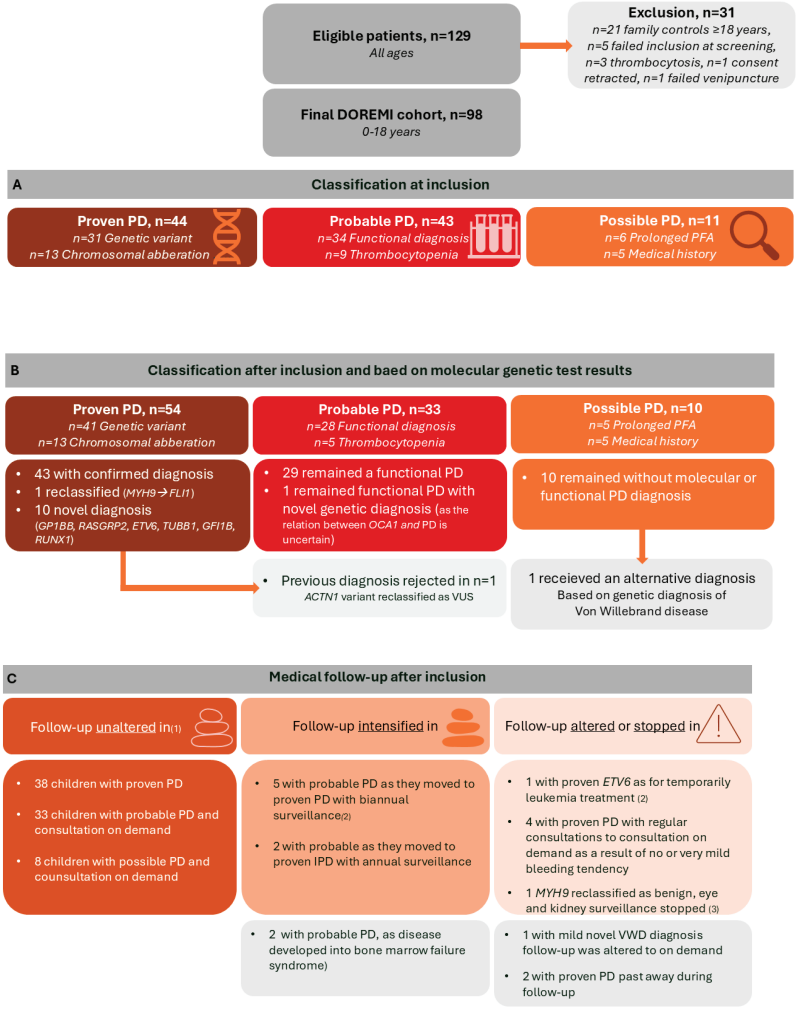
Bleeding symptoms

Overall ISTH-BAT score was 6.1 (SD 4.0, range 0-17); more specific, in the *proven* PD group 5.5 (SD 3.9, range 0-16), in the *probable* PD group 6.7 (3.9, 0-14), and in the *possible* PD group 7.6 (3.8, 3-17). A normal score of ≤2 was found for 15 children, all in the *proven or probable* PD group. Bleeding symptoms, reported by >40% of children, were mainly epistaxis, large hematomas and prolonged or severe bleeding from small wounds (Figure 3A). Less frequently mentioned were 'other' bleedings, predominantly bleeding after venipuncture. Typical PD-associated petechiae were present in 20/98 children (20%), of whom 16 (80%) had a proven PD with mutations in *WAS, MYH9, RUNX1, RASGRP2, ITGA2B,* or *RBM8A; (*sensitivity 80%, specificity 50%*).* Clinically relevant bleeding symptoms requiring surgical intervention, medication and/or blood transfusions occurred in 46/98 (47%) children (Figure 3B). These 46 children reported 70 severe bleeding incidents requiring treatment. This was mainly for severe epistaxis, (post)operative bleeding and HMB. Central nervous bleedings occurred four times in three patients, all with thrombocytopenia (*WAS* and *PTPN11*-related PD, and an unidentified cause).

Mean ISTH-BAT scores per bleeding item differed according to sex and between platelet count-subgroups (Figure 3C). Males reported epistaxis, muscle bleeds, gastro-intestinal bleedings and hematuria more frequently than females. Four of the six (67%) males who underwent a circumcision, reported bleeding after circumcision; all four had platelets ≥150 x 10^9/L. However, circumcision was uneventful in two males with *WAS*-related PD and platelets <50 x 10^9/L. In all six males, circumcision was performed before PD diagnosis, and no preoperative treatment had been given. For females, HMB was reported by 28 of 37 females ≥12 years (76%), who had either platelets <50 x 10^9/L or ≥150 x 10^9/L (p=0.049). Females ≥12 years had higher mean bleeding scores than boys ≥12 years of age, although not significantly different (3.9 vs 7.1, p=0.149). This difference was predominantly due to presence of HMB (Figure 3C). Based on platelet count, a lower score of 4.8 (SD 3.9, p=0.037) was observed for the children with platelet counts of 50-150 x 10^9/L in comparison to children with either platelet counts <50 x 10^9/L (score 6.5, SD 4.1) or ≥150 x 10^9/L (score 6.3, SD 3.9). Postoperative bleeding occurred more frequently in the subgroup with platelets ≥150 x 10^9/L compared to <150 x 10^9/L: respectively 59% versus 38%, although not significantly different (p=0.08). Older age (p=0.001) and bleeding as

presenting symptoms (p<0.001, Table 1) were significantly correlated to significantly higher bleeding scores.

Figure 2 Flowchart inclusion, results of molecular tests and impact on follow-up

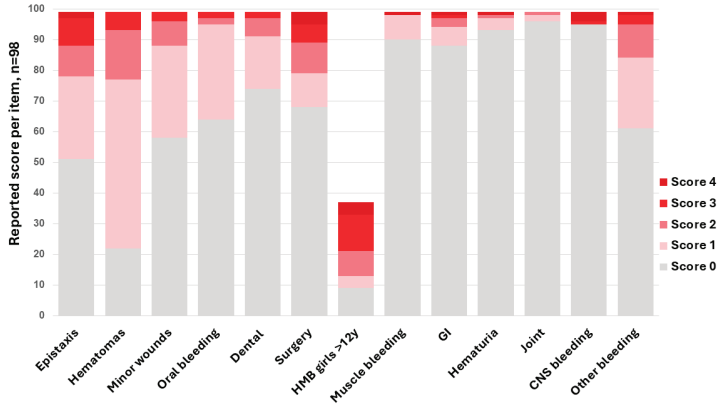


Abbreviations AD = autosomal dominant inheritance, AR = autosomal recessive inheritance, BSS = Bernard-Soulier syndrome, del = deletion, dupl = duplication, HPS2 = Hermansky-Pudlak syndrome type 2, gene *AP3B1*, LTA = light transmission aggregometry, MYH9-RD = MYH9 related disorders, nos = not otherwise specified, n = number, OCA1 = ocular-albinism syndrome type 1, PD = platelet disorder, PFA = Platelet function analyzer, TAR = thrombocytopenia with absent radius, VWD = Von Willebrand disease, VUS = variant of unknown significance, XLT = x-linked thrombocytopenia

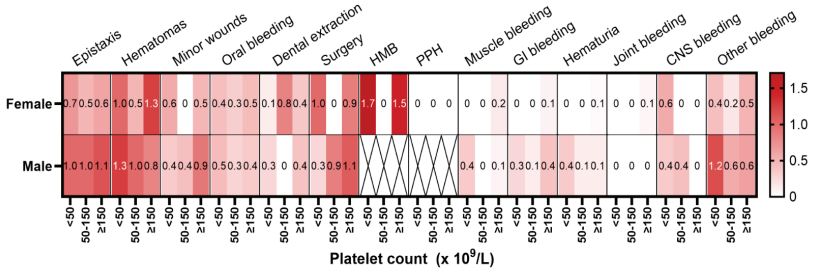
Legend (1) frequency varies from once every year to every 5 years; (2) one *ETV6*-patient mentioned both in the 'visits intensified' group as well as 'visits temporarily ended' group; (3) one patient with formerly diagnosis of PD by pathogenic variant in MYH9-RD was now reclassified as *FLI1* PD. The former variant in *MYH9* was years later reclassified as class 2

Figure 3 Bleeding symptoms in children with platelet defects, scored by ISTH bleeding assessment tool

A



B



Abbreviations CNS = central nervous system bleeding, ENT = ear-nose-throat, GI = gastro-intestinal bleeding, HMB = heavy menstrual bleeding, PPH = postpartum hemorrhage, y = years

Legend (A) Bleeding score is assessed using the ISTH bleeding assessment tool, HMB illustrated in subgroup of 37 females aged 12 years or older, PPH is left out as none of the female patients <18 years had been pregnant, (B) Platelets ≥150 (x 10^9/L) are seen in 39 females, 22 males; 50-150 platelets in 7 females, 11 males; <50 platelets in 9 females and 10 males.

Laboratory results

Overall, the mean platelet count was 210 x 10^9/L (SD 110, range 6-481; Table 2). Of the 98 children, 61 (62%) had a functional defect with normal platelet count. The remaining 37 had a thrombocytopenic PD with or without platelet dysfunction. Mean platelets numbers at inclusion were 74 x 10^9/L (SD 39.8, range 6-141). Of these 37, longitudinal data were available for 34 (92%), showing a median platelet nadir of 30 x 10^9/L (IQR 57, 0-126), that occurred most frequently at neonatal age.

Table 1 Univariate and multivariate model of factors associated with higher bleeding scores

Factor	Beta	95% CI	P-value
Patient characteristics			
Female sex	0.50	-1.012 to 2.12	0.539
Comorbidities	0.29	-1.48 to 2.06	0.743
Age			
Continuous variable, per year	**0.27**	**0.11 to 0.43**	**0.001**
< 12 years vs ≥ 12 years	**2.62**	**1.04 to 4.19**	**0.001**
Reason for referral			
Bleeding as presenting symptom	**2.77**	**1.26 to 4.28**	**<0.001**
Abnormal LTA	0.88	-0.77 to 2.52	0.292
Thrombocytopenia	-0.72	-2.95 to 1.51	0.524
Prolonged PFA	-0.92	-4.05 to 2.20	0.559
Medical History	0.12	-2.34 to 2.58	0.922
Final PD categorization			
Proven	-1.42	-3.01 to 0.170	0.080
Probable	0.82	-0.867 to 2.50	0.338
Possible	1.68	-0.85 to 4.21	0.190
Laboratory results			
TPO	-0.02	-0.03 to 0.01	0.137
Leucocytes lowest	**-0.36**	**-0.69 to -0.02**	**0.037**
Leucocytes highest	0.01	-0.16 to 0.17	0.954
Platelet lowest	0.00	-0.01 to 0.01	0.869
Platelet mean	0.00	-0.01 to 0.01	0.784
Platelet highest	0.00	-0.01 to 0.01	0.842
Lowest platelet groups			
<50	0.44	-1.64 to 2.52	0.677
50-100	0.56	-2.80 to 3.92	0.742
100-150	**-2.47**	**-4.79 to -0.15**	**0.037**
≥150	0.79	-0.86 to 2.45	0.343
Abnormal blood smear	0.15	-1.97 to 2.27	0.885
LTA abnormalities	1.23	-0.74 to 3.20	0.217
Number of abnormal LTA agonists	0.41	-0.03 to 0.85	0.068

Legend Linear Regression analysis: outcome ISTH-BAT score (continuous variable)

Table 1 Multivariate model of factors associated with higher bleeding scores Included (*continued*)

Factor	***Beta***	***95% CI***	***P-value***
Age (continuous variable, py)	0.18	0.02 to 0.34	0.031
Bleeding as presenting symptom	2.57	1.03 to 4.11	0.001

Table 1 Excluded Multivariate model of factors associated with higher bleeding scores Included (*continued*)

Factor	*Beta*	*95% CI*	*P-value*
Lowest Leucocytes	-0.14		0.165
Lowest platelets in the 100-150 range	-0.13		0.200

Abbreviations CI = confidence interval, LTA = Light transmission aggregometry, PD = platelet disorder, PFA = Platelet function analyzer, py = per year, TPO = thrombopoietin, U = unit

Legend Multiple Regression analysis: outcome ISTH-BAT score (continuous variable), forward approach

Table 2 Patient characteristics and laboratory result of hemostatic tests and platelet indices

Laboratory test	**Results**
Platelet measurement, n=98	
Mean platelet count (SD, range; x10^9/L)	209.8 (109.7, 6 - 481)
in PFD, n=61	266.9 (151 - 481)
in CTP, n=37	74 (39.8, 6 - 141)
Platelets <50 x 10^9/L, n=18	23.47 (13.0, 0-48)
Platelets 50-150 x 10^9/L, n=19	106.54 (28.46, 50-145)
Mean MPV, n=89[1], (SD, range, fL)	10.6 (1.36, 6.3 - 13.3)
MPV <8, n (%)	2 (2.0)
MPV ≥12, n (%)	16 (16.3)
Median IPF (IQR, range; %), n=40[1]	8 (9, 0 - 76)
Median TPO (IQR, range; U/mL), n=95	12 (13, 4 -310)
TPO ≥34, n=14	83.9 (31.3)
Median PFA-EPI (sec), n=75[1] (IQR)	152.0 (110, 83 - >300)
Abnormal PFA-EPI ≥150 sec, n=38	235.0 (51.5)
In platelets <50 x 10^9/L, n=18	190.7 (48.6, 115 - 278)
In platelets 50-150 x 10^9/L, n=19	183.0 (77.7, 83 - >300)
In platelets ≥150 x 10^9/L, n= 61	170.7 (72.0, 84 - >300)
Median PFA-ADP (sec), n=38[2]	154.0 (131.9, 80 - >300)
Abnormal blood smear, n=67	**n (%)**
Abnormalities reported	48 (49%)
Anisocytosis of platelets, n	22
Hypogranulation of platelets	12
Macroplatelets	19
Microplatelets	2
Döhle-like inclusions in neutrophils and eosinophils	3
Abnormal or hypogranulation of neutrophils	7
Howell-Jolly bodies or vacuoles in white blood cells	4
Elliptocytosis or poikilocytosis of red blood cells	9

Table 2 Patient characteristics and laboratory result of hemostatic tests and platelet indices (*continued*)

Laboratory test	Results
LTA results, n=84[3]	n (%)
Abnormal LTA	62 (73.8%)
Abnormal agonists, n	
ADP	43
Epinephrin	33
TRAP/thrombin	25
Arachidonic acid	13
U46619	7
Ristocetin	6
Collagen	6
Range of abnormal agonists	
1-3	48
4-6	10
7-9	4
Additional coagulation factor deficiencies, n=91	Mean (range)
Von Willebrand disease[4], n=14 (U/mL)	0.47 (0.34 - 0.57)
Other factor deficiencies[5]	
Factor VII, n=1	0.49
Factor IX, n=2	0.34, 0.40
Factor XI, n=3	0.38 (0.26 - 0.45)
Factor XIII, n=1	0.45
Fibrinogen, n=3 (g/dL)	1.05 (0.60 - 1.40)
Combinations[7], n	
Mild type 1 VWD + FXI or + FXIII	3
FIX + FXI or FIX and fibrinogen	2

Abbreviations Act = activity, ADP = adenosine diphosphate, Ag = antigen, F = Factor, fL = femtoliter, IPD = Inherited Platelet Disorder, L = liter, LTA = Light Transmission Aggregometry, mL = milliliter, PFA = Platelet Function Analyzer, U = unit, VWD = Von Willebrand Disease, VWF = Von Willebrand Factor

Legend (1) missing values for PFA-EPI in n=23, (2) PFA-ADP was only performed when PFA-EPI was abnormal, (3) LTA was not possible due to PRP <50 x 10^9/L as a result of severe thrombocytopenia in n=13, missing value in n=1. PRP was between 100-150 x 10^9/L in 4 of 84 children, (4) Low VWF defined as VWF:Act between 0.30-0.60 U/mL, (5) defined as Factor activity <0.5 U/mL, (6) defined as serum fibrinogen <1.5 g/dL), (7) occurred 4x in patients with Noonan syndrome (3x *PTNN11*, once *RIT1*-mediated) and once in patient with terminal 11q deletion syndrome.

Concerning platelet indices, a low MPV was only seen in two children with an MPV of respectively 6.3 and 7.8 fL. Although considered a hallmark for *WAS*, a low MPV was only present in one of four (25%) children with *WAS*-related PD. High MPV was found in 11 of the 16 thrombocytopenic PD (69%), with a mean of 12.6 fL (SD 0.34; 12.0-13.3).

Decreased IPF ≤1% was seen in all four -*AS*-mediated PD and in one PD as part of Hermansky-Pudlak syndrome type 2. Increased IPF (median 11, IQR 18.3, 8-76%) was seen in

all patients with thrombocytopenia. Significantly increased IPF ≥30% was seen in all three children with *MYH9*-related diseases and one with Bernard-Soulier syndrome.

Blood smear abnormalities were relatively frequent (48/67, 72%) and involved platelet abnormalities, as well as additional white and red blood cells abnormalities in 15 children (31%). In six children (13%) the blood smear hinted to PD diagnose by showing: strongly increased numbers of giant platelets in Bernard-Soulier syndrome,(16) multiple hypogranulated platelets in *GFI1B* grey-platelet syndrome,(17) Döhle-like neutrophil inclusions in *MYH9*-related PD.(18) Only one patient (*TUBB1*-related PD) had a medical history for elliptocytosis without anemia.

For platelet function testing, median PFA was 152 sec (range 83 till >300). In children with platelet counts <50 x 10^9/L only marginally prolonged PFA (190.7 sec) were seen in comparison to samples of children with normal platelet counts (170.7 sec), however variability was large. When we tested the correlation of an abnormal bleeding score with the higher cut-off of a PFA ≥200 sec we correctly identified all PD children in this cohort, LTA was abnormal in 62 (74%) children, most frequently for one till three of the following agonists: ADP, epinephrin, TRAP or arachidonic acid.

TPO levels were elevated in 14 children, of whom 12 (86%) had a proven PD with mutations in either *RBM8A, WAS, PTPN11, FLI1, OCA1, FLNA* or terminal 11q deletion). Elevated TPOs > 200 U/mL (respectively 247 and 310 U/mL) were measured in two patients with thrombocytopenia who developed bone marrow failure during follow-up. An elevated TPO level (≥34 U/mL) showed some correlation with proven PD (sensitivity 86%, but specificity 50%).

Concomitant mild type 1 Von Willebrand disease (VWD) was found in 14 (14%) children (mean VWF:Act 0.47 U/mL). Ten other (combinations of) additional mild coagulation factor deficiencies were seen as well in eight children, of whom six (75%) were diagnosed with Noonan syndrome.

Table 1 shows that none of the laboratory tests correlated with increased bleeding scores in multivariate analysis.

Molecular genetic test results and impact on follow-up

Variants or chromosomal aberrations, confirmed or newly identified in this study, are listed and classified in Supplementary Table S3. Genetic diagnosis was already known in 44 children before inclusion (Figure 2A). The routine use of the tNGS panel resulted in a novel diagnosis in 10, all coming from the 'probable PD' group, reducing this group to 33 children (Figure 2B). Of the now 54 children with proven PD (55%), 23 (42%) had a throm-

bocytopenic PD, representing 79% of all thrombocytopenic PD. The remaining 31 had a functional PD, of which 24 had a syndrome-associated PD (SAPD, 77%, See Table 2B).

In five cases the genetic test outcome impacted the follow-up as the diagnosis of the children changed. First, reclassification of former diagnoses was done three times: a previous diagnosis of *MYH9*-related PD was reclassified to *FLI1*-related PD fitting the phenotype with congenital thrombocytopenia and alopecia. (19) As a result, kidney and ophthalmological surveillance were no longer needed. Also, a clinical diagnosis of Hermansky-Pudlak syndrome was reclassified in ocular-albinism syndrome 1 (OCA1) after referral to a clinical geneticist revealed a homozygous likely pathogenic variant in *TYR*. The role of *TYR* in platelet disorders has not been reported before; and the third had a clinical diagnosis of Glanzmann Thrombasthenia based on LTA pattern, but genetic diagnosis showed homozygous pathogenic variants in *RASGRP2* leading to CalDAG-GEFI deficiency.(20) Secondly, a previous given diagnosis of *ACTN1*-related PD was withdrawn, as it was now reclassified as likely benign (class 2). The patient was reclassified as probable PD based on congenital thrombocytopenia. Additional genetic analysis including open whole-exome sequencing followed by RNA-analysis did not lead to an alternative diagnosis. Lastly, for one patient the diagnosis of PD was changed into VWD since a likely pathogenic variant in *VWF* was found and a nadir of Von VWF:Activity of 0.41 U/mL in follow-up testing despite normal values before and at inclusion.

Table 3 Univariate and multivariate model of factors associated with an identified genetic variant or chromosomal defect for the platelet disorder

Factor	OR	95% CI	P-value
Patient characteristics			
Female sex	0.45	0.20 to1.01	0.054
Comorbidities	**3.15**	**1.29 to 7.68**	**0.012**
Age			
Continuous variable, per year	**0.81**	**0.73 to 0.90**	**<0.001**
< 12 years vs ≥ 12 years	**0.65**	**2.19 to 14.3**	**<0.001**
ISTH-BAT score	0.93	0.84 to 1.03	0.181
Reason for referral			
Bleeding as presenting symptom	**0.11**	**0.05 to 0.28**	**<0.001**
Abnormal LTA	0.78	0.34 to 1.76	0.547
Thrombocytopenia	1.86	0.59 to 5.92	0.293
Prolonged PFA	0.31	0.06 to 1.70	0.179
Medical History	0.83	0.25 to 2.78	0.762
Laboratory results			
TPO	1.00	0.99 to 1.01	0.983
Leucocyte count	**1.24**	**1.01 to 1.52**	**0.040**

Investigating the association between clinical and laboratory test on a definite genetic diagnosis showed that bleeding as presenting symptom (p<0.001), and abnormal response on TRAP in the LTA (p<0.001) were correlated (Table 3).

Table 3 Univariate and multivariate model of factors associated with an identified genetic variant or chromosomal defect for the platelet disorder (*continued*)

Factor	OR	CI	P-value
MCV	**0.93**	**0.87 to 0.99**	**0.029**
Lowest platelet count measured	**0.99**	**0.99 to 1.00**	**<0.001**
Highest platelet count measured	**1.00**	**1.00 to 1.00**	**0.019**
Platelet counts <50	**5.53**	**1.48 to 20.58**	**0.011**
Platelet counts 50-100	4.58	0.52 to 40.78	0.172
Platelet counts 100-150	2.10	0.60 to 7.33	0.247
Platelet counts ≥150	**0.18**	**0.07 to 0.46**	**<0.001**
MCHC	**2.11**	**1.08 to 4.13**	**0.028**
MPV	**0.30**	**0.10 to 0.93**	**0.036**
Abnormal blood smear	0.63	0.21 to 1.89	0.413
PFA EPI 200	**2.86**	**1.04 to 7.89**	**0.043**
Repeatedly abnormal PFA-EPI	**1.02**	**1.01 to 1.04**	**<0.001**
PFA ADP	**1.01**	**1.00 to 1.02**	**0.028**
An LTA abnormality	1.69	0.63 to 4.53	0.294
Specific LTA agonist thrombin or TRAP	**7.20**	**1.88 to 27.51**	**0.004**
Specific LTA agonist ADP 10mcM	**0.20**	**0.04 to 0.91**	**0.038**
Number of abnormal LTA agonists	**1.31**	**1.01 to 1.69**	**0.041**

Legend Logistic Regression analysis: outcome (post) proven IPD (categorical variable)

Table 3 Multivariate model of factors associated with proven PD
All dependent variables (*continued*)

Factor	P-value
Age at inclusion, py	1.000
Comorbidities	0.999
Bleeding as presenting symptom	**<0.001**
Leucocytes lowest	0.999
Abnormal MCV	1.000
Platelet lowest	0.998
MPV	**0.002**
Repeatedly abnormal PFA-EPI	1.000
Abnormal thrombin/TRAP response	**<0.001**
Number of abnormal agonists	**0.010**

Table 3 Included (*continued*)

Factor	OR	95% CI	P-value
Bleeding as presenting symptom	**0.10**	**0.03 to 0.33**	**<0.001**
MPV	0.29	0.06 to 1.35	0.115
Abnormal thrombin response in LTA	**9.63**	**1.40 to 66.50**	**0.022**
Number of abnormal agonists	0.93	0.61 to 1.42	0.734

Abbreviations CI = confidence interval, LTA = Light transmission aggregometry, MCHC = Mean Corpuscular Hemoglobin Concentration, MCV = mean corpuscular volume, MPV = mean platelet volume, OR = Odds ratio, PD = platelet disorder, PFA (ADP or EPI) = Platelet function analyzer (adenosine diphosphate or epinephrin), py = per year, TPO = thrombopoietin, U = unit

Legend Multiple Regression analysis: outcome (post) proven IPD (categorical variable), forward approach

Clinical follow up

The final diagnosis of proven PD had an impact on the clinical follow-up in a proportion of children (Figure 2C). This implied for example intensification of follow-up if there was a risk on development of hematological malignancy.(19, 21, 22) On the other hand, regular follow-up was changed to on demand or even stopped when only mild or no bleeding tendency was present. Or additional screening and surveillance were stopped, such as kidney and ophthalmic screening in *MYH9*-related PD.

Clinical follow up showed a disease or treatment-related event in a small proportion of children. Three children developed platelet alloimmunization with antibodies directed against Human Leukocyte Antigens (2/3, HLA) or Human Platelet Antigen (HPA). Two children were treated for pre-B acute lymphatic leukemia, one with *ETV6*-related PD at age 15 years and one child with unexplained thrombocytopenic PD at age 5. There were no serious bleeding events during chemotherapy in the child with *ETV*-related PD, but the second child developed intracranial bleeding. Both patients fully recovered. Two children progressed to multiple lineage cytopenias during follow-up and were reclassified as bone marrow failure syndrome. In these two children no genetic cause was found. Two children died during follow-up with sudden death (terminal 11q deletion) and cardiac failure cardiomyopathy and lung disease (*FLNA)*.

DISCUSSION

We demonstrated that the presentation and course of disease in children diagnosed with PD is heterogeneous with respect to platelet counts and bleeding symptoms. In the majority of children with PD, clinically relevant bleeding occurs, however with varying levels of severity. The pattern of bleeding strongly resembles that seen in children with VWD.(23-25) Bleeding in children with PD resulted in the need for medical consultation,

treatment or transfusions in nearly 50% of our study population. Factors associated with more serious bleeding events and/or treatment, were associated with bleeding as reason for referral and older age, most probably due to an increased exposure to hemostatic challenges such as medical procedures or presence of menstrual cycles. We identified or confirmed a molecular genetic cause in half of our cohort. The genetic variants found impacted platelet production and/or function and/or function in 40% of our cohort comorbidities were present, affecting other organs and tissues. We demonstrated that an identified genetic cause helps to classify subgroups and affects counseling and follow-up strategies. Therefore, our data underlines the importance of early (molecular genetic) diagnosis and a better classification of PD subgroups.

Using these study results to improve our diagnostic algorithm and follow-up, we propose the following regarding the diagnostic work-up of a child suspected of PD: we first advise performing a clinical examination and assessment of bleeding phenotype. The presence of comorbidities may suggest a specific PD subgroup. Furthermore, family history and sex of affected family members should be taken into account as for example, bleeding symptoms in only male family members can point to an X-linked PD. Sex-related bleeding symptoms in males are postcircumcision bleeding, higher risk on epistaxis, bleeding from small wounds and gastro-intestinal bleeding to a lesser extent. This has not been previously observed in male PD patients, but has been reported in males with VWD.(26, 27) For postpubertal females with PD, we demonstrated that HMB may be the first symptom of PD but also that previous PD diagnosis results in a higher risk on developing HMB later in life. HMB resulted in the need for treatment in >70% of females. HMB as first symptom of PD may explain why females are slightly overrepresented in our and previous cohorts, ranging from 55-61%.(2, 5, 28-32) In line with previous reports (2), our data shows that counseling of young girls with PD is of major importance, especially HMB may be a risk factor for future postpartum bleeding.(2) Although the presence of petechiae, is considered to be a hallmark of PD, these were not often present in our cohort (20%), but strikingly often related to an underlying genetic cause of PD. Serious bleedings such as CNS bleedings were rare (3%), and similar to results from two other studies in PD in which respectively 5/410 children (1.3%) and 2 of 96 (2%) adults were affected.(2,5) However, the occurrence of CNS bleedings was lower than the 10% found in a cohort of 174 patients with only *WAS*-related PD.(33)

The second step is measuring platelet counts and platelet function to assess if there may be a quantitative or qualitative defect. As demonstrated, mild to moderate thrombocytopenia does not necessarily lead to an increased bleeding tendency. This may be due to the fact that some PDs presenting with low platelet counts are more a quantitative than a qualitative disorder, but it is also possible that early recognition of thrombocytopenia in a patient may result in a lower threshold for prophylactic measures, in perioperative settings (SPATA trial), which consequently prevents excessive bleeding.(34, 35)

We observed that MPV, IPF and platelet morphology may be suggestive of a specific PD subgroup such as *MYH9* or *WAS*-related PD, but do not lead to a definite diagnosis. For example, we confirmed the relation between Döhle-like inclusions and *MYH9*-related PD, and observed that very low IPFs (0-1%) occurred in *WAS*-related PD and very high IPFs >30% in *MYH9*-related PD. However, more research is needed to further confirm these associations.

To measure platelet function, we found that PFA is insufficient as a diagnostic tool when the standardized cut-off value of >150 sec is used. Only in cases of a clearly prolonged PFA ≥200 sec the correlation with a diagnosis of PD improves, as reported in other studies.(3) In our cohort, patients with a prolonged PFA in absence of VWD were included in the '*probable*' PD group. However, in none of these patients a mutation was detected in any of the analyzed genes by our tNGS panel. This makes PD suspicion based on only a prolonged PFA debatable. In our opinion, for a probable PD, both prolonged PFA and an aberrant LTA would be necessary. In a similar 'PFA only' group, where whole-blood thrombus formation under shear was investigated, prolonged PFA seemed to be the effect of a combination of low-normal VWF and low-normal platelet aggregation. This shear-dependent platelet function defect was not detected by conventional hemostatic tests. (36) Until more specific techniques for testing are available, we suggest to reclassify this group to bleeding disorder of unknown cause or to classify it as BDUC with a prolonged PFA (BDUC, or even more specific, BDUC-PFA).

For LTA, we demonstrated that an increasing number of abnormal agonist responses are associated with PD diagnosis, as well as abnormal thrombin response. This finding however, may be due to the subgroups of patients with 11q aberrations included in the study, as these patients often present with thrombin abnormalities on LTA.(34) We underline that LTA testing remains essential to diagnose functional defects, despite its limitations. Besides the classically distinctive patterns identified by LTA for Bernard-Soulier syndrome (abnormal ristocetin) and Glanzmann Thrombasthenia or *RASGRP2*-related PD (only ristocetin normal), it does not suffice to identify PD subgroups. Unfortunately, in our cohort LTA application was only possible in ~10% of children due to either very young age or severe thrombocytopenia. Future directions for research should focus on new functional tests needing low blood volumes such as flowcytometry (36-38), or investigate platelet function from a different perspective with new or current diagnostic tools, such validation studies applying lower PRP, ATP/ADP ratio or mepacrine for granule defects.(13, 37, 39-41)

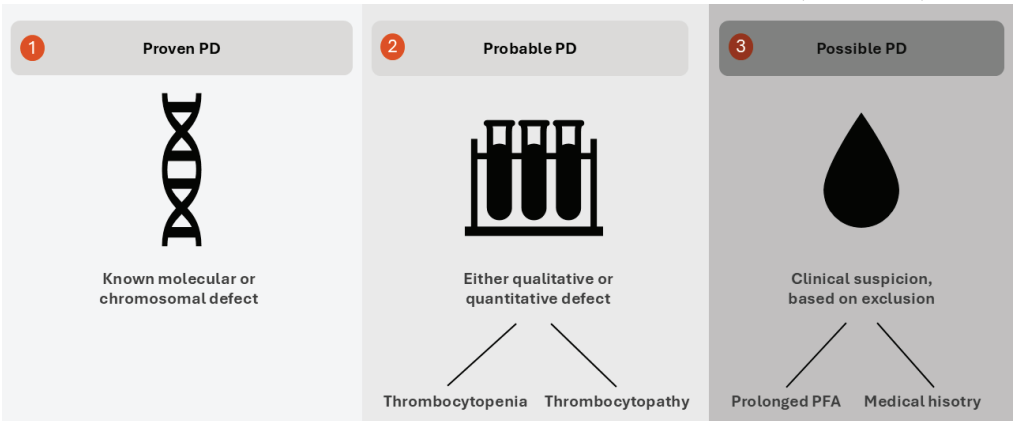
The third step that should be performed is molecular genetic testing. This information is of great value for counseling and follow-up.(3) Although we found a high incidence of 50% of genetic PD diagnoses in our study population, we doubt if this is exemplary of the a priori chance to find a genetic cause in PD patients in general. This is due to the fact that

a known genetic cause was a reason for study inclusion (proven PD). Furthermore, our study was performed in a tertiary care setting with expert care for children with Noonan and 11q-disorders with associated PD.(34, 42) In order to estimate the added value of molecular genetic testing in diagnostic settings, we narrowed the incidence down to the 54 children without known genetic cause at inclusion. In these children, 10 novel diagnoses were made (19%) in children with thrombocytopenic PD and functional PD while using a tNGS of 73 genes. This incidence resembles previous incidences of 17% (5/30 children with thrombocytopenic PD using whole exome sequencing (WES) with a preset filter of PD genes) (43), and 18% (10/56, using WES) in adults with a functional PD, but only 4% (2/22) in patients with thrombocytopenia. To note, this incidence is 31% when the subgroup of Glanzmann Thrombasthenia and Bernard-Soulier patients is not left out of this cohort, n=30/96), as these are relatively over-represented in this paper.(5) However, our incidence is in contrast with the UK-GAPP study where a genetic diagnosis was found in 17/37 (46%) of patients with thrombocytopenic PD using open WES, indicating also that inclusion criteria are of importance.(44) Besides inclusion criteria, choice of molecular techniques are also relevant. We used a tNGS panel of 73 genes, limiting the results, as currently >150 PD related genes are discovered and more will follow. Other molecular techniques, such as WES or whole genome sequencing (WGS) may be superior, as identification panels can be more easily updated to new insights and the addition of CNV analysis to both techniques as well as intron analysis in WGS combines even more potential. Lastly, one needs to keep in mind that a molecular diagnosis may be wrong in hindsight, as more and more data from 'healthy populations' become available in population-based databases, leading to a better understanding and categorization of (single nucleotide) variants. We detected two such cases in our cohort (Table 2B). We advise performing genetic analysis and to thoroughly review the method used if no genetic variation is identified and to continue testing if a genetic cause is strongly suspected.

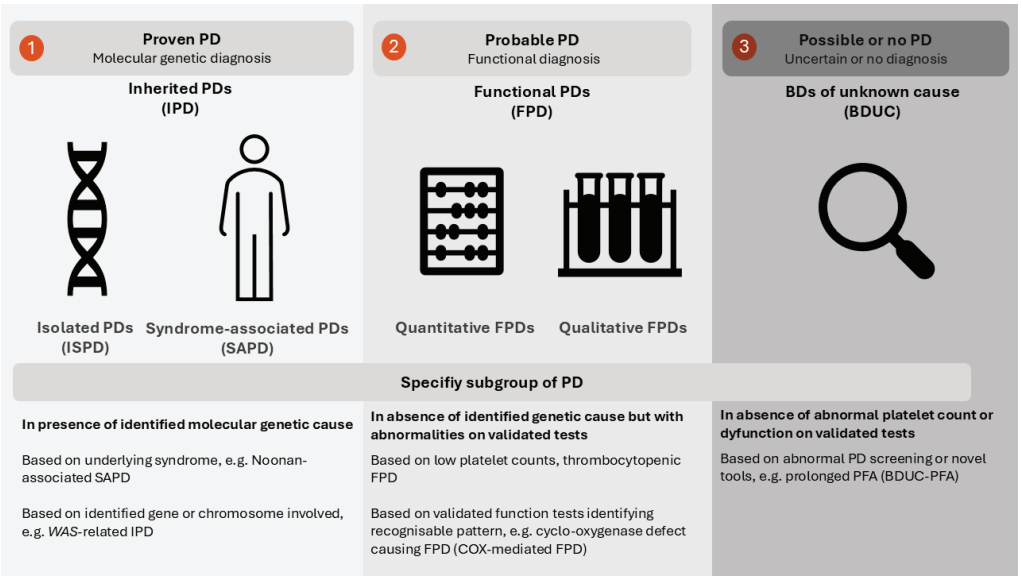
Based on the above, we propose a new PD classification, that will replace our study classification that categorized patients with PD in the three subgroups 'proven', 'probable', or 'possible', see Figure 4A. Based on our findings, we propose a classification that is based on molecular findings and functional analysis and discriminates two main PD groups and a rest group: first, the inherited PD (IPD) group in which a molecular genetic cause is identified, and second, the functional PD (FPD) group in which no such genetic cause is identified but PD diagnosis is based on quantitative defect (thrombocytopenic FPD) or qualitative defect (thrombocytopathic FPD), see Figure 4B. We also propose further categorize the main diagnoses into subgroups as this may impact counseling and follow-up. For example, in IPDs, some mutations will lead to an isolated defect leading to problems in the hematopoietic tract only, for example PDs predisposing for hematological malignancies (isolated PD, ISPD) or will lead to a broad range of defects in which PD is part of a syndrome (syndrome-associated PD; SAPD).

Further specification can be given by adding the gene involved or syndrome associated with PD, e.g. *RUNX1*-regulated IPD or Noonan-associated SAPD. In line with this, subgroups can be made for the FPD, the second main group of PD, by adding the functional mechanism involved, e.g. SPD or COX-mediated FPD in case of a storage pool or cyclo-oxygenase defect. This classification can guide physicians in the diagnostic trajectory and in tailored counseling and surveillance as both phenotype and genotype can be taken into account.

Figure 4 Used and proposed classification of platelet disorders based on genotype-phenotype



A



B

Abbreviations BDUC = bleeding disorders of unknown cause, BDUC-PFA = with prolonged PFA of unknown cause, COX = cyclo-oxygenase defects, FPD = functional platelet disorder, IPD = inherited platelet disorder, ISPD = isolated platelet disorder, PD = platelet disorder, PFA = Platelet function analyzer, SAPD = syndrome-associated platelet disorder

Our study has several limitations. First of all, although we studied a relatively large pediatric cohort, the underlying heterogeneity of PD may have negatively impacted the results of reported incidences as well as sensitivity or specificity of analyzed laboratory tools. Future research in larger cohorts is required. The tertiary setting of our cohort may have also impacted incidence numbers of comorbidities and identified genetic causes. Furthermore, there are missing values for some of our test results, frequently the result of technical requirements is too difficult to meet in young or thrombocytopenic patients. Finally, although validated for PD, the use of bleeding scores in general in childhood has limitations, as it only scores the bleeding event that needed the most treatment. This does not predict the chances of a future bleeding.(11) Nor does the ISTH-BAT score take repetitive bleedings into account, while a frequent recurrence of bleeding complications may negatively impact the disease burden, as impact is based on both severity (measured) and frequency of symptoms (not-measured). This limits the use of bleedings scores in assessing future bleeding risks.(45)

In conclusion, we observed that bleeding tendency is increased in most children with a diagnosed or suspected PD, but not in all. Comorbidities are present in almost half of these children, and genetic results can predispose for hematological malignancies in a small proportion. To provide a basis for counseling and follow up, we categorized patients according to probability and origin of diagnosis, and subsequently propose a classification based on routinely performed molecular genetics and functional analysis, differentiating between inherited PD (IPD) and functional PD (FPD) as two main groups. Within these two main groups, we advocate to annotate additional information to highlight the fact that PDs can be part of a syndrome (SAPD) or isolated (ISPD) or result from platelet dysfunctions impacting platelet number or specific functional pathways (e.g. COX-mediated FPD). Such a classification can serve as a base for more individualized counseling and treatment and enables pooling of subgroups in future research.

ACKNOWLEDGMENTS

We thank Resi Spaendonk and Berna Beverloo for their assistance in DNA variant classification.

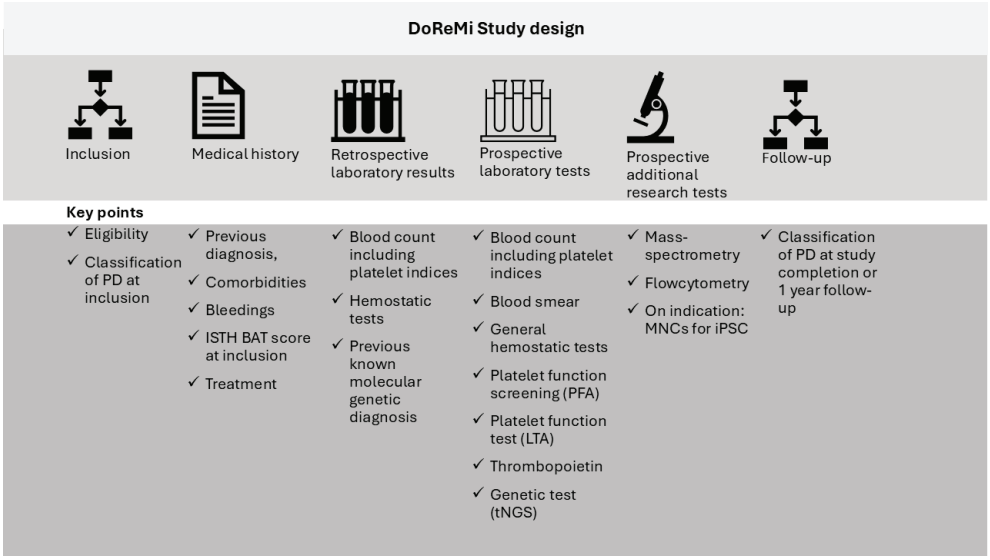
REFERENCES

1. Palma-Barqueros V, Revilla N, Sanchez A, Zamora Canovas A, Rodriguez-Alen A, Marin-Quilez A, et al. Inherited Platelet Disorders: An Updated Overview. Int J Mol Sci. 2021;22(9).
2. Gresele P, Orsini S, Noris P, Falcinelli E, Alessi MC, Bury L, et al. Validation of the ISTH/SSC bleeding assessment tool for inherited platelet disorders: A communication from the Platelet Physiology SSC. J Thromb Haemost. 2020;18(3):732-9.
3. Alhaj D, Hagedorn N, Cuntz F, Reschke M, Schuldes J, Ruthenberg J, et al. ISTH bleeding assessment tool and platelet function analyzer in children with mild inherited platelet function disorders. Eur J Haematol. 2024;113(1):54-65.
4. Blaauwgeers MW, Kruip M, Beckers EAM, Coppens M, Eikenboom J, van Galen KPM, et al. Bleeding phenotype and diagnostic characterization of patients with congenital platelet defects. Am J Hematol. 2020.
5. Israels SJ, Kahr WH, Blanchette VS, Luban NL, Rivard GE, Rand ML. Platelet disorders in children: A diagnostic approach. Pediatr Blood Cancer. 2011;56(6):975-83.
6. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. Journal of Thrombosis and Haemostasis. 2013;11(6):1183-9.
7. Lentaigne C, Freson K, Laffan MA, Turro E, Ouwehand WH, Consortium B-B, the ThromboGenomics C. Inherited platelet disorders: toward DNA-based diagnosis. Blood. 2016;127(23):2814-23.
8. Alessi MC, Sie P, Payrastre B. Strengths and Weaknesses of Light Transmission Aggregometry in Diagnosing Hereditary Platelet Function Disorders. J Clin Med. 2020;9(3).
9. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis. 2008;46(12):1813-21.
10. James PD, Connell NT, Ameer B, Di Paola J, Eikenboom J, Giraud N, et al. ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. Blood Advances. 2021;5(1):280-300.
11. Adler M, Kaufmann J, Alberio L, Nagler M. Diagnostic utility of the ISTH bleeding assessment tool in patients with suspected platelet function disorders. J Thromb Haemost. 2019;17(7):1104-12.
12. Elbatarny M, Mollah S, Grabell J, Bae S, Deforest M, Tuttle A, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. Haemophilia. 2014;20(6):831-5.
13. https://www.sanquin.org/products-and-services/molecular-diagnostics-ngs/available-tests/thrombocytopathies/thrombobasthenia-and-thrombocytopenia
14. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-24.
15. Almomani MH, Mangla A. Bernard Soulier Syndrome. 2022.
16. Monteferrario D, Bolar NA, Marneth AE, Hebeda KM, Bergevoet SM, Veenstra H, et al. A dominant-negative GFI1B mutation in the gray platelet syndrome. N Engl J Med. 2014;370(3):245-53.

17. https://imagebank.hematology.org/image/3383/dohle-bodies-in-the-mayhegglin-anomaly--1
18. Anguita E, Candel FJ, Chaparro A, Roldan-Etcheverry JJ. Transcription Factor GFI1B in Health and Disease. Front Oncol. 2017;7:54.
19. Di Paola J, Porter CC. ETV6-related thrombocytopenia and leukemia predisposition. Blood. 2019;134(8):663-7.
20. Stockley J, Morgan NV, Bem D, Lowe GC, Lordkipanidze M, Dawood B, et al. Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects. Blood. 2013;122(25):4090-3.
21. Rosenberg N, Dardik R, Hauschner H, Nakav S, Barel O, Luboshitz J, et al. Mutations in RASGRP2 gene identified in patients misdiagnosed as Glanzmann thrombasthenia patients. Blood Cell Mol Dis. 2021;89.
22. Sanders YV, Fijnvandraat K, Boender J, Mauser-Bunschoten EP, van der Bom JG, de Meris J, et al. Bleeding spectrum in children with moderate or severe von Willebrand disease: Relevance of pediatric-specific bleeding. Am J Hematol. 2015;90(12):1142-8.
23. Heijdra JM, Atiq F, Al Arashi W, Kieboom Q, Wuijster E, Meijer K, et al. Desmopressin testing in von Willebrand disease: Lowering the burden. Res Pract Thromb Haemost. 2022;6(6):e12784.
24. Saini S, Zhang S, Yates S, Garcia J, Sharma R, Formella J, et al. High annualized bleeding rates in pediatric patients with inherited platelet function disorders. Haematologica. 2024;109(7):2351-4.
25. Schwaiger M, Edmondson SJ, Rabensteiner J, Prüller F, Gary T, Zemann W, Wallner J. Gender-specific differences in haemostatic parameters and their influence on blood loss in bimaxillary surgery. Clin Oral Invest. 2022;26(4):3765-79.
26. Abe K, Dupervil B, O'Brien SH, Oakley M, Kulkarni R, Gill JC, et al. Higher rates of bleeding and use of treatment products among young boys compared to girls with von Willebrand disease. American Journal of Hematology. 2020;95(1):10-7.
27. Pai M, Liu Y, Whittaker S, Arnold E, Seecharan JL, Moffat KA, et al. Gender differences in bleeding problems and implications for the assessment of a bleeding disorder. Blood. 2007;110(11):639a-a.
28. Delaney A, Kritsotakis EI, Horner K, Kitchen S, Sedcole J, Baxter T, et al. High Prevalence of Platelet Function Disorders in Women Referred for Surgical Management of Refractory Heavy Menstrual Bleeding. Haemophilia. 2025.
29. Zia A, Jain S, Kouides P, Zhang S, Gao A, Salas N, et al. Bleeding disorders in adolescents with heavy menstrual bleeding in a multicenter prospective US cohort. Haematologica. 2020;105(7):1969-76.
30. Mills HL, Abdel-Baki MS, Teruya J, Dietrich JE, Shah MD, Mahoney D, Jr., et al. Platelet function defects in adolescents with heavy menstrual bleeding. Haemophilia. 2014;20(2):249-54.
31. Philipp CS, Dilley A, Miller CH, Evatt B, Baranwal A, Schwartz R, et al. Platelet functional defects in women with unexplained menorrhagia. Journal of Thrombosis and Haemostasis. 2003;1(3):477-84.
32. Albert MH, Bittner TC, Nonoyama S, Notarangelo LD, Burns S, Imai K, et al. X-linked thrombocytopenia (XLT) due to WAS mutations: clinical characteristics, long-term outcome, and treatment options. Blood. 2010;115(16):3231-8.
33. Huisman EJ, Dalm V, Joosten M, Smiers FJ, Porcelijn L, Hoogendijk A, et al. Interstitial 11q Deletions and Terminal 11q Duplications Cause a Bleeding Tendency due to Platelet Dysfunction That Is Similar to 11q Deletions Causing Jacobsen Syndrome. Eur J Haematol. 2025.

34. Orsini S, Noris P, Bury L, Heller PG, Santoro C, Kadir RA, et al. Bleeding risk of surgery and its prevention in patients with inherited platelet disorders. Haematologica. 2017;102(7):1192-203.
35. Heubel-Moenen FCJI, Brouns SLN, Herfs L, Boerenkamp LS, Jooss NJ, Wetzels RJH, et al. Multiparameter platelet function analysis of bleeding patients with a prolonged platelet function analyser closure time. Brit J Haematol. 2022;196(6):1388-400.
36. van Asten I, Blaauwgeers M, Granneman L, Heijnen HFG, Kruip MJHA, Beckers EAM, et al. Flow cytometric mepacrine fluorescence can be used for the exclusion of platelet dense granule deficiency. Journal of Thrombosis and Haemostasis. 2020;18(3):706-13.
37. Navred K, Martin M, Ekdahl L, Zetterberg E, Andersson NG, Strandberg K, Norstrom E. A simplified flow cytometric method for detection of inherited platelet disorders-A comparison to the gold standard light transmission aggregometry. PLoS One. 2019;14(1):e0211130.
38. Huskens D, Li L, Florin L, de Kesel P, de Laat B, Roest M, Devreese KMJ. Flow cytometric analysis of platelet function to improve the recognition of thrombocytopathy. Thromb Res. 2020;194:183-9.
39. Jourdi G, Ramstrom S, Sharma R, Bakchoul T, Lordkipanidze M, group F-PiTs. Consensus report on flow cytometry for platelet function testing in thrombocytopenic patients: communication from the SSC of the ISTH. J Thromb Haemost. 2023;21(10):2941-52.
40. Gresele P, Falcinelli E, Bury L, Alessi MC, Guglielmini G, Falaise C, et al. Association of laboratory test results with the bleeding history in patients with inherited platelet function disorders (the Bleeding Assesment Tool- LABoratory tests substudy): communication from the Platelet Physiology ISTH-SSC. Res Pract Thromb Hae. 2024;8(1).
41. Stoffman JM, Chodirker BN, Israels SJ. Coagulation abnormalities in patients with Noonan syndrome - a single centre case series. Blood. 2004;104(11):295a-6a.
42. Andersson NG, Rossing M, Fager Ferrari M, Gabrielaite M, Leinoe E, Ljung R, et al. Genetic screening of children with suspected inherited bleeding disorders. Haemophilia. 2020;26(2):314-24.
43. Johnson B, Lowe GC, Futterer J, Lordkipanidze M, MacDonald D, Simpson MA, et al. Whole exome sequencing identifies genetic variants in inherited thrombocytopenia with secondary qualitative function defects. Haematologica. 2016;101(10):1170-9.
44. Fasulo MR, Biguzzi E, Abbattista M, Stufano F, Pagliari MT, Mancini I, et al. The ISTH Bleeding Assessment Tool and the risk of future bleeding. J Thromb Haemost. 2018;16(1):125-30.

Supplementary Figure S1 Study protocol



Abbreviations iPSC = induced pluripotent stem cells, ISTH-BAT = bleeding assessment tool from the International Society of Thrombosis and Hemostasis, LTA = light-transmission aggregometry, MNCs = mononuclear cells, PD = platelet disorder, PFA = Platelet function analyzer, tNGS = targeted next generation sequencing

Supplementary Table S1 Overview of genes included in used panel platelet disorders

Gene	Name	OMIM	Location
ABCG5	ATP-Binding cassette, subfamily G, member 5	605459	2p21
ABCG8	ATP- Binding cassette, subfamily G, member 8	605460	2p21
ACTN1	Actinin, alpha-1	102575	14q24,1
ADRA2A	Apha-2A-adrenergic receptor	104210	10q25,2
ANKRD26	Ankyrin repeat domain-containing protein 26	610855	10p12,1
ANO6	Anoctamin 6	608663	12q12
AP3B1	Adaptor-related protein complex 3, beta-1 subunit	603401	5q14,1
BLOC1S1	Biogenesis of lysosome-related organelles complex 1, subunit 1	601444	12q13,2
BLOC1S2	Biogenesis of lysosome-related organelles complex 1, subunit 2	609768	10q24,31
BLOC1S3	Biogenesis of lysosome-related organelles complex 1, subunit 3	609762	19q13,32
BLOC1S4	Biogenesis of lysosome-related organelles complex 1, subunit 4	605695	6p16,1
BLOC1S5	Biogenesis of lysosome-related organelles complex 1, subunit 5	607289	6p24,3
BLOC1S6	Biogenesis of lysosome-related organelles complex 1, subunit 6	604310	15q21,1
BLOC1S7 / SNAPIN	SNAP-associated protein	607007	1q21,3
CYCS	Cytochrome C	123970	7p15,3
DTNBP1	Dystrobrevin-binding protein 1	607145	6p22,3
ETV6	ETS variant gene 6	600618	12p13,2
F2R	Coagulation factor II receptor	187930	5q13,3

Supplementary Table S1 Overview of genes included in used panel platelet disorders (*continued*)

Gene	Name	OMIM	Location
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor for	146790	1q23,3
FERMT3	Fermitin family (Drosophilia) homolog 3	607901	11q13,1
FLI1	Friend leukemia virus integration 1	193067	11q24,3
FLNA	Filamin A	300017	Xq28
GATA1	GATA-binding protein 1	305371	Xp11,23
GATA2	GATA- binding protein 2	137295	3q21,3
GFI1B	Growth factor-independent 1B	604383	9q34,13
GNA12	Guanine nucleotide-binding protein, Alpha-12	604394	7p22-3-p22,2
GNA13	Guanine nucleotide-binding protein, Alpha -13	604406	17q24,1
GNAI1	Guanine nucleotide-binding protein, Alpha -inhibiting activity polypeptide 1	139310	7q21,11
GNAQ	Guanine nucleotide-binding protein, Q polypeptide	600998	9q21,2
GNAS	GNAS complex locus	139320	22q13,32
GNB3	Guanine nucleotide-binding protein, Beta -3	139130	12p13,31
GP1BA	Glycoprotein Ib, platelet, Alpha polypeptide	606672	17p13,2
GP1BB	Glycoprotein Ib, platelet, Beta polypeptide	138720	22q11,21
GP6	Glycoprotein VI, platelet	605546	19q13,42
GP9	Glycoprotein IX, platelet	173515	3q21,3
HOXA11	Homeobox A11	142958	7p15,2
HPS1	HPS1 gene	604982	10q24,2
HPS3	HPS3 gene	606118	3q24
HPS4	HPS4 gene	606682	22q12,1
HPS5	HPS5 gene	607521	11p15,1
HPS6	HPS6 gene	607522	10p24.32
ITGA2	Integrin, Alpha -2	192974	5q11,2
ITGA2B	Integrin, Alpha -2B	607759	17q21.31
ITGB1	Integrin, Beta-1	135630	10p11.22
ITGB3	Integrin, Beta -3	173470	17q21.32
LYST	Lysosomal trafficking regulator	606897	1q42.3
MASTL	Microtubule-associated Serine/Threonine kinase-like	608221	10p12.1
MPL	Myeloprolifrative leukemia virus oncogene	159530	1p34.2
MYH9	Myosin, heavy chain 9, nonmuscle	160775	22q12.3
NBEA	Neurobeachin	604889	13q13.3
NBEAL2	Neurobeachin -like 2	614169	3p21.31
P2RY1	Purinergic receptor P2Y, G protein-coupled, 1	601167	3q25.2
P2RY12	Purinergic receptor P2Y, G protein-coupled, 12	600515	3q25.1
PEAR1	Platelet endothelial aggregation receptor 1	610278	1q23.1
PIK3CG	Phosphatidylinositol 3-kinase, catalytic, Gamma	601232	7q22.3
PLAU	Plasminogen activator, urinokinase	191840	10q22.2

Supplementary Table S1 Overview of genes included in used panel platelet disorders (*continued*)

Gene	Name	OMIM	Location
PLCB2	phospholipase C, Beta -2	604114	15q15.1
PLCB3	phospholipase C, Beta -3	600230	11q13.1
PLCG2	phospholipase C, GAMMA-2	600220	16q23.3
PRKACG	Protein kinase, cAMP-dependent, catalytic, Gamma	176893	9q21.11
PTGS1	Prostaglandin-endoperoxide synthase 1	176805	9q33.2
RASGRP2	RAS Guanyl nucleotide-releasing protein 2	605577	11q13.1
RBM8A	RNA-binding motif protein 8A	605313	1q21.1
RHOA	RAS Homolog gene family, member A	165390	3p21.31
RUNX1	RUNT-related transcription factor 1	151385	21q22.12
TBX1	T-box 1	602054	22q11.21
TBXA2R	Thromboxane A2 receptor, PLATELET	188070	19p13.3
TBXAS1	Thromboxane A synthase 1	274180	7q34
THPO	Thrombopoietin	600044	3q27.1
TUBB1	Tubulin, Beta -1	612901	20q13.32
VIPAS39	VPS33B-interacting protein, apical-basolateral polarity regulator SPE39 homolog	613401	14q24.3
VWF	Von Willebrand Factor	613160	12p13.31
WAS	WAS gene	300392	Xp11,23

Supplementary Table S2 Overview of reported comorbidities

Reported comorbidities in n=98 children	
Hematological, not coagulation-related	**number (n)**
Pediatric ITP, transient but requiring treatment	2
Leukemia	2
Elliptocytosis	1
Skin and hair	
Moderate-severe eczema	7
Alopecia	2
Albinism	1
Brittle hair	1
Thoracic	
Congenital cardiac malformations	15
(Obstructive) cardiomyopathy	1
Asthma	1
Intrathoracic lipoma	1
Progressive pulmonary fibrosis	1
Congenital pulmonary malformations	1
Eye & ear	
Cataract	2
Nystagmus	2
Coloboma	1
Congenital deafness	2
Kidney	
Progressive proteinuria	1
Horse shoe kidney	1

Supplementary Table S2 Overview of reported comorbidities (*continued*)

Reported comorbidities in n=98 children	
Hematological, not coagulation-related	**number (n)**
Pediatric ITP, transient but requiring treatment	2
Leukemia	2
Elliptocytosis	1
Skin and hair	
Moderate-severe eczema	7
Alopecia	2
Albinism	1
Brittle hair	1
Thoracic	
Congenital cardiac malformations	15
(Obstructive) cardiomyopathy	1
Asthma	1
Intrathoracic lipoma	1
Progressive pulmonary fibrosis	1
Congenital pulmonary malformations	1
Eye & ear	
Cataract	2
Nystagmus	2
Coloboma	1
Congenital deafness	2
Kidney	
Progressive proteinuria	1
Horse shoe kidney	1
Inborn error of immunity	
Hypogammaglobulinemia	6
Abnormal T-cell function	6
Abnormal neutrophil function	1
Gastro-intestinal	
Feeding difficulties in infancy	4
Intestinal pseudo-obstruction	3
GERD	2
Pylorus hypertrophy	1
Ossal structures	
Craniosynostosis	3
Abnormal limbs	2
Unstable girdle	2
Scoliosis	2
Congenital hip dysplasia	2
Cleft palate	1
Development delay	
Psychomotor retardation	9
Autism spectrum disorder	3
Speech and language delay	2
Epilepsia	2
Fever fits	1
Other	
Lymphedema	1
Hypospadia	1
Type 1 IDDM	1

Supplementary Table S3 Molecular diagnosis of children with inherited or syndrome-associated platelet disorders

N	Gene	Nucleotide	Protein	Inheritance	Class [1]	Syndrome name
Monogenetic, inherited platelet disorders						
Transcription factor and early megakaryopoiesis						
1	*NM_005105.5 (RBM8A)*	c.67+32G>C, homozygous	p.?	AR	5	Thrombopenia with absent radius
2	*NM_005105.5 (RBM8A)*	Del1q21/c.-21G>A, homozygous	p.?	AR	5	Thrombopenia with absent radius
3-4; F1	*NM_004188.4 (GFI1B)*	c.814+1G>A	p.?	AD	5	
5-6; F2	*NM_001987.4 (ETV6)*	c.1105C>T	p.(Arg369Trp)	AD	4	
7	*NM_001754.4 (RUNX1)*	c.319C>A	p.(Arg107Ser)	AD	4	Familial PD with associated myeloid malignancy
8	*NM_002017.4 (FLI1)*	c.297del	p.(Met100*)	AD	4	
Late megakaryopoiesis						
9	*AP3B1*[†]	-	-	-	5	Hermansky-Pudlak syndrome type 2
Pro-platelet formation and cytoskeleton regulation						
10	*NM_030773.3 (TUBB1)*	c.721C>T	p.(Arg241Trp)	AD	4	
11-12; F3	*NM_030773.3 (TUBB1)*	c.751C>A	p.(Arg251Ser)	AD	4	
13-14; F4	*NM_002473.4 (MYH9)*	c.5797C>T	p.(Arg1933*)	AD	5	May-Hegglin anomaly
15	*NM_002473.4 (MYH9)*	c.2104C>T	p.(Arg702Cys)	AD	5	Fechtner syndrome
16; F5.1	*NM_001110556.1 (FLNA)*	c.7941_7942 del	p.(*2648Ser ext*100)	XL	5	Filamin A - Intestinal pseudo-obstruction
17-18; F5.2	*NM_001110556.1 (FLNA)*	c.7941_7942 del	p.(*2648Ser ext*100)	XL	5	Filamin A - Intestinal pseudo-obstruction
19	*NM_001110556.1 (FLNA)*	c.7078delG	p.(Ala2360fs)	XL	4	Filamin A - Cardiac dysplasia
20-21; F6	*NM_000377.2 (WAS)*	c.360+3A>T	p.?	XL	4	X-linked thrombocytopenia
22	*NM_000377.2 (WAS)*	c.137C>A	p.(Pro58His)	XL	4	X-linked thrombocytopenia
23	*NM_000377.2 (WAS)*	c.399G>C	p.(Glu133Asp)	XL	4	

Supplementary Table S3 Molecular diagnosis of children with inherited or syndrome-associated platelet disorders (*continued*)

N	Gene	Nucleotide	Protein	Inheritance	Class [1]	Syndrome name
Monogenetic, inherited platelet disorders						
Platelet glycoprotein or receptor signaling						
24	*NM_000419.4 (ITGA2B)*	c.2875G>C, homozygous	p.(Ala959Pro)	AR	4	Glanzmann Thrombasthenia; Reduced expression (<5%, type 1)
25	*NM_001098670.1 (RASGRP2)*	c.706C>T	p.(Gln236*)	AR	5	
26	*NM_000407.4 (GP1BB)*	c.281A>G, homozygous	p.(Asp94Gly)	AR	5	Bernard-Soulier syndrome; Reduced expression (<5%, type 1)
27	*NM_000407.4 (GP1BB)*	c.410T>C	p.(Leu137Pro)	AD	5	Mildly reduced expression (50-60%, type 3)
28	*NM_001001548.3 (CD36)*	c.430-2A>G c.1254+4dupT	p.? p.?	AR	5	Platelet glycoprotein IV deficiency; Reduced expression (<1% CD36, type 1)
Platelet dysfunction						
29	*NM_001204803.1 (ANO6)*	c.1318C>T, homozygous	p.(Arg440*)	AR	4	Scott syndrome
30	*NM_002834.5 (PTPN11)*[†]	c.172A>G	p.(Asn58Asp)	AD	5	Noonan syndrome
31-32; F7	*NM_002834.5 (PTPN11)*	c.922A>G	p.(Asn308Asp)	AD	5	Noonan syndrome
33	*NM_002834.5 (PTPN11)*[†]	c.317A>C	p.(Asp106Ala)	AD	5	Noonan syndrome
34	*NM_002834.5 PTPN11*	-	-	AD	5	Noonan syndrome
35	*NM_002834.5 (PTPN11)*	c.179G>C	p.(Gly60Ala)	AD	4	Noonan syndrome
36	*NM_002834.5 PTPN11*	-	-	-	4	Noonan syndrome
37	*NM_002834.5 (PTPN11)*	c.1504C>A	p.(Ser502Thr)	AD	4	Noonan syndrome
38	*NM_005633.4 (SOS1)*	c.1656G>T	p.(Arg552Ser)	AD	4	Noonan syndrome
39	*NM_006912.5 (RIT1)*	c.280G>A	p.(Ala77Thr)	AD	5	Noonan syndrome
40	*NM_000372.5 (TYR)*	c.898dupG, homozygous	p.(Val300fs)	AR	4	Oculocutaneous albinism type 1

Supplementary Table S3 Molecular diagnosis of children with inherited or syndrome-associated platelet disorders (*continued*)

N	Gene	Nucleotide	Protein	Class [(1)]	Syndrome name
Syndrome-associated PD as a result of chromosomal abnormality					
42	target (hg19)	11q14.2-q22.1(del)	(79,729,111 - 98,148,532) x1	-	Interstitial 11q deletion
43	SNP (GRCh37)	11q14.1-q23.3(del)	(79,729,111 - 98,148,532) x1	32 / 34144	Interstitial 11q deletion
44	Karyo	46,XX,+der(11).ish dup(11)(q21q23.3)	x3	-	Interstitial 11q duplication
45	Karyo	47,XX,+der(22) t(11;22)(q23;q11)	x3	-	Emanuel syndrome
46	SNP (hg19)	11q24.2q25(del)	(124,552,720 - 134,934,063) x1	-	Paris-Trousseau syndrome Jacobsen syndrome
47	SNP (GRCh37)	11q24.1q25(del) 12q24.21q24.33 (dupl)	(122,893,213 - 134,450,518) x1 (114,633,820 - 133,779,217) x3	12 / 755 19.2 / 1167	
48	Micro (GRCh37)	11q24.2q25(del) 5q35.1q35.3(dupl)	(126,456,874 - 129,671,201) x1 (171,063,816 - 180,099,598) x3	10 9	
49	Karyo	46,XX, 11Xq23.3q25 (del)	x1	15.5	Paris-Trousseau syndrome Jacobsen syndrome
50	WGA (hg18)	11q24.3q25(del)	(127,727,172 - 134,452,384) x1	-	Paris-Trousseau syndrome Jacobsen syndrome
51	SNP (GRCh37)	11q24.2q25(del)	(126,908,896 - 134,938,470) x1	8 / 9498	Paris-Trousseau syndrome Jacobsen syndrome
52	SNP (GRCH37)	11q24.2q25(del)	(126,728,342 - 134,938,470) x1		Paris-Trousseau syndrome Jacobsen syndrome
53	SNP (GRCh37)	11q24.1q25(del)	(123,307,968 - 134,938,470) x1	11.6 / 13938	Paris-Trousseau syndrome Jacobsen syndrome
54	SNP (?)	22q11.21(del)	(18,893,831 - 18,894,140) x1	1.4	Velo-cardiofacial or DiGeorge syndrome

Abbreviations del = deletion, dup =duplication, fs = frame shift, Karyo = karyogram, Micro = micro-array, MYH9-RD = MYH9 related diseases, n = (patient) number, PD = platelet disorder, SNP = SNP array, target = targeted array, WGA = whole genome array

Legend (1) Classification according to American College of Medical Genetics (ACMG) guidelines with 4 = likely pathogenic, 5 = pathogenic. (2) test results performed in another center, no genomic data available, but conclusions in correspondence of clinical geneticist

For me, a landscape does not exist in its own right,
since its appearance changes at every moment

Claude Monet (1840 – 1926 AD)

4 Proteomic landscapes of inherited platelet disorders with different etiologies

C. Kreft*
E.J. Huisman*
M.H. Cnossen
F.P.J. van Alphen
C. van der Zwaan
K. van Leeuwen
R. van Spaendonk
L. Porcelijn
C.S.B. Veen
M. van den Biggelaar
M. de Haas
A.B. Meijer
A.J. Hoogedijk

Journal of Thrombosis and Haemostasis 2023; 21(2):359-372
*shared 1st authors

ABSTRACT

Background

Inherited Platelet Disorders (IPDs) are a heterogeneous group of rare diseases that are caused by defects in early megakaryopoiesis, pro-platelet formation and/or mature platelet function. Although genomic sequencing is increasingly used to identify genetic variants underlying IPD, this technique does not disclose resulting molecular changes that impact platelet function. Proteins are the functional units that shape platelet function, however insights into how variants that cause IPDs impact platelet proteomes is limited.

Objectives

To profile the platelet proteomics signatures of inherited platelet disorders.

Methods

We performed unbiased label free quantitative mass spectrometry (MS)-based proteome profiling on platelets of 34 IPD patients with variants in 13 ISTH TIER1 genes that affect different stages of platelet development.

Results

In line with the phenotypical heterogeneity between IPDs, proteomes were diverse between IPDs. We observed extensive proteomic alterations in patients with a *GFI1B* variant and for genetic variants in genes encoding proteins that impact cytoskeletal processes (*MYH9*, *TUBB1* and *WAS*). Utilizing the diversity between IPDs, we clustered protein dynamics, revealing disrupted protein-protein-complexes. This analysis furthermore grouped proteins with similar cellular- function and location, classifying mitochondrial protein constituents and identifying both known and putative novel alpha granule associated proteins.

Conclusions

With this study we demonstrate a MS-based proteomics perspective to IPDs. By integrating the effects of IPDs that impact different aspects of platelet function, we dissected the biological contexts of protein alterations to gain further insights into the biology of platelet (dys)function.

Key words

Blood platelets, Inherited Blood Coagulation Disorders, Mass spectrometry, Multiprotein Complexes, Proteomics

Essentials

- Inherited Platelet Disorders (IPDs) are a heterogeneous group of rare diseases with various etiologies.
- Mass spectrometry (MS)-based proteomic profiling of 13 different IPDs with variants in ISTH TIER1 genes.
- Protein dynamics allow for annotation of platelet alpha granule proteins. MS-based proteomics gives insight into the molecular backgrounds of IPDs.

INTRODUCTION

Platelets are anucleated cell fragments that are released by megakaryocytes into the circulation and play a crucial role in primary hemostasis. Activation of platelets induces granule release, shape change and aggregation, ultimately resulting in the formation of a platelet plug that restores vascular integrity and limits blood loss.(1) Inherited platelet disorders (IPDs) are a heterogeneous group of rare bleeding disorders with varying clinical expression and an increased bleeding tendency as common denominator.(2) Gene variants causing IPDs may disrupt hematopoietic stem cell commitment to megakaryocyte lineage, megakaryocyte proliferation and terminal differentiation, proplatelet formation or platelet shedding. Therefore IPDs may exhibit impairments in the production of sufficient platelet numbers (*i.e.* thrombocytopenia) and in the function of circulating platelets (*i.e.* thrombocytopathy).(3) Diagnosis of IPDs is increasingly performed using high-throughput DNA sequencing.(4-7) This development is supported by establishment of curated disease-causing (ISTH TIER1) genes and the gold variant resources for inherited bleeding, platelet, or thrombotic disorders.(6) However, for variants for which no curated evidence is available, interpretation of clinical or biological relevance remains challenging.

It is generally recognized that proteins are the functional units that shape platelet function in both healthy individuals and IPD patients.(2, 8) Therefore, quantitative high-resolution mass spectrometry (MS)-based proteomics holds great potential to provide a deeper understanding of the affected molecular mechanisms of both novel and curated genetic variants associated with platelet disorders.(9, 10) A first step herein is to assess proteomic alteration in platelets of patients with pathogenic variants in well curated genes (*i.e.* TIER1 genes). However, thus far MS-based studies have been restricted to a limited variety of IPDs.(9, 11) Using label-free quantitative MS-based proteomics, we here investigated the platelet proteomic impact of IPDs with variants in 13 TIER1 genes.(11) We compared these IPDs of different etiologies spanning from early megakaryopoiesis, pro-platelet formation and/or mature platelet function.(3)

Overall, we observed a large heterogeneity between IPDs ranging from 1 to 250 differentially abundant proteins. We found limited overlap between IPDs affecting transcription factors and a considerable overlap in affected proteomes between IPDs with genetic variants in cytoskeletal proteins. Furthermore, we demonstrate that protein dynamics clustered across a wide range of IPDs according to cellular function, location and protein-complex membership. Taken together, this study demonstrates that a proteomics point of view provides insight into the molecular backgrounds of platelet (dys)function.

METHODS

Study population

The study population consisted of children with a DNA-based IPD diagnosis and affected relatives. The study was approved by the Medical Ethical Review Board of the Erasmus University Medical Center (MEC-2016-218). Patients were included Patients were included when the following criteria suggested a platelet disorder 1) abnormal bleeding tendency and abnormal platelet indices, 2) prolonged Platelet Function Analyzer (PFA) and/or 3) abnormal LTA results and/or 4) abnormal platelet morphology, combined with a genetic variant in a TIER1 gene as determined by targeted Next Generation Sequencing (tNGS). Affected relatives were also included into our analysis.(12) All participants were included after obtaining written informed consent by legal representatives and patients (if ≥12 years). Due to the rarity of IPDs, for proteomic analysis we selected all patients and affected relatives with variants in ISTH TIER1 genes. Additional blood was drawn from healthy, anonymized volunteers in accordance with Dutch regulations and after approval from the Sanquin Ethical Advisory Board. The study was performed according to the Declaration of Helsinki.

Hematological assays

Blood was obtained by venipuncture and collected in 9 mL EDTA Vacutainer tubes (Becton Dickinson, Plymouth, UK), except for the LTA analysis citrated blood was collected. Study participants did not fast prior to blood drawing. Per patient or control a single tube was used for platelet isolation. 1.8 mg/mL EDTA-blood was used for blood cell indices (Sysmex XN-9100®), thrombopoietin (TPO) analysis (in-house ELISA) and citrated blood (0.109 mol/L) was taken for Collagen-ADP (C-ADP) and collagen-epinephrine (C-Epi) cartridges (PFA-200, Siemens), and the light transmission aggregometry (LTA). PFA was performed on whole blood drawn by venipuncture. Collagen-ADP (C-ADP) and collagen-epinephrine (C-Epi) cartridges were used to measure closure times (CT, seconds) on the PFA-200 (Siemens). Abnormal closure time is defined as >155 seconds.

LTA was performed on a Chrono-Log aggregometer 490 (Stago Benelux) within 20 minutes after venipuncture, following current guidelines (Cattaneo et al., 2013; Munnix et al., 2021). In summary, citrated platelet-rich plasma (PRP) was used, obtained by centrifugation at 260 g for 7 minutes at room temperature. A minimal PRP of 150 x 10^9/L was considered adequate for analysis. Platelet aggregation was assessed using the following agonists and concentrations: adenosine diphosphate (ADP, 5 and if abnormal: 10 µM), Archidonic acid (AA, 0,5 mM), Collagen (2 and 5 and if abnormal: 10 µg/mL), Epinephrine (5 µM and if abnormal epinephring/ADP mix 1µM/5 µM mix), Thrombin 1 U/mL (before 2021) and TRAP-6 (30 µM from 2021), and U46619 (4µM) and Ristocetin (0,31 and 1.25 mg/mL). An abnormal LTA was defined as ≤ 50% response to ≥ 1 of the above-mentioned agonists or a reversible response defined as a short time >50% response, but no steady

state reached >50%. All PFA and LTA-tests were performed at one central laboratory, "Diagnostic laboratory of hemostasis" Erasmus MC, Rotterdam, NL according to standardized protocol.(13, 14)

DNA-analysis

DNA was isolated from EDTA-blood according to manufacturer's instructions using the Gentra Puregene kit or the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). An in-house developed Ampliseq custom thrombocyte panel (Thermo Fisher Scientific, Waltham, Mass) was used to sequence the coding regions of 73 genes (Supplementary Table S1). Library preparation and sequencing were performed according to manufacturer protocols on a S5 system (Thermo Fisher Scientific). Ion Reporter software (Thermo Fisher Scientific) was used to analyze genetic sequence data.

Sample preparation and mass spectrometry analysis

For mass spectrometry analysis a single 9 ml EDTA BD Vacutainer tube was used for platelet isolation. EDTA-blood was centrifuged two times for 20 min at 120g to obtain platelet-rich plasma. Next, platelets were isolated by centrifuging for 10 min at 2,000g. Platelets were washed three times with platelet wash buffer (36 mM citric acid, 103 mM NaCl, 5 mM KCl, 5 mM EDTA, 5.6 mM D-glucose, 10% (v/v) acid-citrate-dextrose-A (Becton Dickinson, Plymouth, UK), pH 6.5) and aliquots of 1×10^8 platelets in 25 µL were stored at -80°C until further processing. One aliquot of platelets was lysed in 8 M urea in 100 mM Tris-HCl (pH 8). The protein concentration was determined and 5 µg of protein was used for sample preparation for mass spectrometry. Disulphide bonds were reduced with 10 mM dithiotreitol (DTT, Thermo Fisher) for 60 minutes at 20°C, alkylated with 55 mM iodoacetamide (IAM, Thermo Fisher) for 45 minutes at 20 °C, and samples were digested overnight at 20°C with MS-grade trypsin Gold (Promega, Madison, WI, US) (in a protein: trypsin ratio of 20:1). Tryptic peptides were desalted and concentrated using Empore-C18 StageTips,(15) and eluted with 0.5% (v/v) acetic acid, 80% (v/v) acetonitrile. Samples were dried by SpeedVac and dissolved in 2% acetonitrile, 0.1% trifluoroacetic acid. Tryptic peptides were separated by nanoscale C18 reverse phase chromatography coupled online to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) via a nano electrospray ion source (Nanospray Flex Ion Source, Thermo Fisher Scientific). Peptides were loaded on a 20 cm 75 µm-360 µm inner-outer diameter fused silica emitter (New Objective, Bremen, Germany) packed in-house with ReproSil-Pur C18-AQ, 1.9 µm resin (Dr Maisch GmbH, Ammerbuch-Entringen, Germany). The column was installed on a Dionex Ultimate 3000 RSLC nanoSystem (Thermo-Fisher Scientific) using a MicroTee union formatted for 360 µm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15 kV. Buffer A was composed of 0.5% acetic acid and buffer B of 0.5% acetic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nL/min at 5% buffer B, equilibrated for 5 minutes at 5% buffer B (17-22 min) and eluted by increasing buffer B from 5 to 15% (22-87 min) and 15 to 38% (87-147 min), followed by a

10 min wash to 90% and a 5 min regeneration to 5%. Survey scans of peptide precursors from 400 to 1500 m/z were performed in the orbitrap at 120K resolution with a 4×10^5 ion count target. Tandem mass spectrometry was performed by isolation with a quadrupole with isolation window 1.6, HCD fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS2 ion count target was set to1.5×10^4 and the max injection time was 35 ms. Precursors with charge state 2–7 were sampled for MS2. The dynamic exclusion duration was set to 60s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3s-cycles. The raw MS files and search/identification files obtained with MaxQuant have been deposited in the ProteomeXchange consortium,(16) via the PRIDE partner repository with identifier PXD027463.(17) Previously published GFI1B and RUNX1 proteome data were obtained from PXD003912.

Data analysis

Raw MS data files were acquired with XCalibur software (Thermo Fisher Scientific) and processed with MaxQuant 1.6.2.10 software.(18) Peptides were searched against the homo sapiens Uniprot database (downloaded March 2019, 73,932 entries). An additional database sequence was added to allow for detection of a C-terminal extension event (NP _001447.2.p.Ter26487Serext101) in FLNA.(19) Enzyme specificity was set to trypsin with a maximum of 2 missed cleavages, and carbamidomethylation on cysteine residues was used as fixed modification. The false discovery rate (FDR) was 0.01 for peptide and protein levels with a minimum length of 7 amino acids. The peptide precursor MS tolerance was set to 10 ppm and fragment ion mass tolerance at 0.6 Dalton. Label-free quantitation (LFQ) was performed with a minimum ratio count of 2 based on unique peptides for quantification. Absolute protein abundances were estimated using intensity-based absolute quantification (iBAQ). MaxQuant output files were loaded in R 4.1.2 for further analysis. Proteins were filtered by "reverse" and "only identified by side" and LFQ intensities were $\log_2$ transformed and iBAQ values were $\log_{10}$ transformed. Proteins were considered quantified based on the presence of 100% valid values in at least one condition. Missing values were imputed by normal distribution (width = 0.3, shift = 1.8). CVs were calculated per protein using all sample intensities. Statistical analysis was performed by comparing each IPD with controls resulting in 13 contrasts using moderated t-tests using the limma package.(20) To limit potential false positives due to the applied imputation strategy, results were censored for comparisons in which all conditions contained imputed values. Gene ontology and pathway overrepresentation analyses were performed using the cluster profiler package.(21) All p-values were corrected for multiple testing using the Benjamini-Hochberg method. An adjusted P value < 0.05 was considered statistically significant, for proteomic analyses, an adjusted P value < 0.05 and absolute log fold change of >1 was considered statistically significant and relevant. To query the data for IPD proxy marker proteins, we defined theoretical profiles for each IPD in which intensities

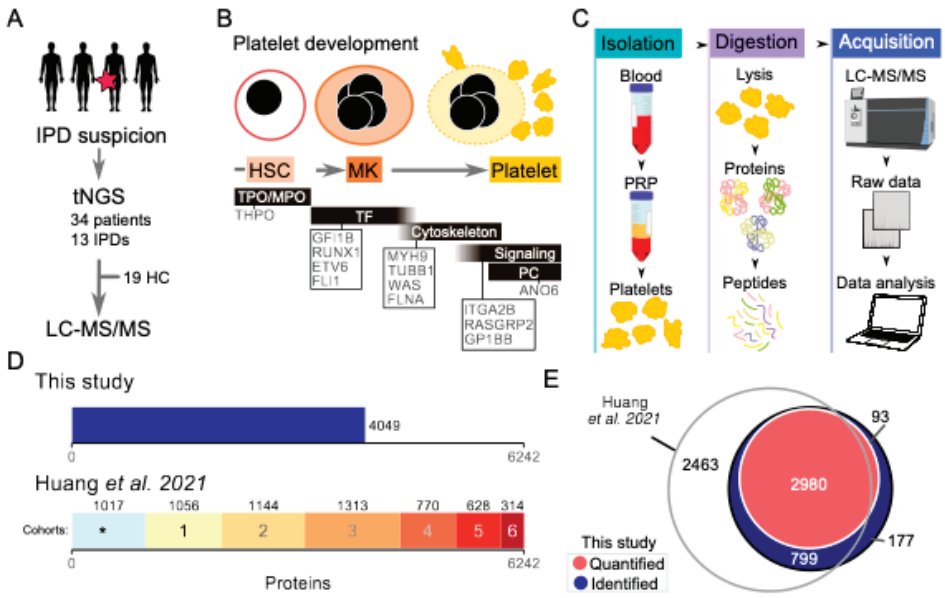
are high for a given IPD and low in all other samples. These profiles were correlated with all quantified proteins, and a selection was made based on an absolute Pearson correlation coefficient threshold of 0.7. Protein dynamics were evaluated by means of weighted gene co-expression network analysis (WGCNA) using a soft-power of 7 signed network, modules were defined by dynamic tree cut.(22) Correlation between data and disorder specific theoretical protein profiles was performed using Pearson correlation applying a coefficient threshold of 0.7.

RESULTS

Patient cohort and platelet proteomes

To study platelet proteomes in IPDs, we included 34 patients and relatives with targeted Next Generation Sequencing (tNGS) variants in 13 TIER1 genes and 19 healthy individuals as controls (Figure 1A, Tables S2 and Table1). IPDs were categorized into 5 groups according to molecular function: 1) Thpo/Mpl signaling: THPO (N=2), 2) transcription regulation: ETV6 (N=2), GFI1B (N=3), FLI1, (N=1), RUNX1 (N=1), 3) cytoskeleton regulation: MYH9 (N=4), WAS (N=5), FLNA c-terminal extension (N=5), TUBB1 (N=6), 4) glycoprotein (GP) signaling: GP1BB (N=2), ITGA2B (N=1), RASGRP2 (N=1), and 5) procoagulant function: ANO6 (N=1). Figure 1B shows these categories in the context of megakaryopoiesis and platelet function, note that some variants may affect more than one phenotypic outcome, i.e. GP1BB and ITGA2B 'signaling' category members also have impact on platelet formation. We applied high resolution quantitative label free MS-based proteomics on platelets isolated from peripheral blood (Figure 1C). To assess if the isolation protocol restricted inclusion of large (>12 fL) platelets we compared mean platelet volumes (MPV) prior and post isolation in all patients and patients with a MPV >12 fL at inclusion (Supplementary Figure S1). This showed that although large platelets (>12 fL) were included, sample MPV was reduced by the isolation ($P<0.03$). MS analysis resulted in the identification of >58,000 peptides corresponding to 4,049 proteins of which 3,073 proteins were reliably quantified (Figure 1D-E). Comparison with a recently published in depth compilation of seven platelet proteome studies showed that although our dataset contained less protein identifications (Figure 1D), there was a large overlap between proteomes (Figure 1E).(11)

Figure 1 Proteomic landscapes of inherited platelet disorders with different etiologies



Abbreviations HC = healthy control, IPD = inherited platelet disorders, LC-MS = liquid chromatographic mass-spectrometry, MS = mass-spectrometry, tNGS = target next generation sequencing

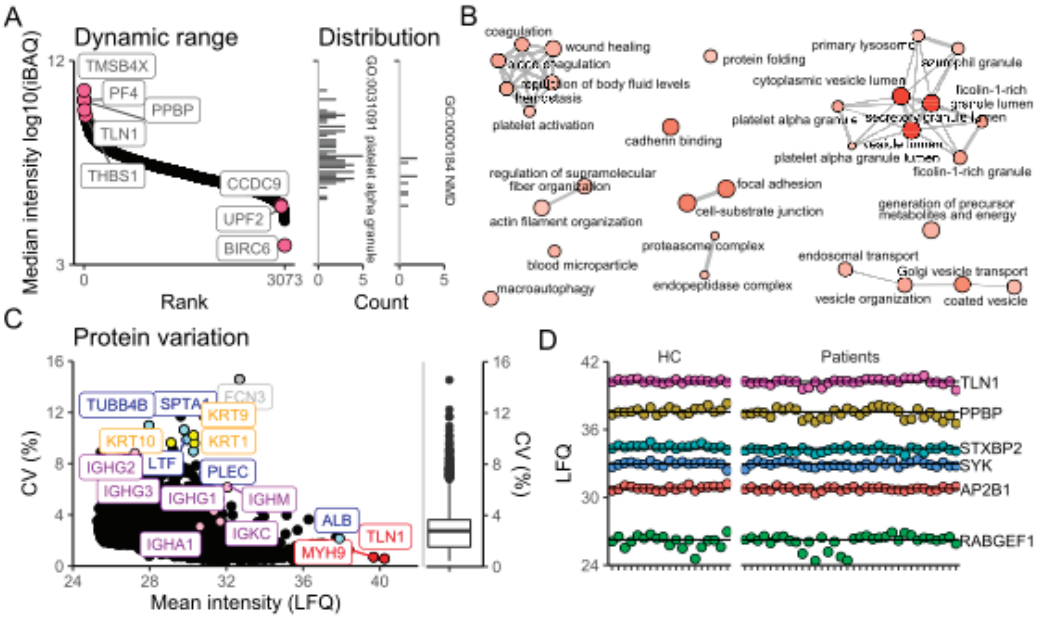
Legend (A) Flow chart of sample inclusion for spectrometric analysis. (B) Schematic representation of platelet development and categorization of IPDs investigated in this study. *GP1BB and ITGA2B are categorized as singling molecules, in addition, variants in these genes also affect platelet formation. (C) Overview of experimental setup and analysis. Platelets were isolated from whole blood and lysed. Proteins were digested intro tryptic peptides and analyzed by quantitative label-free LC-MS/MS. (D) Comparison of the proteins identified in the present study with those identified in the meta-analysis and proteomic study by Huang et al.(11) Bar colors/numbers indicate amount of studies describing a set of proteins. #indicates proteins directly measured by Huang et al.(191) (E) Overlap of proteins identified by Huang et al.(11) and protein identified (blue) and quantified (coral) in the present study.

Platelet proteomic depth and stability

Plotting the median protein iBAQ intensities (a proxy of absolute quantities) against their ranked abundance showed that these intensities spanned more than six orders of magnitude between the most and least abundant proteins. Proteins with established functions in platelets, *i.e.* Thymosin beta-4 (TMSB4X), Talin-1 (TLN1), Platelet factor 4 (PF4), pro-platelet basic protein (PPBP) and thrombospondin-1 (THBS1)(23-25) were highly abundant. Proteins involved with nonsense-mediated mRNA decay (Regulator of nonsense transcripts; UPF2 and Coiled-coil domain-containing protein 9; CCDC9) were measured in the mid to low range of detection (Figure 2A). Gene ontology term enrichment on all quantified proteins revealed overrepresentation of platelet associated terms (*e.g.* granules and hemostasis, Figure 2B). Next, we determined the variability and

reproducibility of protein quantifications by assessing the coefficient of variation (CV) versus median label free relative quantification intensities (LFQ). Although an inverse relation between protein intensities and variability was present ($P<2 \times 10^{16}$, ANOVA; Figure 2C), the highest observed CV was within 20% and the median CV was below 4%. Proteins with high CVs included possible exogenous contaminants (*i.e.* keratins, likely due to sample handling), plasma proteins (immunoglobulins) as well as cellular contaminations from red- and white blood cells (Lactotransferrin, LTF and cytoskeletal associated proteins). Apart from its high relative abundance, TLN1 exhibited the lowest variability.(23-25) Inspection of six proteins associated with platelets and/or signaling transduction demonstrated high reproducibility across the measurement range quantiles (Figure 2D), *i.e.* TLN1 (Talin 1) and RABGEF1 (RAB Guanine Nucleotide Exchange Factor 1) which are part of integrin-mediated platelet function and signaling.(25-27) PPBP (Pro-Platelet Basic Protein) a platelet alpha granule constituent,(28) STXBP2 (Syntaxin Binding Protein 2), which plays a role in platelet granule secretion,(29) AP2B1 (Adaptor Related Protein Complex 2 Subunit Beta 1) which is involved in the endosomal vesicle trafficking network.(30) Together this highlights the overall stability of platelet proteins across IPDs and controls, and shows the robustness of label free MS for comparing protein abundances between IPD patient platelets.

Figure 2 Platelet proteomic depth and stability



Abbreviations CV = coefficient of variation, GO = gene ontology, LFQ = label-free quantifications

Legend (A) Dynamic range of quantified proteins (iBAQ intensities are shown). Right panels show intensity distribution of protein annotated with platelet alpha granules (GO:00.1091) and non-sense-mediated mRNA decay (NMD, GO:0000184) gene ontology terms. (B) Gene ontology term enrichment network of all quantified proteins. Node color indicated adjusted p-values, edge width

indicates term similarities based on Jaccard's similarity. (C) Scatterplot of coefficients of variance (depicted as CV in %) and mean protein abundance (LFQ). Red labels and dots represent hallmark platelet proteins with highest abundances and inter-individual stability, purple labels and dots show immunoglobulins, yellow dots and labels indicated potential keratin contamination, and teal labels and dots depict proteins with highest coefficients of variance and albumin. (D) LFQ intensities of proteins covering the rang of measurements. Solid lines represent mean LFQ intensities. X-axis ticks delimit samples.

Label free quantitative MS reveals diverging and overlapping IPD proteomes

Comparing IPD patient and healthy individual platelet proteomes resulted in a total of 705 (23% of quantified) significantly differentially abundant proteins (Benjamini Hochberg adjusted P<0.05 & log fold change >1) across all IPDs. The impact on platelet proteomes varied greatly between different IPDs (Figure 3A). *TUBB1*, *MYH9, WAS* and *GFI1B* variants induced major proteomic changes resulting in >200 differentially abundant proteins whereas the *RASGRP2* variant only impacted the RASGRP2 protein itself. Not all variants translated into measurable differences in corresponding protein abundances (Figure 3B). For genetic variants in transcription factors *ETV6*, *FLI1*, *GFI1B*, *RUNX1* and growth factor *THPO*, no corresponding proteins could be detected in our data, which was most likely due to their low abundances in circulating platelets. In contrast, MYH9 protein could be detected, although no concordance was observed between the variants in *MYH9* and corresponding protein levels. For *TUBB1* a minor reduction (0.42 fold) in protein levels could be observed. These findings were not unexpected as all individuals with *MYH9* or *TUBB1* variants were heterozygous. Notably, for *GP1BB* variants we found that only the homozygous patient (DOM92, Table 1) exhibited reduced GP1BB protein levels. For *ANO6, ITGA2B*, *TUBB1* and *WAS* variants we could observe that variants resulted in altered abundances of corresponding proteins. For *FLNA* variant patient platelets, canonical FLNA protein (P21333) abundances did not differ from healthy controls. Because the *FLNA* variant in our study results in a protein with a C-terminal extension we inspected all detected FLNA peptides. In addition to peptides mapping to the canonical sequence, patient peptides also mapped to the p.Ter26487Serext101 extension (Figure 3C), thus allowing identification of this proteoform that is not present in healthy individuals. For the other cases, the protein coverages did not allow for analysis of variant peptides. Due the low probability for quantifying THPO (which is produced in the liver) or transcription factor proteins in circulating platelets we proceeded to interrogate the dataset for the presence of other IPD-specific proteins that could serve as proxy marker proteins. Correlating platelet proteome data with theoretical specific profiles for these IPDs revealed putative marker proteins for *THPO*, *ETV6*, *GFI1B*, *RUNX1* and *MYH9* consisting of Prostamide/prostaglandin F synthase (FAM213B), Insulin-like growth factor-binding protein 2 (IGFBP2), Hematopoietic progenitor cell antigen CD34 (CD34), Ras and Rab interactor 3 (RIN3), and Ras-related protein R-Ras (RRAS) respectively (Figure 3D and Supplementary Figure S2).

To assess whether different IPDs share common proteomic signatures we investigated similarities between patient proteomes. Although proteomes were diverse, heatmap visualization indicated overlap between IPDs (Figure 4A). Of the 705 differentially abundant proteins, 301 proteins were shared between multiple IPDs (Supplementary Table S2). Undirected clustering of the differentially abundant proteins showed that GFI1B, RUNX1 and THPO samples clustered together, whereas for other affected genes this clustering was not as evident (Figure 4B). To further investigate similarities between biological processes, we performed gene ontology enrichment (Figure 4C and Supplementary Table S3). In this analysis *WAS, MYH9* and *TUBB1* also enriched for similar gene ontology terms. Terms and pathways most enriched in *MYH9* and *TUBB1* samples were mitochondrial associated terms, whereas *ITGA2B* and *GFI1B* samples highly enriched for 'alpha-granule' associated GO terms. The latter is in line with the established impact of *GFI1B* deficiency on α-granules.(31)

In addition to gene-centric approaches, we compared the impact of individual variants within a single gene. Both TUBB1 (c.721C>R; R214W, N=4 versus c.751C>A; R251S, N=2), and WAS (c.360+3A>T; p.?, N=2 versus c.173C>A; P58H, N=2) variants correlated well with summarized gene ratios (figure 4D). We extended this by comparing our *GFI1B* and *RUNX1* variant proteomes with independent patient platelet samples from another study (Figures 4E and Figure S4).(32) Between *GFI1B* variants there was an overlap of 121 proteins (~50%), which included proteins that were previously shown to be affected in *GFI1B* patients (*e.g.* CD34, CXCL5, IQGAP1, NT5E).(32) For *RUNX1* the overlap with the data by van Bergen *et al.* was considerably less. It should be noted that within the van Bergen study different *RUNX1* variants also showed limited overlap. This indicates that not every variant has the same proteomic impact.

Table 1 DNA diagnosis of patients with inherited platelet disorders

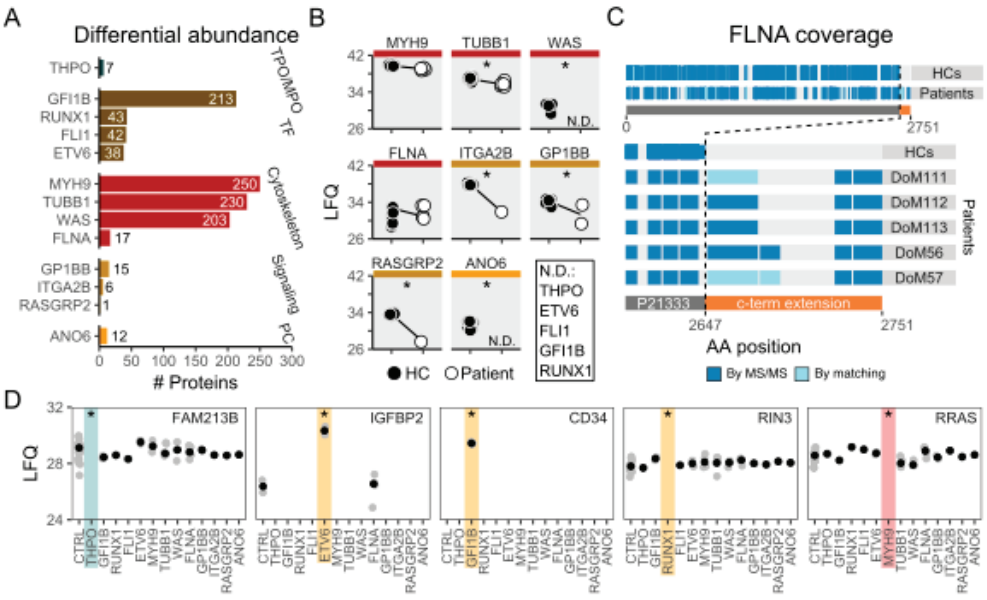
Patient	Family	Gene name	Transcript	Nucleotide	Protein	Molecular consequence	Inheritance
1	1	*TUBB1*	NM_030773.3	c.721C>T	p.(Arg241Trp)	Heterozygous missense	AD
88		*ANO6*	NM_001204803.1	c.1318C>T	p.(Arg440*)	Homozygous nonsense	AR
103	10	*ETV6*	NM_001987.4	c.1105C>T	p.(Arg369Trp)	Heterozygous missense	AD
104	10	*ETV6*	NM_001987.4	c.1105C>T	p.(Arg369Trp)	Heterozygous missense	AD
40		*FLI1*	NM_002017.4	c.297del	p.(Met100*)	Heterozygous nonsense	AD
56	5.1	*FLNA*	NM_001110556.1	c.7941_7942del	p.(*2648Ser ext.*100)	Hemizygous extension	XLR
57	5.1	*FLNA*	NM_001110556.1	c.7941_7942del	p.(*2648Ser ext.*100)	Heterozygous extension	XLD
111	5.2	*FLNA*	NM_001110556.1	c.7941_7942del	p.(*2648Ser ext.*100)	Heterozygous extension	XLD
112	5.2	*FLNA*	NM_001110556.1	c.7941_7942del	p.(*2648Ser ext.*100)	Hemizygous extension	XLR
113	5.2	*FLNA*	NM_001110556.1	c.7941_7942del	p.(*2648Ser ext.*100)	Hemizygous extension	XLR
19	3	*GFI1B*	NM_004188.4	c.814+1G>A	p.?	Heterozygous splice site variant	AD
20	3	*GFI1B*	NM_004188.4	c.814+1G>A	p.?	Heterozygous splice site variant	AD
94	3	*GFI1B*	NM_004188.4	c.814+1G>A	p.?	Heterozygous splice site	AD
66		*GP1BB*	NM_000407.4	c.410T>C	p.(Leu137Pro)	Heterozygous missense	AD
92		*GP1BB*	NM_000407.4	c.281A>G	p.(Asp94Gly)	Homozygous missense	AR
73		*ITGA2B*	NM_000419.3	c.2875G>C	p.(Ala959Pro)	Homozygous missense	AR
10	2	*MYH9*	NM_002473.4	c.5797C>T	p.(Arg1933*)	Heterozygous nonsense	AD
11	2	*MYH9*	NM_002473.4	c.5797C>T	p.(Arg1933*)	Heterozygous nonsense	AD
12	2	*MYH9*	NM_002473.4	c.5797C>T	p.(Arg1933*)	Heterozygous nonsense	AD
43		*MYH9*	NM_002473.4	c.2104C>T	p.(Arg702Cys)	Heterozygous missense	AD
84		*RASGRP2*	NM_001098670.1	c.706C>T	p.(Gln236*)	Homozygous missense	AR
86		*RUNX1*	NM_001754.4	c.319C>A	p.(Arg107Ser)	Heterozygous missense	AD

Table 1 DNA diagnosis of patients with inherited platelet disorders (*continued*)

Patient	Family	Gene name	Transcript	Nucleotide	Protein	Molecular consequence	Inheritance
51	4	*THPO*	NM_009379	c.13+1G>C	p.?	Heterozygous splice site	AD
52	4	*THPO*	NM_009379	c.13+1G>C	p.?	Heterozygous splice site	AD
47	1	*TUBB1*	NM_030773.3	c.721C>T	p.(Arg241Trp)	Heterozygous missense	AD
48	1	*TUBB1*	NM_030773.3	c.721C>T	p.(Arg241Trp)	Heterozygous missense	AD
49	1	*TUBB1*	NM_030773.3	c.721C>T	p.(Arg241Trp)	Heterozygous missense	AD
64	6	*TUBB1*	NM_030773.3	c.751C>A	p.(Arg251Ser)	Heterozygous missense	AD
65	6	*TUBB1*	NM_030773.3	c.751C>A	p.(Arg251Ser)	Heterozygous missense	AD
30		*WAS*	NM_000377.2	c.399G>C	p.(Glu133Asp)	Hemizygous missense	XLR
77	8	*WAS*	NM_000377.2	c.360+3A>T	p.?	Hemizygous splice site	XLR
78	8	*WAS*	NM_000377.2	c.360+3A>T	p.?	Hemizygous splice site	XLR
79	9	*WAS*	NM_000377.7	c.173C>A	p.(Pro58His)	Hemizygous missense	XLR
80	9	*WAS*	NM_000377.7	c.173C>A	p.(Pro58His)	Hemizygous missense	XLR

Abbreviations AD = Autosomal dominant, AR = Autosomal recessive, XLD = x-linked dominant, XLR= X-linked recessive, XLT= X-linked thrombocytopenia

Figure 3 Proteomic impact of platelet disorders on platelet proteome



Abbreviations DOM = study patients identifier, IPD = inherited platelet disorder, MS = mass-spectrometry, ND = not detected, tNGS = targeted next-generation sequencing

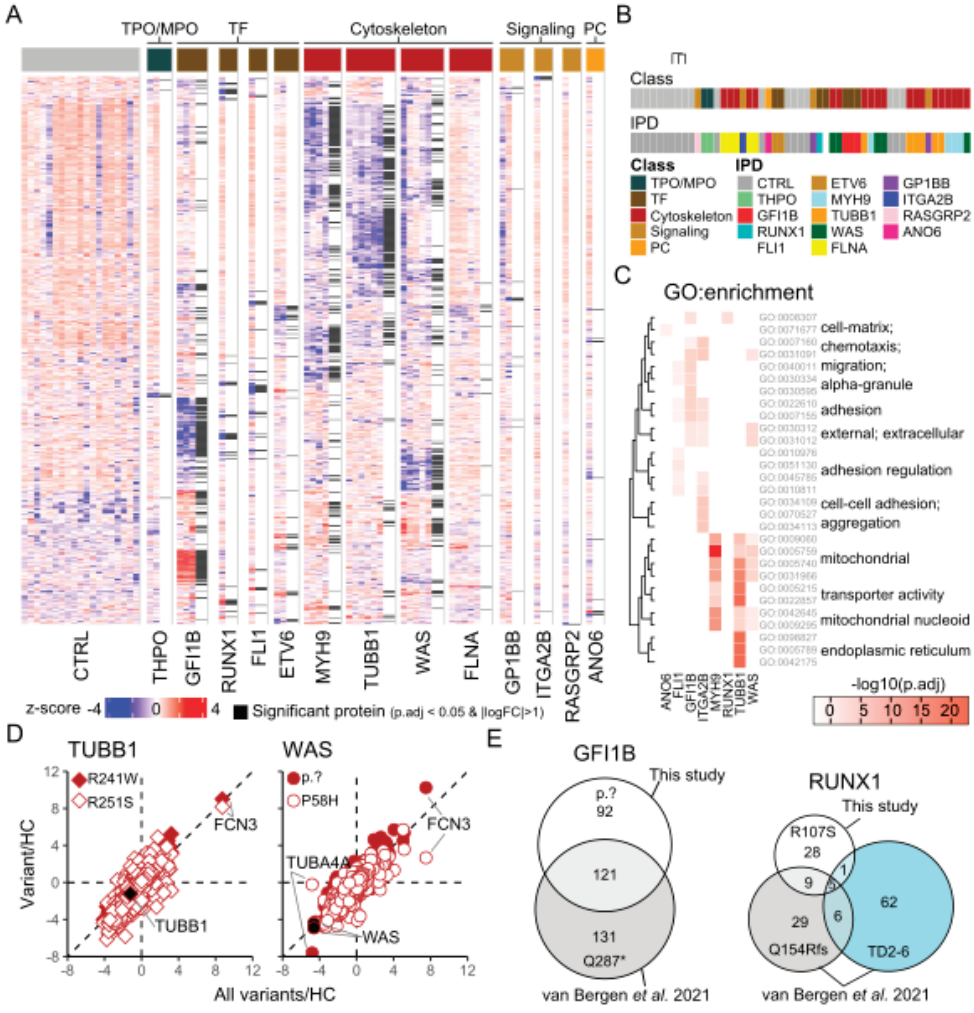
Legend (A) Bar plots of the amount of statistically differentially abundant proteins between platelets of inherited platelet disorders and controls per affected *TIER1* gene. (B) Dot plot of proteins in which a variant was determined by tNGS. Closed circles: healthy individuals, and open circles: patients. (C) Graphic of protein coverage of FLNA (p21333, gray) and the c-terminal extension (orange) in FLNA patients. Zoom shows c-terminal region with peptides mapping to p.Ter2648SerextTer1010. Blue represents peptides identified by MS/MS and light blue identified by MaxQuant matching. (D) Dot plots of putative marker proteins for IPDs for *THPO, GFI1B*, *RUNX1*, *ETV6* and *MYH9*. Selections of proteins that exhibiting absolute correlation coefficients >0.7 with IPD-specific theoretical profiles are shown (See Figure S2). Shaded rectangles indicated for which IPD a protein was considered specific. Gray dots show individual data points; black dots represent medians. *Benjamin Hochberg adjusted p>.05 and |logFC|>1

Protein dynamics cluster functional aspects and complex memberships

Based on our previous observation that protein dynamics in contrasting conditions can reveal/infer protein function or complex membership,(22) we clustered the dynamics of all 3073 quantified proteins using co-expression analysis. Weighted gene co-expression network analysis (WGCNA) resulted in 18 modules (MEs) consisting of 23 to 1,073 proteins. By combining the WGCNA module allocations and 9,443,329 pairwise Pearson coefficients we constructed a correlation map of the platelet's proteomes in this study (Figure 5A). To determine which biological processes are associated with these 18 modules, we performed GO term enrichment (Figure 5B and Supplementary Table S4). This showed that the largest module (ME1) was enriched for mitochondrial and membrane GO terms, and that the second largest module (ME2) enriched for catabolic process terms, indicating that a large amount of platelet proteins was associated with these functions. Both modules ME8 and ME10 were enriched for the GO-term alpha granule proteins, which was greatest for module ME8 ($P<2.7 \times 10^{-17}$ versus $P<1.1 \times 10^{-4}$ for ME8 and ME10, respectively). Based on the importance of alpha granules for platelet effector functions, we further investigated module ME8. Proteins in this module with and without alpha granule annotations showed a similar range of module membership scores (Figure 5C), indicating that latter proteins also include putative alpha granule constituents or associated proteins (*e.g*. Latent-transforming growth factor beta-binding protein 1 (LTBP1), Angiopoietin-1 (ANGPT1) and C-C motif chemokine 5 (CCL5).(33, 34)

By annotating our data using the 'Complex Portal' resource,(35) we observed that the Pearson coefficient distribution of proteins of known complexes was shifted towards higher positive correlation coefficients ($P<2 \times 10^{-16}$, Supplementary Figure S3). Next, we selected proteins with correlation coefficients >0.6 and visualized these in a network (Figure 5D). In line with previous reports,(36) we found reduced levels of fibrinogen complex (FGA, FGB, FGG, CPX-1922) and Integrin alphaIIb-beta3 complex (ITGA2B and ITGB3, CPX-1799) members in the *ITGA2B* variant patient (Figure 5E). We also observed disruption of GP5 complex (GP1BA, GP1BB, GP5 and GP9, CPX-114) in *GP1BB* patients (Figure 5E), which was most pronounced for the homozygous *GP1BB* patient (DOM92). Therefore, these analyses show that harnessing the diversity of IPDs provides a solid basis for inferring platelet protein function and uncovers the current limitations in gene ontology annotations and protein classification.

Figure 4 Shared and IPD specific response and processes



Abbreviations GO = gene ontology, HC = healthy control

Legend (A) Heatmap of 705 differentially abundant proteins (Benjamin Hochberg adjust p<.05 and |logFC|>1. Gradient indicated z-score (low: blue, high: red). Black tiles indicate for which IPD a protein was differentially abundant. Column annotations show IPD class (Teal: TPO/MPO, brown: TF, red: cytoskeleton, beige: signaling, orange: PC). (B) Dendrogram of the differentially abundant proteins between IPDs. Color blocks indicate IPD class of *TIER1* genes (Light green: THPO, red: GF1B, light pink: RASGRP2, pink: ANO6). (C) Tile plot of GO-enrichment terms. Row annotations show GO IDs and high frequency keywords. Lines indicate clusters. Gradient indicates p-adjusted value (low: white, high: red). (D) Scatter plot showing variant specific ratios (y-axis) compared with gene-centric (x-axis) ratios between patients and controls. Diamonds indicated *TUBB1* variants (open: R251S variant, closed: R241W variant). Circles indicated *WAS* variants (open: P58H, closed p.?). (E) Comparison of proteomic impact of variants of GFI1B and RUNX1 in the present study and van Bergen et al.(33) Venn-diagram shows the number of overlapping significant proteins. Scatter plot of the median log-fold change of patients vs controls of overlapping proteins

DISCUSSION

Here we applied MS-based label free proteomics to study the impact of IPDs on the platelet proteome. In contrast to current literature on IPDs using MS-based proteomics,(36-38) our study includes a wide range of IPDs that enables the concurrent investigation of proteomic effects of platelet disorders with different etiologies. We found that the phenotypical heterogeneity between IPDs is also present at the proteome level. Although ~1/3 of the platelet proteome was altered across all IPDs in our study, most IPDs exhibited a limited impact on the platelet proteome.

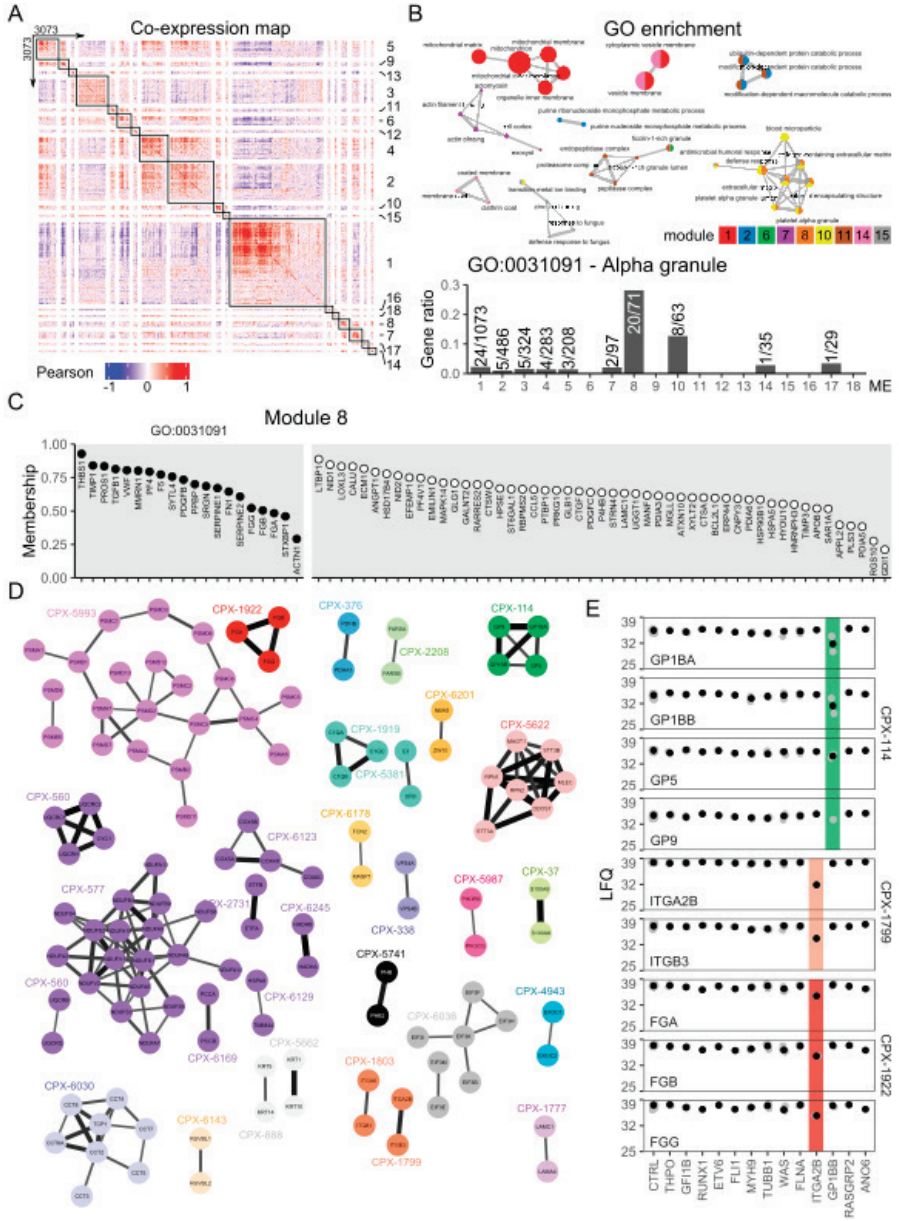
The largest effects were due to variants in cytoskeletal proteins, which altered up to ~8% of quantified proteins. MYH9, WAS and TUBB1 are associated with proplatelet formation,(39) which may explain their large impact. In contrast, *FLNA* resulted in only 17 altered protein abundances. Whereas *FLNA* variants may either impact platelet development or activation,(40) the *FLNA c-terminal extension* genetic variant in our study was previously associated with enhanced effector functions rather than disrupted platelet formation.(19) In a recent study, the proteomic effects of variants in *GFI1B*, *RUNX1* and *GATA2* transcription factors were assessed.(32) In line with this study, we show that the *GFI1B* variant had a larger impact than other transcription factor defects. GFI1B deficiency is well established in regard to its effects on the biosynthesis of protein rich platelet alpha-granules.(41) In contrast, deficiencies in *RUNX1* and *FLI1* were described to impact dense granules,(42, 43) which are low in protein content. Furthermore, ETV6 was recently suggested to play a role in terminal megakaryopoiesis, although no large phenotypic effects were observed other than thrombocytopenia.(44) This distinction between monogenetic to mono/oligo-proteomic versus poly-proteomic effects implies that the extent of anomalies in a platelet proteome is not necessarily indicative of disease severity. Illustrative of this, in a recent study that included 38 patients from 9 families with *TUBB1* variants (with 230 significant proteins in our study) no severe bleeding phenotype was observed.(45) In contrast, Glanzmann thrombasthenia (*ITGA2B*, 6 significant proteins in our study) is associated with a severe bleeding phenotype.(46) In addition, the impact on the platelet proteome differed between homozygous versus heterozygous variant carriers, illustrated by the *GP1BB* patients in which GP-complex proteins were less affected in the heterozygous individual.

The platelet proteomic profiles of the diverse array of IPDs incorporated in our study provided a fundament to study inter-protein dynamics that underly platelet functions. This allowed us to identify disruption of protein complexes. In Glanzmann Thrombasthenia platelets, we observed reduced ITGA2B and ITGB3 abundances. Consequently we also found fibrinogen (FGA, FGB and FGG) in reduced amounts in line with established findings that this complex is internalized from the circulation by these integrin receptors.(36, 47)

Moreover, co-expression clustering combined with GO term analysis highlighted distinct protein groups and enriched processes. The largest set of highly correlating proteins described mitochondrial processes, which emphasizes the integral role mitochondria play in platelet metabolism and function.(48) The dynamics summarized by module ME8 highly enriched for alpha granule annotated proteins (Figure 5). Apart from well-known platelet alpha granule constituents (*e.g.* Thrombospondin-1, von Willebrand factor and platelet factor 4), this module included proteins that despite lack of prior annotation have been associated with alpha granules. Using MS analysis to investigate alpha-granule enriched subcellular fractions, Maynard *et al.*(49) previously identified ANGPT1, CCL5, CTFG as putative alpha granule constituents, which in our analysis were all members of ME8. In another study, these authors convincingly showed that latent-transforming growth factor beta-binding protein 1 (LTBP1) is present within platelet alpha granules.(50) Therefore, the dynamics in module ME8 are indicative of alpha granule associated proteins.

Our study has limitations. Our centrifugation-based isolation protocol, although not excluding large platelets, exhibited a bias towards platelets with a smaller volume. Although the study population consisted of children and affected relatives, all control samples were obtained from adults. Therefore, apart from IPD impact age may have influenced platelet proteomes. Depending on the IPD, we were able to include a limited number of individuals, which impacts the findings we present. A shortcoming of our approach is the relatively limited recovery of low-abundant proteins due to constraints in the dynamic range of current untargeted MS methods. In cases of low abundance, proteins may be below the detection limit (*e.g.* transcription factors). In addition, proteins that have functions during platelet formation in the bone marrow may also be absent in mature platelets. Targeted proteomics may partially overcome this and have proven to be reliable in terms of accuracy and quantification. However, the amount of simultaneously measured target proteins is substantially less than in discovery based untargeted MS.(37) Recently, Malchow *et al.* applied targeted MS to quantify 99 IPD related proteins.(51) To expand upon this, the platelet proteomes we present in this study may be used as a guide for refinement. For instance, for IPDs that lack a match between a genetic variation and the corresponding protein, putative marker proteins could be targeted to identify these disorders (*e.g.* RIN3 for *RUNX1* and CD34 for *GFI1B*). Although needing further validation, *e.g.* the observation of enhanced platelet CD34 levels in the GFI1B variant is in line with multiple other studies in GFI1B patients.(52, 53) Furthermore it should be noted that applying MS-based proteomics is more suited to elucidate thrombocytopathy rather than thrombocytopenia, in which platelet numbers are affected but not their composition.

Figure 5 Protein dynamics cluster according to IPD and platelet functional aspects.



Abbreviations GO = gene ontology, WGCNA = weighted gene co-expression network analysis

Legend (A) Heatmap of Pearson correlation coefficient of the pairwise comparison of 3073 quantified proteins in this study. Column and row splits are based on WGCNA defined functional modules, indicated with numbers. Color gradient indicated Pearson correlation coefficients. (blue: -1, white: 0, red: +1). (B) GO-term enrichment on functional modules. Colors indicated module. Node size indicates amount of proteins per GO term annotations. (C) The importance of alpha granules for platelet effector functions was further investigated in the ME8 module with and without alpha granule annotations. It showed a similar range of module membership scores, indicating that latter proteins also include putative alpha granule constituents or associated proteins (*e.g.* Latent-transforming growth factor beta-binding protein 1 (LTBP1), Angiopoietin-1 (ANGPT1) and C-C motif chemokine 5 (CCL5)) (D) Selection of proteins with correlation coefficients >0.6. (E) Visualization in a network reveals reduced levels of fibrinogen complex (FGA, FGB, FGG, CPX-1922) and Integrin alphaIIb-beta3 complex (ITGA2B and ITGB3, CPX-1799) members in the *ITGA2B* variant patient; as well as a disruption of GP5 complex (GP1BA, GP1BB, GP5 and GP9, CPX-114) in Bernard-Soulier patient with a homozygous *GP1BB* variant

In conclusion, in this study we demonstrated a proteomics perspective that complements DNA-based IPD diagnosis. Moreover, by integrating disorders that span the genetic landscape of platelet development, we dissected biological contexts of protein alterations that may aid in the development of deeper insights into the molecular backgrounds of IPDs and platelet biology in general.

ACKNOWLEDGMENTS

The work presented here was supported by the Landsteiner Foundation for Blood Transfusion Research [LSBR-1517 to M.v.d.B., LSBR-1923 to M.v.d.B. and A.J.H]. We acknowledge all the patients and family members who contributed to this research. The authors would like to thank the people from the research facility of Sanquin for helping with isolation of platelets.

REFERENCES

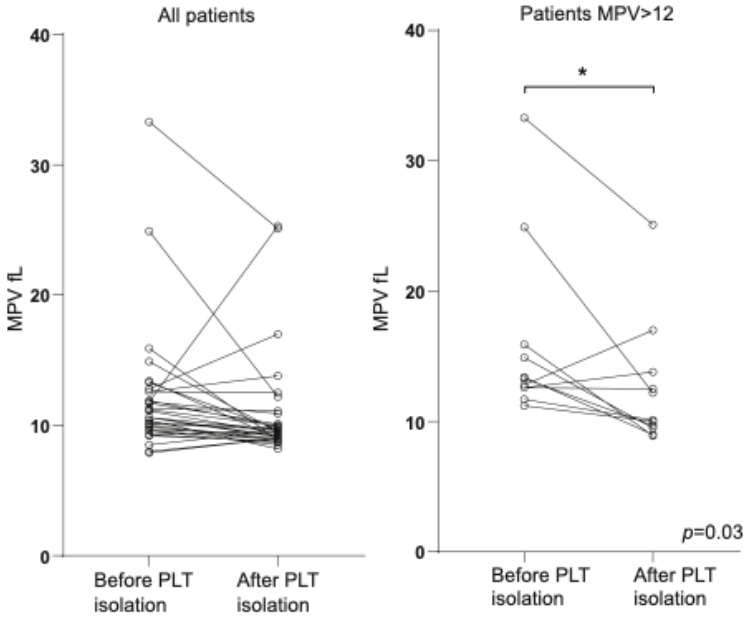
1. Golebiewska EM, Harper MT, Williams CM, Savage JS, Goggs R, von Mollard GF, Poole AW. Syntaxin 8 Regulates Platelet Dense Granule Secretion, Aggregation, and Thrombus Stability. J Biol Chem. 2015;290(3):1536-45.
2. Gianazza E, Brioschi M, Baetta R, Mallia A, Banfi C, Tremoli E. Platelets in Healthy and Disease States: From Biomarkers Discovery to Drug Targets Identification by Proteomics. International Journal of Molecular Sciences. 2020;21(12).
3. Lentaigne C, Freson K, Laffan MA, Turro E, Ouwehand WH, Consortium B-B, the ThromboGenomics C. Inherited platelet disorders: toward DNA-based diagnosis. Blood. 2016;127(23):2814-23.
4. Downes K, Megy K, Duarte D, Vries M, Gebhart J, Hofer S, et al. Diagnostic high-throughput sequencing of 2396 patients with bleeding, thrombotic, and platelet disorders. Blood. 2019;134(23):2082-91.
5. Bastida JM, Lozano ML, Benito R, Janusz K, Palma-Barqueros V, Del Rey M, et al. Introducing high-throughput sequencing into mainstream genetic diagnosis practice in inherited platelet disorders. Haematologica. 2018;103(1):148-62.
6. Megy K, Downes K, Morel-Kopp MC, Bastida JM, Brooks S, Bury L, et al. GoldVariants, a resource for sharing rare genetic variants detected in bleeding, thrombotic, and platelet disorders: Communication from the ISTH SSC Subcommittee on Genomics in Thrombosis and Hemostasis. Journal of Thrombosis and Haemostasis. 2021;19(10):2612-7.
7. Smedley D, Smith KR, Martin A, Thomas EA, McDonagh EM, Cipriani V, et al. 100,000 Genomes Pilot on Rare-Disease Diagnosis in Health Care - Preliminary Report. New Engl J Med. 2021;385(20):1868-80.
8. Stalker J timothy, Newman K debra, Ma Peisong, Wannemacher M Kenneth BFL. Platelet Signaling. Handb Exp Pharmacol. 2012;210(210):59-85.
9. Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, et al. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. Blood. 2012;120(15):E73-E82.
10. McRedmond JP, Park SD, Reilly DF, Coppinger JA, Maguire PB, Shields DC, Fitzgerald DJ. Integration of proteomics and genomics in platelets - A profile of platelet proteins and platelet-specific genes. Mol Cell Proteomics. 2004;3(2):133-44.
11. Huang JN, Swieringa F, Solari FA, Provenzale I, Grassi L, De Simone I, et al. Assessment of a complete and classified platelet proteome from genome-wide transcripts of human platelets and megakaryocytes covering platelet functions. Sci Rep-Uk. 2021;11(1).
12. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine. 2015;17(5):405-24.
13. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. Journal of Thrombosis and Haemostasis. 2013;11(6):1183-9.
14. Munnix ICA, Van Oerle R, Verhezen P, Kuijper P, Hackeng CM, Hopman-Kerkhoff HIJ, et al. Harmonizing light transmission aggregometry in the Netherlands by implementation of the SSC-ISTH guideline. Platelets. 2021;32(4):516-23.

15. Rappsilber J, Ishihama Y, Mann M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem. 2003;75(3):663-70.
16. Deutsch EW, Csordas A, Sun Z, Jarnuczak A, Perez-Riverol Y, Ternent T, et al. The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. Nucleic Acids Res. 2017;45(D1):D1100-D6.
17. Perez-Riverol Y, Csordas A, Bai JW, Bernal-Llinares M, Hewapathirana S, Kundu DJ, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Research. 2019;47(D1):D442-D50.
18. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: A Peptide Search Engine Integrated into the MaxQuant Environment. J Proteome Res. 2011;10(4):1794-805.
19. Berrou E, Adam F, Lebret M, Planche V, Fergelot P, Issertial O, et al. Gain-of-Function Mutation in Filamin A Potentiates Platelet Integrin alpha(IIb)beta(3) Activation. Arterioscler Thromb Vasc Biol. 2017;37(6):1087-97.
20. Phipson B, Lee S, Majewski IJ, Alexander WS, Smyth GK. Robust Hyperparameter Estimation Protects against Hypervariable Genes and Improves Power to Detect Differential Expression. Ann Appl Stat. 2016;10(2):946-63.
21. Yu GC, Wang LG, Han YY, He QY. clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters. Omics. 2012;16(5):284-7.
22. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. Bmc Bioinformatics. 2008;9.
23. Scheller I, Beck S, Göb V, Gross C, Neagoe RAI, Aurbach K, et al. Thymosin β4 is essential for thrombus formation by controlling the G-actin/F-actin equilibrium in platelets. Haematologica. 2022;107(12):2846-58.
24. Wijten P, van Holten T, Woo LL, Bleijerveld OB, Roest M, Heck AJR, Scholten A. High Precision Platelet Releasate Definition by Quantitative Reversed Protein Profiling-Brief Report. Arterioscl Throm Vas. 2013;33(7):1635-8.
25. Lagarrigue F, Paul DS, Gingras AR, Valadez AJ, Sun H, Lin J, et al. Talin-1 is the principal platelet Rap1 effector of integrin activation. Blood. 2020;136(10):1180-90.
26. Petrich BG, Marchese P, Ruggeri ZM, Spiess S, Weichert RAM, Ye F, et al. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. J Exp Med. 2007;204(13):3103-11.
27. Stefanini L, Bergmeier W. RAP GTPases and platelet integrin signaling. Platelets. 2019;30(1):41-7.
28. El-Gedaily A, Schoedon G, Schneemann M, Schaffner A. Constitutive and regulated expression of platelet basic protein in human monocytes. J Leukocyte Biol. 2004;75(3):495-503.
29. Al Hawas R, Ren QS, Ye SJ, Karim ZA, Filipovich AH, Whiteheart SW. Munc18b/STXBP2 is required for platelet secretion. Blood. 2012;120(12):2493-500.
30. Lee H, Chae S, Park J, Bae J, Go EB, Kim SJ, et al. Comprehensive Proteome Profiling of Platelet Identified a Protein Profile Predictive of Responses to An Antiplatelet Agent Sarpogrelate. Mol Cell Proteomics. 2016;15(11):3461-72.
31. Noris P, Pecci A. Hereditary thrombocytopenias: a growing list of disorders. Hematol-Am Soc Hemat. 2017:385-99.
32. Van Bergen MGJM, Marneth AE, Hoogendijk AJ, Van Alphen FPJ, Van den Akker E, Laros-Van Gorkom BAP, et al. Specific proteome changes in platelets from individuals with GATA1-, GFI1B-, and RUNX1-linked bleeding disorders (vol 138, pg 86, 2021). Blood. 2021;138(8):739-.

33. Machlus KR, Johnson KE, Kulenthirarajan R, Forward JA, Tippy MD, Soussou TS, et al. CCL5 derived from platelets increases megakaryocyte proplatelet formation. Blood. 2016;127(7):921-6.
34. Chanzu H, Lykins J, Wigna-Kumar S, Joshi S, Pokrovskaya I, Storrie B, et al. Platelet α-granule cargo packaging and release are affected by the luminal proteoglycan, serglycin. Journal of Thrombosis and Haemostasis. 2021;19(4):1082-95.
35. Combe CW, Sivade M, Hermjakob H, Heimbach J, Meldal BHM, Micklem G, et al. ComplexViewer: visualization of curated macromolecular complexes. Bioinformatics. 2017;33(22):3673-5.
36. Loroch S, Trabold K, Gambaryan S, Reiss C, Schwierczek K, Fleming I, et al. Alterations of the platelet proteome in type I Glanzmann thrombasthenia caused by different homozygous delG frameshift mutations in ITGA2B. Thromb Haemost. 2017;117(3):556-69.
37. Solari FA, Mattheij NJ, Burkhart JM, Swieringa F, Collins PW, Cosemans JM, et al. Combined Quantification of the Global Proteome, Phosphoproteome, and Proteolytic Cleavage to Characterize Altered Platelet Functions in the Human Scott Syndrome. Mol Cell Proteomics. 2016;15(10):3154-69.
38. Winkler W, Zellner M, Diestinger M, Babeluk R, Marchetti M, Goll A, et al. Biological variation of the platelet proteome in the elderly population and its implication for biomarker research. Mol Cell Proteomics. 2008;7(1):193-203.
39. Ghalloussi D, Dhenge A, Bergmeier W. New insights into cytoskeletal remodeling during platelet production. Journal of Thrombosis and Haemostasis. 2019;17(9):1430-9.
40. Rosa JP, Raslova H, Bryckaert M. Filamin A: key actor in platelet biology. Blood. 2019;134(16):1279-88.
41. Stevenson WS, Morel-Kopp MC, Chen Q, Liang HP, Bromhead CJ, Wright S, et al. GFI1B mutation causes a bleeding disorder with abnormal platelet function. J Thromb Haemost. 2013;11(11):2039-47.
42. Glembotsky AC, Bluteau D, Espasandin YR, Goette NP, Marta RF, Oyarzun CPM, et al. Mechanisms underlying platelet function defect in a pedigree with familial platelet disorder with a predisposition to acute myelogenous leukemia: potential role for candidate RUNX1 targets. Journal of Thrombosis and Haemostasis. 2014;12(5):761-72.
43. Saultier P, Vidal L, Canault M, Bernot D, Falaise C, Pouymayou C, et al. Macrothrombocytopenia and dense granule deficiency associated with FLI1 variants: ultrastructural and pathogenic features. Haematologica. 2017;102(6):1006-16.
44. Melazzini F, Palombo F, Balduini A, De Rocco D, Marconi C, Noris P, et al. Clinical and pathogenic features of ETV6-related thrombocytopenia with predisposition to acute lymphoblastic leukemia. Haematologica. 2016;101(11):1333-42.
45. Palma-Barqueros V, Bury L, Kunishima S, Lozano ML, Rodríguez-Alen A, Revilla N, et al. Expanding the genetic spectrum of related thrombocytopenia. Blood Advances. 2021;5(24):5453-67.
46. Botero JP, Lee K, Branchford BR, Bray PF, Freson K, Lambert MP, et al. Glanzmann thrombasthenia: genetic basis and clinical correlates. Haematologica. 2020;105(4):888-94.
47. Disdier M, Legrand C, Bouillot C, Dubernard V, Pidard D, Nurden AT. Quantitation of platelet fibrinogen and thrombospondin in Glanzmann's thrombasthenia by electroimmunoassay. Thromb Res. 1989;53(6):521-33.
48. Melchinger H, Jain K, Tyagi T, Hwa J. Role of Platelet Mitochondria: Life in a Nucleus-Free Zone. Front Cardiovasc Med. 2019;6:153.
49. Maynard DM, Heijnen HF, Horne MK, White JG, Gahl WA. Proteomic analysis of platelet alpha-granules using mass spectrometry. J Thromb Haemost. 2007;5(9):1945-55.

50. Maynard DM, Heijnen HFG, Gahl WA, Gunay-Aygun M. The α-granule proteome: novel proteins in normal and ghost granules in gray platelet syndrome. Journal of Thrombosis and Haemostasis. 2010;8(8):1786-96.
51. Malchow S, Loosse C, Sickmann A, Lorenz C. Quantification of Cardiovascular Disease Biomarkers in Human Platelets by Targeted Mass Spectrometry. Proteomes. 2017;5(4).
52. Rabbolini DJ, Morel-Kopp MC, Chen Q, Gabrielli S, Dunlop LC, Chew LP, et al. Thrombocytopenia and CD34 expression is decoupled from -granule deficiency with mutation of the first growth factor-independent 1B zinc finger. Journal of Thrombosis and Haemostasis. 2017;15(11):2245-58.
53. Palareti G, Legnani C, Cosmi B, et al. Thrombocytopenia and CD34 expression is decoupled from α-granule deficiency with mutation of the first GFI1B zinc finger. Int J Lab Hematol. 2016;38(1):42-49.

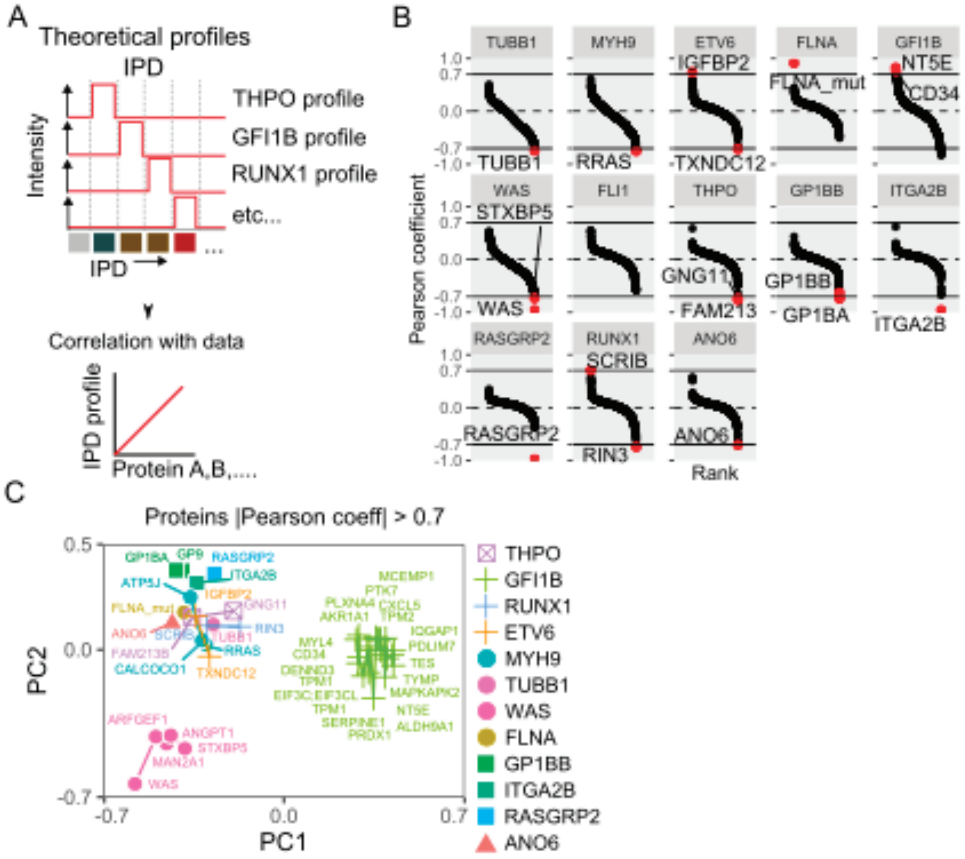
Supplementary Figure S1 Levels of platelet's mean platelet volume



Abbreviation MPV = mean platelet volume

Legend MPV levels on arrival of blood in our laboratory and after platelet isolation of entire patient population (left panel) and of patients with MPV >12 fL at inclusion (right panel)

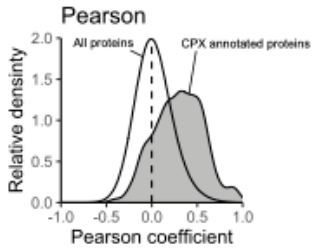
Supplementary Figure S2 Scheme showing theoretical profile approach



Abbreviations IPD = inherited platelet disorder, LFQ = label-free quantification, PC(A) = principle component (analysis)

Legend (A) To identify proteins of which the intensity values associate with a given IPD, we defined theoretical profiles in which intensities are high for a given IPD and low in all other samples (e.g. a LFQ value of 30 in *GFI1B* samples and a LFQ value of 20 in alle other samples). (B) Theoretical profiles defined in (A) were correlated with all quantified proteins. (C) PCA of proteins with absolute Pearson correlation coefficient >0.7 for an IPD specific profile

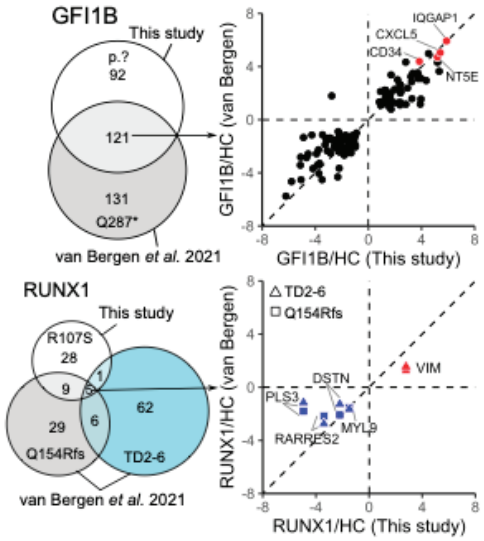
Supplementary Figure S3 Density plot of Pearson correlation coefficient for protein-protein correlations



Abbreviations Carboxy-terminal domain protein kinase complex

Legend White: distribution of all correlation coefficients. Gray: distribution of correlation coefficients or proteins that are part of protein complexes, annotated using EMBL-EBI complex portal resource.

Supplementary Figure S4 Comparison of two studies investigating the impact of genetic variation in *GFI1B* and *RUNX1* on platelet proteomes



Abbreviations HC = healthy control

Legend Venn-diagram shows overlap between differentially abundant proteins of this study and Van Bergen et al. Scatter plots show the median log-fold change of patients vs controls of overlapping proteins

All things excellent are as difficult as they are rare

Baruch Spinoza (1632 - 1677)

5 Interstitial 11q deletions and terminal 11q duplications cause a bleeding tendency due to platelet dysfunction that is similar to 11q deletions causing Jacobsen syndrome

E.J. Huisman
V.A.S.H. Dalm
A.M. Joosten
F.J. Smiers
L. Porcelijn
A. Hoogendijk
H. Janssen
N.N. Van der Wel
I.C.L. Kremer Hovinga
M. de Haas
M.H. Cnossen

European Journal of Haematology 2025 Jul 22 – Online ahead of print

ABSTRACT

Introduction

Jacobsen syndrome, resulting from a terminal deletion of chromosome 11 (11q), may lead to an increased bleeding tendency due to low platelet counts or platelet dysfunction. Currently, information on bleeding tendency and platelet function in patients with nonterminal 11q aberrations such as larger deletions, interstitial 11q deletions or 11q duplications is lacking.

Methods

We investigated the bleeding symptoms in relation to platelet numbers and function in 14 patients: 10 patients with a terminal 11q deletion, one terminal 11q duplication, two interstitial 11q deletions, and one with an interstitial 11q duplication.

Results

Twelve patients reported bleeding complications (12/14, 86%), most frequently perioperative bleeding (21%), hematomas (17%), epistaxis (13%), prolonged bleeding and severe bruising (11%) and heavy menstrual bleeding in 4/5 postmenarcheal women (80%). Seven (50%) had platelet counts below normal values (median 62 x 10^9/L). Strikingly, the seven patients with a normal platelet count reported more frequent perioperative bleeding complications as well as atypical bleeding such as intramuscular hematoma after injection than the group with low platelet counts. Platelet dysfunction was detected in all (100%) patients by Light Transmission Aggregometry. Mass-spectrometry analysis showed an overrepresentation of NUBP2 and ATP5S, both involved in mitochondrial ATP synthesis. Electron-microscopy confirmed abnormal granules in some.

Conclusions

We demonstrated increased bleeding tendency due to platelet disorders in the majority of patients with terminal 11q deletions but also in nonterminal 11q aberrations. This is confirmed by abnormal platelet function tests and mass-spectrometry. We therefore recommend screening of patients with all forms of 11q disorders and, when indicated, preventive preoperative measurements and counselling for heavy menstrual bleeding in females.

Key words

Blood coagulation disorders [MESH], Blood platelet disorders [MESH], Chromosome 11q, trisomy [MESH]; Jacobsen Distal 11q Deletion Syndrome [MESH], Mass-spectrometry [MESH], microscopy, electron [MESH]; Platelet granule deficiency disorder [MESH]

Novelty statements

- New aspects of this study involve the finding that: "Patients with non-terminal 11q deletions as well as terminal 11q duplications have a similar haematological phenotype with increased bleeding tendency and platelet abnormalities as the better-known terminal 11q deletions (Jacobsen syndrome)"
- Central finding of this study is that: "Platelet dysfunction is highly prevalent in patients with any kind of 11q aberration leading to a spectrum of bleeding symptoms, as well as common once like hematomas or perioperative bleedings, as well as uncommon once as intramuscular bleeding"
- Clinical relevance of this study is that: "despite a normal platelet number, platelet dysfunction should always be taken into account in patients with any form of 11q aberrations unless platelet dysfunction is ruled out."

INTRODUCTION

Partial deletions or duplications of the long arm of chromosome 11 (11q) are rare and may involve various chromosomal regions. These chromosomal abnormalities range in size from 1-20 Mb,(1) and can occur with concomitant abnormalities in other chromosomes.(2, 3) Most frequently a terminal 11q deletion occurs, but interstitial deletions as well as interstitial and terminal duplications of 11q are reported as well.(4) Breakpoints arising within or distal to subband 11q23.3 with a deletion extending to the telomere lead to a clinical entity known as Jacobsen syndrome.(5, 6) The estimated incidence of Jacobsen syndrome is 1:100 000 births with a female:male ratio of 2:1.(1) Incidences of other 11q aberrations are unknown but considered to occur even more rarely. The clinical phenotype of terminal 11q deletions depends on the location and extent of the chromosomal abnormality and includes: cognitive impairment, cardiac malformation, increased susceptibility to infections and an increased bleeding tendency.(1) This bleeding tendency is most probably due to low platelet counts and/or platelet dysfunction, and generally leads to mucosal and perioperative bleedings, but cerebral bleeding has also been reported.(7) Typically giant alpha granules may be observed by electron microscopy (EM); known as Paris-Trousseau syndrome.(8-11) Abnormal dense granules (12), as well as micro-karyocytes have also been described in bone marrow specimens in a few cases with Jacobsen syndrome.(10, 13) Detailed data on bleeding phenotype, platelet numbers and platelet function in other 11q disorders is lacking. We therefore investigated the clinical phenotype and laboratory results of patients with a variety of 11q aberrations.

METHODS

Patient population and study design

Patients were invited for this study by their treating physician or by the Dutch Chromosome 11 Network. Medical Ethical Committee approval was obtained (MEC-2013-026, Erasmus MC, University Medical Center Rotterdam). The study was performed according to the Declaration of Helsinki. All patients were included after patient or parental written informed consent according to national law and regulations.

Clinical data included patient characteristics including diagnosis based on historical molecular analysis, and medical history including comorbidities, bleeding symptoms, hemostatic treatment and transfusions. Data were derived from electronic patient files. Bleeding symptoms were documented in detail but also scored using the validated bleeding assessment tool of the International Society of Thrombosis and Hemostasis (ISTH-BAT). The ISTH-BAT contains 13 items. Per item a score is given. This score reflects the presence (none = score 0) and severity of bleeding (score 1-4). The cut-off values for an abnormal bleeding score per individual is validated as the sum of all scored items

and are defined as ≥3 in children, ≥4 in adult males, and ≥6 in adult females.(14) We also determined the sum of all individual bleeding scores in a predefined subgroup, leading to a bleeding score for the subgroup as a whole, covering both frequency and severity of the bleeding item.

Laboratory tests

Retrospectively, platelet counts and hemostatic test results were collected. Prospectively the following laboratory results were measured at inclusion: blood count, platelet indices, thrombopoietin (TPO), hemostatic tests, platelet function, and platelet proteome. Blood cell count and platelet indices (Sysmex XN-9100, Netherlands) were measured in EDTA. Serum was used for measuring TPO analyzed with an in-house analysis (ELISA-assay, Sanquin). Citrate-blood was used for aPTT (Actin FS), PT (Thromborel S) and fibrinogen (Thrombin Reagent; all three Sysmex CS5100, Siemens Healthcare, Germany), von Willebrand factor antigen (VWF:Ag) levels were measured by an in-house ELISA assay using polyclonal rabbit antihuman VWF antibodies (DakoCytomation, Denmark) for capturing and detection; VWF activity (VWF:Act) on the Innovance VWF Act-assay (Sysmex CS-5100, Siemens); and VWF collagen binding (VWF:CB) activity by an in house ELISA assay using bovine achilles tendon collagen type I for capturing (Sigma-Aldrich) and polyclonal rabbit antihuman VWF antibodies (DakoCytomation) for detecting. Factor VIII activity (FVIII:Act) was measured using one-stage clotting assays (APTT-based, Sysmex CS-5100, Siemens). Results were considered normal when test results were in p5-p95 interval of the age-references indicated. Platelet function analyzer (PFA-200) was measured for collagen-epinephrine (PFA-EPI) and, following in house protocol, when closure time of the PFA-EPI was prolonged, collagen-ADP (PFA-ADP; INNOVANCE® PFA-200® System, Siemens, Germany). Light transmission Aggregometry (LTA) was performed on a Chrono-Log aggregometer 490 (Stago Benelux) using the following agonists: collagen (2 µg/mL), U46619 (4 µM), thrombin (40 U/mL) before July 2019 and TRAP (30 µM) from July 1st 2019, ADP (5 and 10 µM), epinephrin (5 µM), low and high concentration of ristocetin (0.31 en 1.25 mg/mL), arachidonic acid (1 µM).(15) Response was considered normal when the activation wave reached ≥50% as final amplitude for weak agonists (ADP and epinephrin) and ≥70% for strong agonists (Collagen, TRAP6, high Ristocetin, Arachidonic acid, U46199) and the response was not reversible, no second wave was visible and response to Ristocetin low was <10%.(15)

Mass-spectrometry and Electron Microscopy

Methods of Mass-spectrometry (MS) and Electron Microscopy (EM) imaging are described in detail in Supplement 1.

Statistical analysis

Descriptive statistics were used to summarize baseline characteristics of the study population. In case of a skewed distribution, data are presented as median and

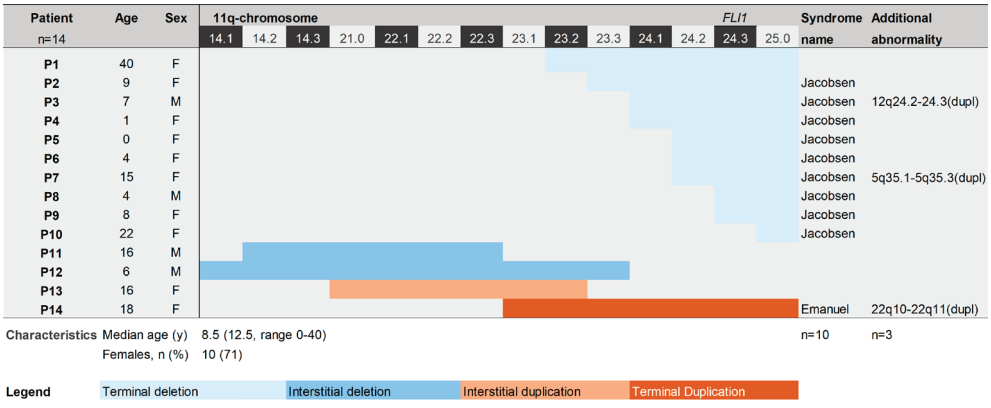
interquartile range (IQR). In case of a normal distribution, data are presented as mean and standard deviation (SD) or range. Categorical data are presented as numbers with percentages and range of minimum and maximum value. To identify the largest variation in platelet proteome, a principal component analysis (PCA) is performed. Statistical data analyses were performed using SPSS version 21.0 (IBM, Armonk, NY, USA). For MS-data, files acquired from XCalibur software (Thermo Fisher Scientific) and processed with MaxQuant 1.6.2.10 software for MS-analysis were used.

RESULTS

Patient characteristics

Fourteen patients were included with a median age of 8.5 years (IQR 12.5; range 0-40). Ten were female (71%, Figure 1). Ten patients had a terminal 11q deletion with variable size of deletion (71%), and four had other 11q aberrations: interstitial deletion, n=2 (14%); terminal duplication, n=1 (7%) and interstitial duplication, n=1. The localization and size of the chromosomal abnormalities varied, and in three patients, additional chromosomal abnormalities were observed (See Figure1, details in Supplement 1). Comorbidities were present in all patients; neurodevelopmental disabilities, cardiac abnormalities and immunodeficiency were the most common (Supplement 2). During follow-up one patient passed away unexpectedly.

Figure 1 Patient characteristics and genetic distribution of 14 patients with 11q disorders



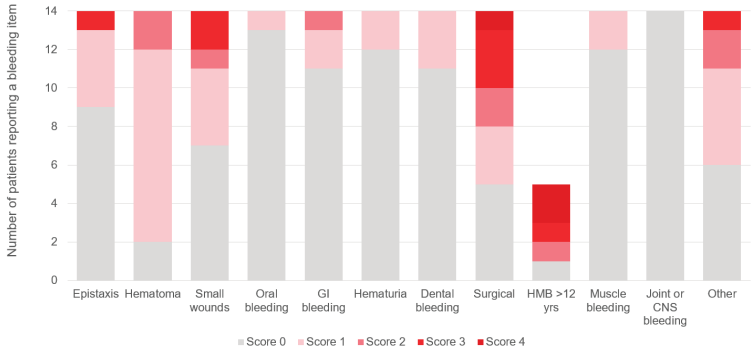
Abbreviations dupl = duplication, F = female, M = male, P = patient

Bleeding symptoms

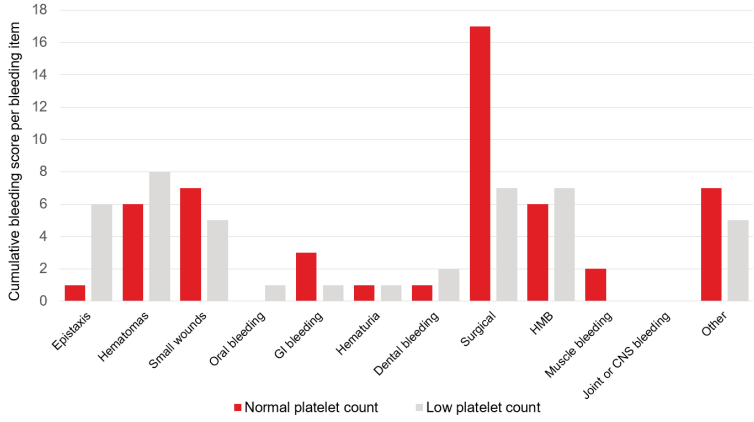
Bleeding symptoms are summarized in Figure 2, details in Supplement 2. Mean bleeding score was 6.7 (3.96 SD; range 0-13). Only 2/14 (14%) patients had a bleeding score below the age-related cut-off: one patient with an interstitial 11q duplication did not report any bleeding symptoms. She also had a normal platelet count. One child with a terminal 11q deletion had a score of 2 and showed only a mildly lowered platelet count (range 82-105 x 10^9/L) as well as only a mild abnormal response in the LTA.

Figure 2 Bleeding symptoms per bleeding item

A



B



Abbreviations CNS = Central nervous system, GI = Gastro-intestinal, HMB = Heavy menstrual bleeding

Legend * treatment required for fibrinogen glue, stitching, local and oral tranexamic acid; ** consultation required in for postoperative bleeding after craniosynostosis repairment surgery or eye-lid correction; treatment required for postoperative bleeding after cardiac surgery and circumcision and blood transfusion needed after adenotonsillectomy; *** consultation required in 1 of 5 post-menarcheal females with heavy menstrua bleeding and treatment required in 3 of 5 females with contraceptive treatment, curettage (1/5, score 3), and hysterectomy for HMB (1/5, score 4); **** consultation required for prolonged bleeding after venipuncture, treatment required for a spontaneous vitreous bleeding causing loss of sight despite timely ablation (1/14, score 3)

Twelve out of 14 (86%) patients with increased bleeding tendency had a mean bleeding score of 7.1 (range 4-13). Bleeding symptoms that were reported most frequently, were large hematomas (12/14), bleeding from small wounds (7/14), increased and prolonged bleeding during or after surgery (9/14), heavy menstrual bleeding (4/10 postmenarcheal women) and other bleedings (8/14). Ten patients (10/14) reported a total of 20 bleeding episodes that required consultation and even treatment (Figure 2A); reported bleeding for large hematomas, epistaxis, increased or prolonged bleeding during or after surgery as well as prolonged bleeding from small wounds, gastro-intestinal bleeding,heavy menstrual bleeding and one eye-bleeding classified as 'other'.

To illustrate these bleeding scores in more detail, the large hematoma (ISTH-BAT score ≥ 2) occurred in two patients after subcutaneous immunoglobulin replacement therapy. The perioperative bleeding complications were after adenotonsillectomy, circumcision, eye lid surgery, cardiac and craniosynostosis repairment surgery (scores 2-4). Concerning only 5 of 10 female patients were postmenarcheal and could score on this item, it is remarkable that 4 out of 5 (80%) scored 2-4 and reported the need for medical consultation, tranexamic acid, anticonceptives, and twice a gynecological intervention (curettage for heavy menstrual bleeding and hysterectomy after failure of anticonceptives). These four patients had terminal 11q deletions (3/4, 75%) or a terminal 11q duplication (1/4). Concerning the 'other' bleedings, one patient with interstitial 11q deletion had a serious spontaneous bleeding in the vitreous gel of one eye leading to loss of vision despite timely laser-photocoagulation). In our cohort 2/14 reported an intramuscular bleeding, both after intramuscular vaccination was given at young age, which is very atypical in patients with platelet disorders. After this bleeding complication, further vaccinations were given subcutaneously. In our cohort, joint or cerebral bleedings did not occur.

When comparing the bleeding scores of the seven patients (50%) with normal platelet counts with the score of the seven patients with low platelet counts, there is a small difference in the mean bleeding score: 6.1 in low platelet counts group versus 7.3 in the group with normal platelet counts. Remarkably, this is mainly due to an increased frequency and/or severity of perioperative bleeding. In the seven patients with normal platelet counts, postsurgical bleeding occurred more frequently and/or more severe than in the group with low platelet counts (sum of all bleeding scores is 17 versus 7). Also, the uncommon intramuscular bleed occurred in 2/7 patients with normal platelet counts. Vice versa, there is a higher frequency and/or severity of epistaxis in the group with the low platelet counts). We report the presence of heavy menstrual bleeding in 4/5 of the postmenarcheal female patients: 2/5 in the group with low platelet counts and 2/5 in the group with normal platelet counts (scores of 2 and 4, Figure 2B). Only the patient with the interstitial 11q duplication reported no heavy menstrual bleeding.

Platelet count and indices and thrombopoietin levels

Results of most relevant laboratory findings are summarized in Figure 3. Median lowest platelet count is 149 x 10^9/L (range 20-340), with seven patients (50%) having congenital thrombocytopenia, ranging between 20-50 x 10^9/L in neonatal period and increasing during childhood towards a range of 80-140 x 10^9/L at time of inclusion. All seven patients with low platelet counts had a terminal 11q deletion. All thrombocytopenic patients had an increased immature platelet count (IPF) with a mean of 15.3% (range 11-29%, normal value <5%), compared to a mean IPF of 5.6% for the 7/14 with normal platelet counts (range 3- 8). This suggests an increased production of young platelets.

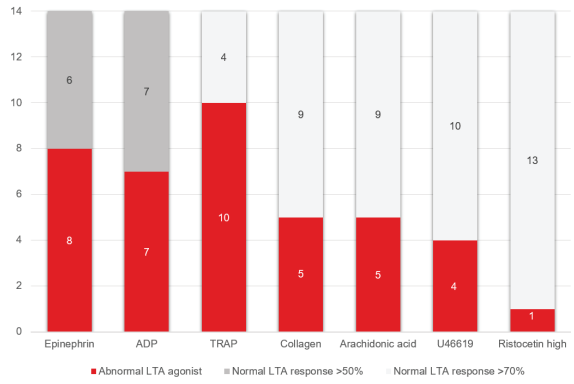
Mean platelet volume (MPV) was normal in 6/7 patients with normal platelet counts (mean 10.6, only in one patient with interstitial 11q duplication MPV >12 fL was seen). In only three patients with low platelet counts MPV was measured due to measurement restrictions in severe thrombocytopenia, and in 2 MPV was >12 (mean 12.2 fL). The three patients with elevated MPVs ≥12 fL had no macro-platelets in the blood smear (3/10). Macro-platelets were reported on blood smeared in 3/14 (21.4%), but this did not lead to an elevated MPV. TPO was measured in 12 patients and elevated in two patients (16.7%) with congenital thrombocytopenia (TPO 46 and 59, normal range <34 U/mL, Figure 3A). One patient underwent bone marrow aspiration to rule out (pre)malignant causes of neonatal thrombocytopenia before the diagnosis of terminal 11q deletion was known (Figure 4). These images showed dysplasia in all megakaryocytes (>10%), ranging from micro-megakaryocytes to abnormal nuclei. Platelet ATP/ADP-ratio was not routinely performed in this study but historically performed in three patients. The ratio was abnormal in 2/3 (66.7%), but results cannot be extrapolated to all patients due to possible selection bias.

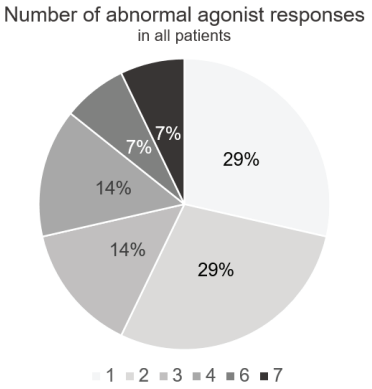
Figure 3 Laboratory results of platelet count, indices and function test

A

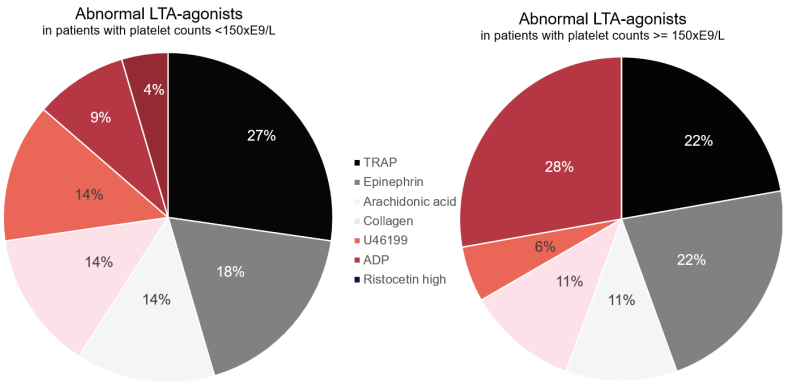
Platelet count	**<150 x 10^9/L, n = 7**	**≥150 x 10^9/L, n = 7**
11q aberration	Terminal 11q deletion, n=7	Terminal 11q deletion, n=3 Non 11q deletion, n=4
Platelet indices and TPO		
Platelet count, x 10^9/L	67 (20-136)	230.9 (180-340)
IPF, %	15.3 (11-29)	5.6 (3-8)
MPV, fL	12.2 (11.8-12.5)	10.6 (9.4-12.2)
PFA EPI, sec	175.2 (91 - >300)	147.6 (79 - >292)
TPO, IU/mL	32 (18-59)	11.1 (7-13)
ATP/ADP ratio	7.8 (6.8-8.8)	-
Light transmission aggregometry		
Abnormalities present, n	7	7

B

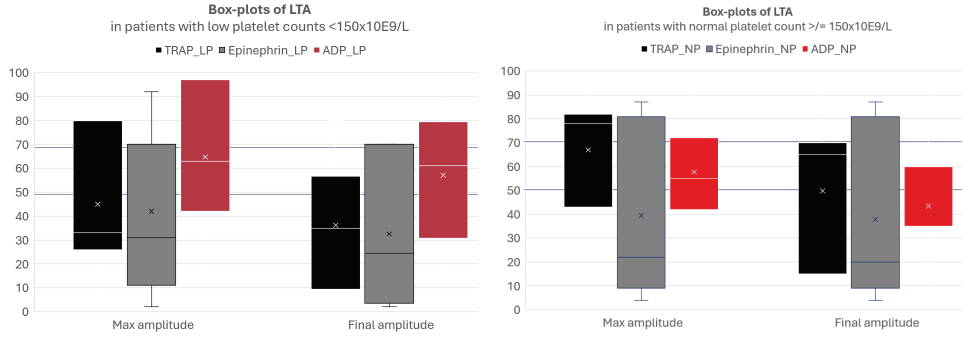




C



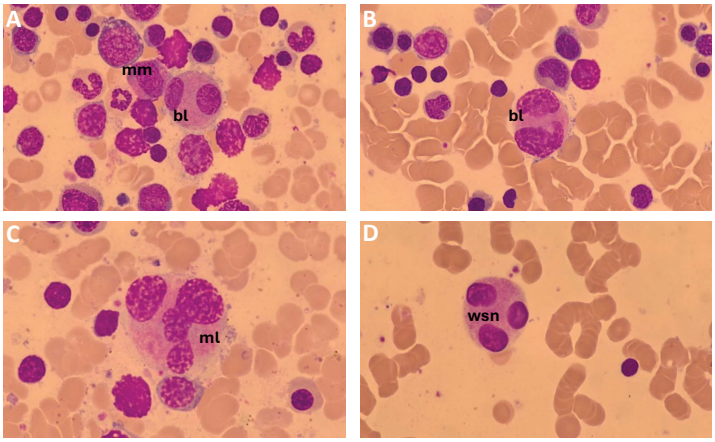
D



Abbreviations ADP = Adenosine Diphosphate, ATP = Adenosine Triphosphate, fL = femtoliter, IPF = Immature Platelet Fraction, IU = international unit, L = liter, LTA = Light transmission Agonist, mL = milliliter, MPV = Mean Platelet Volume, n = number, PFA-EPI, = Platelet Function Analyzer-epinephrin cartridge, sec = seconds, TPO = thrombopoietin, TRAP = Thrombin receptor-activating peptide

Legend Blue lines in the boxplots visualize the 50 or 70% response lines which are defined as the minimal adequate response

Figure 4 Bone marrow morphology of one patient with a terminal 11q deletion



Abbreviations bl = bilobed megakaryocytes, ml = multilobed megakaryocytes, mm = micro-megakaryocytes, wsn = widely separated nuclei

Legend Bone marrow aspiration cytology (May-Grünwald Giemsa staining, magnification 1000x) shows >10% of dysplastic megakaryocytes: micro-megakaryocytes <12µm, mono, bi-lobed or multi-lobed megakaryocytes, as well as non-lobed nuclei megakaryocytes (not on image), and megakaryocytes with widely separated nuclei

Platelet function analyzer, light transmission aggregometry and additional coagulation factor deficiencies as disease modifier

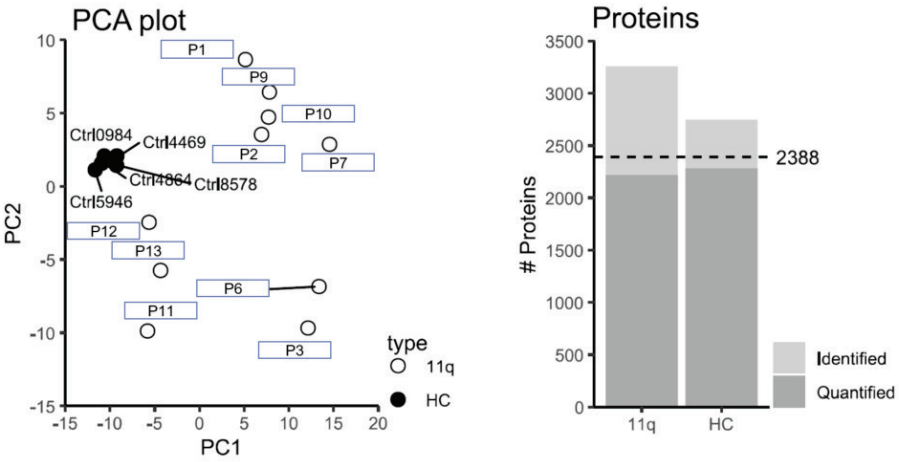
Results are visualized in Figure 3. PFA-EPI and ADP were performed in 11/14. It was prolonged ≥150 sec in 3/11 (27.3%), without a clear correlation to platelet counts ≤80 x 10^9/L as this may bias the closure time. In all patients an LTA was performed for study purposes regardless of platelet count at inclusion. This resulted in a PRP that did not meet the standard of >150 x 10^9/L in patients P6, P7 and P8 (PRP of respectively 115, 51 and 98, with a platelet count of 80, 51, and 133 x 10^9/L). LTA was abnormal for all 14 patients (100%; Figure 3A). In 13/14 LTA test results were available for analysis of amplitudes (Figure 3B-D). On average a combination of three abnormal responses on LTA agonists was seen (range 1-7). The LTA was most often abnormal for TRAP in 10/14 (71%), for epinephrin in eight (57%), ADP in seven (50%), for collagen and arachidonic acid in five (36%), U46619 in four (29%), and ristocetin high in one patient (7%), but this patient had a PRP with only 51 x 10^9 platelets/L. In the seven patients with low platelet counts, the response on TRAP was more frequently abnormal than in the group with normal platelet counts. While abnormal ADP release was most often abnormal in the group with normal platelet counts. Details are depicted in Figure 3C. Additional factor deficiencies were also seen in 2/14 (14%): one patient with a low von Willebrand factor deficiency (mean VWF activity of 0.50 U/L with normal FVIII activity) and one patient with FIX activity of 0.41 U/mL, which may be still physiologically, as she was <1 year at time of inclusion.

Mass-spectrometry results

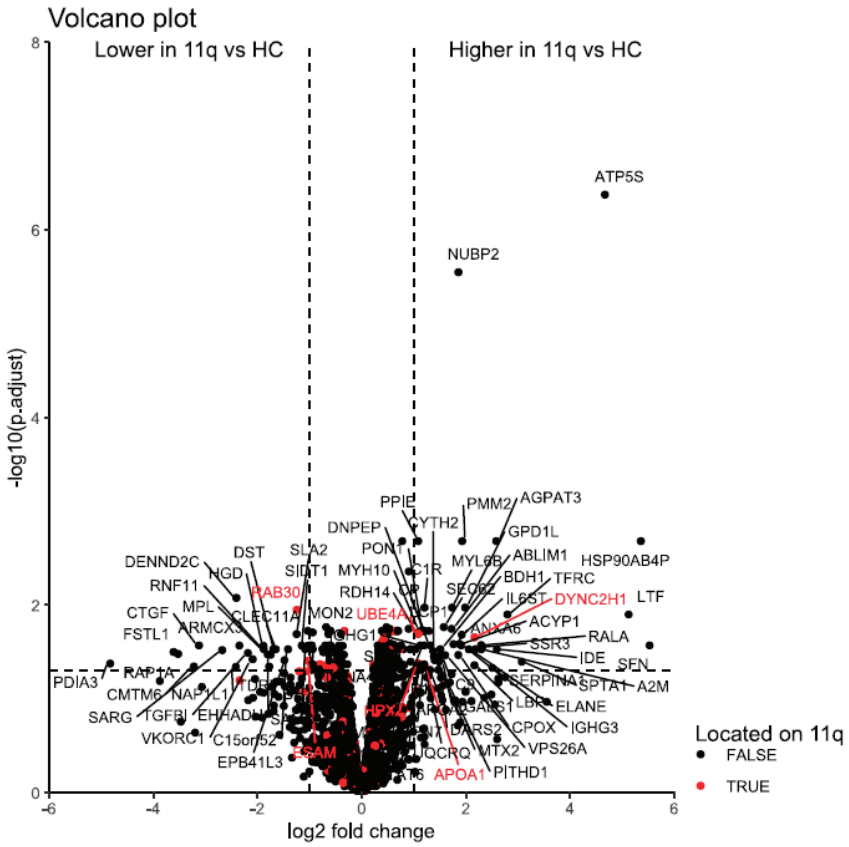
We performed MS in the platelets of 10 patients (P1-3, 6-7, 9-13 (DOM 58, 115-121, 123, Figure 5). Platelet proteomics showed that in patients with 11q disorders the total amount of platelet proteins is significantly more abundant than in healthy controls. This did not correlate with clinical bleeding tendency, as patient 12 (DOM 124) had the highest ISTH-BAT score and patient 13 (DOM 118) the lowest, both were plotted near the healthy controls. (Figure 5A). A volcano plot of identified proteins showed that multiple proteins were measured in lower or higher abundancy than in healthy controls. Only two proteins stood out: NUBP2 and ATP5S were significantly more present in platelets of patients with 11q disorders than in controls (Figure 5B). Both are not located on 11q. NUBP2 is a member of the NUBP/MRP gene subfamily of ATP-binding proteins and involved in mitochondrial function.(16) ATP5S correlates for H(+)-ATP synthase, a multi-subunit membrane-bound enzyme complex consisting of segments F0 and F1. F1 provides the catalytic activity for the interconversion of ADP to ATP and the H+-conduction of ATP-synthase, a very relevant step in platelet function.(17) Although dysfunction of this process can be measured in abnormal ATP/ADP ratios, as is seen in 2/3 patients in which this ratio is tested, the exact role of NUBP2 and ATP5S in 11q disorders need more research.

Figure 5 Mass spectrometry of platelets from patients with 11q aberrations

A



B



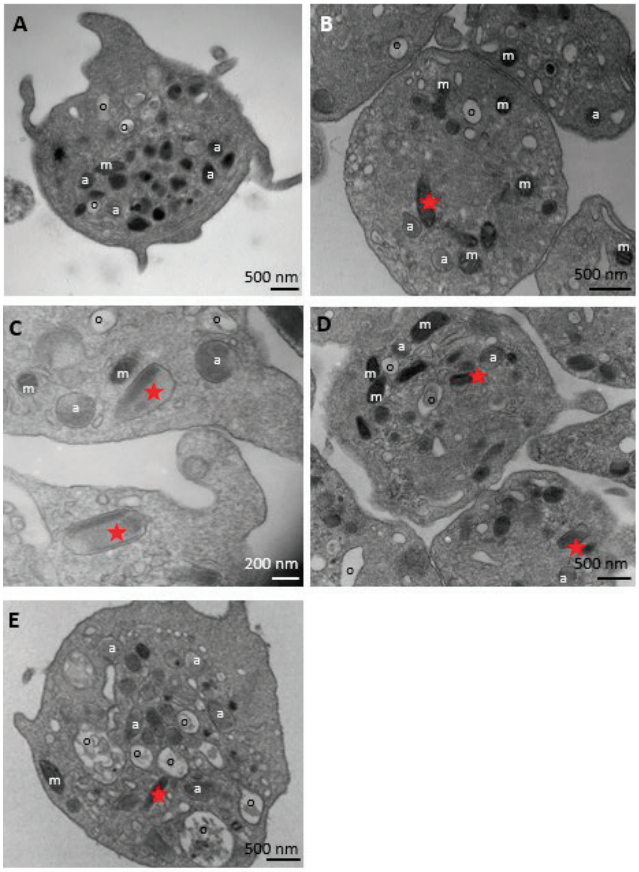
Abbreviations HC = healthy control, P = patient, PC = principal component, PCA = principal component analysis (*continued*)

Electron microscopy results

We performed EM-images of platelets in 2/14 patients, one with a terminal 11q deletion (P2) and one with an interstitial deletion (P11), Figure 6. In comparison with healthy controls, platelets of both patients showed multiple giant alpha-granules (a) as is observed in Paris-Trousseau thrombocytopathy, as well as enlarged compartments containing crystal-like structures of unknown origin (Figure 6C). These compartments are membrane enclosed, have an irregular shape and content. Because the role of *FLI1* has been mentioned in literature as the cause of the platelet dysfunction, we compared the EM-images of a patient with a pathogenic *FLI1* variant (c.279del, p.(Met100*)) with a low platelet count as well as platelet dysfunction to patients in our cohort. Transmission electron microscopy (TEM) analysis demonstrated the same unidentified structure and in addition, increased amounts of open canalicular system with many fusion profiles. To our knowledge, the origin of the crystal filled compartment is unknown, but it has been observed before by Saultier et al.(13) Based on the description by Neumüller et al,(18) we

hypothesize this is an abnormal shaped (alpha) granule, but more research is needed to confirm this hypothesis.

Figure 6 Electron microscopic images of two patients with 11q aberrations



Abbreviations a = alfa granule, m = mitochondrium; o = Open canicular system, Star = crystal like structure

Legend (A) healthy control, (B) patient with terminal 11q deletion, (C-D) patient with interstitial 11q deletion, (E) patient control with pathogenic variant in FLI1 (p.(Met100*))

DISCUSSION

We present a case-series with results on bleeding symptoms in patients with a variety of 11q disorders occurring in both female and male patients, with a 2:1 female:male ratio that is described by other authors as well.(19, 20). Our results confirm that there is an increased bleeding tendency which can already become apparent directly after birth as a result of platelet dysfunction and low platelet count in patients with terminal 11q deletions.(1, 10, 21-23) We also demonstrate that these bleeding complications occur in

nonterminal 11q aberrations as well, by showing a platelet dysfunction in the patients with interstitial 11q deletion and terminal 11q duplication. So far, only one case-report reported low platelet counts in a neonate with interstitial 11q deletion (30-50 x 10^9/L), but platelet function was not analyzed.(24)

The bleeding complications for this cohort range between mild to severe. A special focus should be on the possibility of heavy menstrual bleeding in girls and on the risk of perioperative bleeding. This risk seems even higher in children with normal platelet counts. Rare but severe complications were a spontaneous vitreous bleeding and intramuscular hematoma after intramuscular vaccinations. No intracranial bleedings were reported in our cohort, but this is reported as a risk.(7)

In our cohort it is remarkable that TRAP-response was abnormal in 8 out of 12 abnormal LTA-tests. TRAP is considered a strong agonist reflecting the cleavage of thrombin of platelet membrane proteases PAR1 and 2 and glycoprotein 1bα and V.(25) Abnormal responses in TRAP have been correlated with abnormal alpha granules function.(26, 27) We also hypothesize that there is also an abnormal ATP-ADP formation in platelet mitochondria and delta granules, as we have found significantly increased NUBP2 and ATP5S in the platelet proteome, as well as the abnormal ATP/ADP-ratio in three patients.(9, 13) More research to confirm this finding is needed.

The platelet dysfunction in patients with terminal 11q deletion has been reported more often and is classified as Paris-Trousseau thrombocytopathy due to abnormal alpha-granules.(1, 8, 10, 12, 28) Our EM-images confirm this relation in a patient with terminal 11q deletion, but also demonstrate abnormal alpha granules in a patient with interstitial 11q deletion. This has not yet been described, and it confirms the clinical and hemostatic diagnosis of platelet dysfunction in the two patients in this cohort.

It has been suggested that the gene *FLI1,* located on 11q24.3, could be associated with platelet dysfunction as well as abnormal megakaryopoiesis leading to low platelet counts.(28, 29, 30) For the role of *FLI1* in the abnormal alpha granules formation, there is one case report that demonstrated abnormal alpha granules in a patient with a homozygous pathogenic single nucleotid variant in *FLI1* as well.(30) What remains unclear, is why platelet dysfunction and abnormal alpha granules' formation occurred in the patients with an interstitial 11q deletion not involving the 11q23.3 region. For platelet numbers, monosomy of *FLI1* has been related to thrombocytopenia as well.(29, 31, 32) In our cohort, monosomy of *FLI1* correlated with thrombocytopenia in only 67% (6/9) of patients with a terminal 11q deletion involving 11q23.2. And, one patient with a microdeletion after the locus of *FLI1* (11q25.0 deletion) also had low platelet counts. This discrepancy of *FLI1* and both platelet number and function is reported by other authors as well.(1, 5, 33). Some authors therefore suggest that more than one critical region or gene is involved

and that thrombocytopenia only occurs when at least 3 out of the following 4 genes are involved: *FLI1, ETS1, JAM3* and *NFRKB*.(6, 33) But to date, it remains unclear and more research is needed.

Our study has several limitations. This study comprises a small cohort with a variety of chromosomal defects, as could be expected with a rare continuous gene syndrome. For the even more rare non 11q deletion syndromes, more research is needed before definitive conclusions can be drawn. We can only report on reported incidences. Due to the low numbers of patients, we cannot correct for confounders as gender, age or frequency of surgery performed. Lastly, although we had followed the present protocol, we have missing values. Especially when venipuncture was complicated choices for further analysis had to be made. However, for most patients we obtained information on the clinical course of disease and for the majority of patients we could perform LTA and MS to further explain their disease trajectory.

To conclude, based on this case series, we recommend screening patients with all forms of chromosome 11q disorders as soon as diagnosis is made and certainly before surgery is indicated. Screening should include platelet count and platelet function. When platelet function tests are difficult to obtain or results are still pending at young age, we recommend subcutaneous vaccinations and avoidance of drugs that influence platelet function like non-steroid anti-inflammatory drugs. A special attention should be paid for counseling of female patients with 11q disorders on potential heavy menstrual bleeding.

ACKNOWLEDGEMENTS

We thank the patients and their parents as well as the Dutch 11q Patients associations for their warm support. We thank Tjardo Paardenkoper (Senior analyst cytomorphology laboratory, Leiden University Medical Center) for kindly providing the bone marrow images.

REFERENCES

1. Grossfeld PD, Mattina T, Lai Z, Favier R, Jones KL, Cotter F, Jones C. The 11q terminal deletion disorder: a prospective study of 110 cases. Am J Med Genet A. 2004;129A(1):51-61.
2. Hagen A, Bigl A, Wand D, Klopocki E, Heller R, Siekmeyer M, et al. Combined partial trisomy 11q and partial monosomy 10p in a 19-year-old female patient: phenotypic and genotypic findings. Am J Med Genet A. 2011;155A(12):3075-81.
3. Maruyama K, Koizumi T, Ikeda H. Partial monosomy 10q with partial trisomy 11q due to paternal balanced translocation. J Paediatr Child Health. 2001;37(2):198-200.
4. Emanuel BS, Zackai EH, Medne L. Emanuel Syndrome. 1993.
5. Penny LA, Dell'Aquila M, Jones MC, Bergoffen J, Cunniff C, Fryns JP, et al. Clinical and molecular characterization of patients with distal 11q deletions. Am J Hum Genet. 1995;56(3):676-83.
6. Su J, Chen R, Luo J, Fan X, Fu C, Wang J, et al. de novo interstitial deletions at the 11q23.3-q24.2 region. Mol Cytogenet. 2016;9:39.
7. Grossfeld P. Brain hemorrhages in Jacobsen syndrome: A retrospective review of six cases and clinical recommendations. Am J Med Genet A. 2017;173(3):667-70.
8. Breton-Gorius J, Favier R, Guichard J, Cherif D, Berger R, Debili N, et al. A new congenital dysmegakaryopoietic thrombocytopenia (Paris-Trousseau) associated with giant platelet alpha-granules and chromosome 11 deletion at 11q23 [see comments]. Blood. 1995;85(7):1805-14.
9. White JG. Platelet storage pool deficiency in Jacobsen syndrome. Platelets. 2007;18(7):522-7.
10. Favier R, Jondeau K, Boutard P, Grossfeld P, Reinert P, Jones C, et al. Paris-Trousseau syndrome : clinical, hematological, molecular data of ten new cases. Thromb Haemost. 2003;90(5):893-7.
11. Sheth FJ, Datar C, Andrieux J, Pandit A, Nayak D, Rahman M, Sheth JJ. Distal Deletion of Chromosome 11q Encompassing Jacobsen Syndrome without Platelet Abnormality. Clin Med Insights-Pe. 2014;8:45-9.
12. Krishnamurti L, Neglia JP, Nagarajan R, Berry SA, Lohr J, Hirsch B, White JG. Paris-Trousseau syndrome platelets in a child with Jacobsen's syndrome. Am J Hematol. 2001;66(4):295-9.
13. Saultier P, Vidal L, Canault M, Bernot D, Falaise C, Pouymayou C, et al. Macrothrombocytopenia and dense granule deficiency associated with FLI1 variants: ultrastructural and pathogenic features. Haematologica. 2017;102(6):1006-16.
14. Elbatarny M, Mollah S, Grabell J, Bae S, Deforest M, Tuttle A, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. Haemophilia. 2014;20(6):831-5.
15. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. Journal of Thrombosis and Haemostasis. 2013;11(6):1183-9.
16. https://www.ebi.ac.uk/interpro/entry/InterPro/IPR019591/
17. Richman TR, Ermer JA, Baker J, Sira SJ, Kile BT, Linden MD, et al. Mitochondrial gene expression is required for platelet function and blood clotting. Cell Rep. 2023;42(11).
18. Neumüller J, Ellinger A, Wagner T: Transmission electron microscopy of platelets FROM aphersis and buffy-coat-derived platelet concentrates. From: The transmission electron microscope - theory and applications by Maaz K. IntechOpen 2015 (open e-book https://www.intechopen.com/chapters/48569)
19. Dalm VA, Driessen GJ, Barendregt BH, van Hagen PM, van der Burg M. The 11q Terminal Deletion Disorder Jacobsen Syndrome is a Syndromic Primary Immunodeficiency. J Clin Immunol. 2015;35(8):761-8.
20. Mattina T, Perrotta CS, Grossfeld P. Jacobsen syndrome. Orphanet J Rare Dis. 2009;4:9.

21. Ichimiya Y, Wada Y, Kunishima S, Tsukamoto K, Kosaki R, Sago H, et al. 11q23 deletion syndrome (Jacobsen syndrome) with severe bleeding: a case report. J Med Case Rep. 2018;12(1):3.
22. Serra G, Memo L, Antona V, Corsello G, Favero V, Lago P, Giuffre M. Jacobsen syndrome and neonatal bleeding: report on two unrelated patients. Ital J Pediatr. 2021;47(1):147.
23. Shin, J, Kim, G, Lee, R, Jung N, Shim Y.J., Ha, J-S, A case of Jacobsen syndrome presenting with a huge cephalohematoma and thrombocytopenia after birth. Clin Pediatr Hematol Oncol 2018;25:56-60
24. Wenger SL, Grossfeld PD, Siu BL, Coad JE, Keller FG, Hummel M. Molecular characterization of an 11q interstitial deletion in a patient with the clinical features of Jacobsen syndrome. Am J Med Genet A. 2006;140(7):704-8.
25. Soslau G, Class R, Morgan DA, Foster C, Lord ST, Marchese P, Ruggeri ZM. Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. J Biol Chem. 2001;276(24):21173-83.
26. Lages B, Sussman II, Levine SP, Coletti D, Weiss HJ. Platelet alpha granule deficiency associated with decreased P-selectin and selective impairment of thrombin-induced activation in a new patient with gray platelet syndrome (alpha-storage pool deficiency). J Lab Clin Med. 1997;129(3):364-75.
27. Blair P, Flaumenhaft R. Platelet α-granules: Basic biology and clinical correlates. Blood Reviews. 2009;23(4):177-89.
28. Losos, M., Kahwah, B.M., Conces, M., Thompson, J., Kumar, R., Kahwash, S.B.: Jacobsen/Paris-Trousseau Syndrome: Report of a Case with Emphasis on Platele's Light Microscopic and Ultrastructure Findings. Open J of Path 2016;6:8-13
29. Raslova H, Komura E, Le Couedic JP, Larbret F, Debili N, Feunteun J, et al. FLI1 monoallelic expression combined with its hemizygous loss underlies Paris-Trousseau/Jacobsen thrombopenia. J Clin Invest. 2004;114(1):77-84.
30. Stevenson WS, Rabbolini DJ, Beutler L, Chen Q, Gabrielli S, Mackay JP, et al. Paris-Trousseau thrombocytopenia is phenocopied by the autosomal recessive inheritance of a DNA-binding domain mutation in FLI1. Blood. 2015;126(17):2027-30.
31. Hart A, Melet F, Grossfeld P, Chien K, Jones C, Tunnacliffe A, et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. Immunity. 2000;13(2):167-77.
32. Vo KK, Jarocha DJ, Lyde RB, Hayes V, Thom CS, Sullivan SK, et al. FLI1 level during megakaryopoiesis affects thrombopoiesis and platelet biology. Blood. 2017;129(26):3486-94.
33. Tyson C, Qiao Y, Harvard C, Liu XD, Bernier FP, McGillivray B, et al. Submicroscopic deletions of 11q24-25 in individuals without Jacobsen syndrome: re-examination of the critical region by high-resolution array-CGH. Molecular Cytogenetics. 2008;1.

Supplement 1 Methods of platelet mass-spectrometry and electron microscopy

Mass-spectrometry

For mass-spectrometry (MS), samples were alkylated with 55 mM iodoacetamide for 45min at 20°C and digested overnight at 20°C with MS-grade trypsin (Promega). Peptides were desalted and concentrated with Empore-C18 StageTips and eluted with 0.5% (v/v) acetic acid, 80% (v/v) acetonitrile. Sample volume was reduced by SpeedVac and supplemented with 2% acetonitrile, 0.1% trifluoroacetic acid to a final volume of 5µL. For high pressure liquid chromatography and MS-analysis, tryptic peptides were separated by nanoscale C18 reverse phase chromatography coupled online to an Orbitrap Fusion Tribrid mass-spectrometer (Thermo Fisher Scientific) via a nano electrospray ion source (Nanospray Flex Ion Source, Thermo Fisher Scientific). Peptides were loaded on a 20 cm 75µm-360µm inner-outer diameter fused silica emitter (New Objective, Bremen, Germany) packed in-house with ReproSil-Pur C18-AQ, 1.9µm resin (Dr Maisch GmbH, Ammerbuch-Entringen, Germany). The column was installed on a Dionex Ultimate 3000 RSLC nanoSystem (Thermo-Fisher Scientific) using a MicroTee union formatted for 360µm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15kV. Buffer A was composed of 0.5% acetic acid and buffer B of 0.5% acetic acid, 80% acetonitrile. Peptides were loaded for 17min at 300 nL/minute (min) at 5% buffer B, equilibrated for 5min at 5% buffer B (17-22min) and eluted by increasing buffer B from 5-15% (22-87min) and 15-38% (87-147min), followed by a 10min wash to 90% and a 5min regeneration to 5%. Survey scans of peptide precursors from 400 to 1500 m/z were performed in the orbitrap at 120K resolution with a 1.510^5 ion count target. Tandem MS was performed by isolation with a quadrupole with isolation window 1.6. Higher-energy collisional dissociation (HCD) fragmentation with normalized collision energy of 30, and rapid scan MS-analysis in the ion trap. The MS2 ion count target was set to $1.5x10^4$ and the max injection time was 35ms. Precursors with charge state 2-7 were sampled for MS2. The dynamic exclusion duration was set to 60s with a 10ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3s cycles. Peptides were compared with the homo sapiens Uniprot database. Enzyme specificity was set to trypsin with a maximum of 2 missed cleavages, and carbamidomethylation on cysteine residues was used as fixed modification. The false discovery rate (FDR) was 0.01 for peptide and protein levels with a minimum length of 7 amino acids. The peptide precursor MS tolerance was set to 10ppm and fragment ion mass tolerance at 0.6 Dalton. Label-free quantitation (LFQ) was performed with a minimum ratio count of 2. MaxQuant output files were loaded in R 3.6.2 for further analysis. Proteins were filtered by "contaminants", "reverse" and "only identified by side" and LFQ intensities were $\log_2$ transformed. Technical replicates were summarized to median values.

Electron microscopy

For EM, epon was prepared by mixing Agar 100 resin 24g (±21 mL), *Dodecenyl Succinic Anhydride* (EM grade) 13g (±13 mL), Methylnadic anhydride (EM grade) 13g (±12 mL), *Benzyldimethylamine* 1ml (added in fumehood). Before the Epon embedding, the cells were washed and fixed with 2% Paraformaldehyde, 2,5% Gluteraldehyde in 0.1M cacodylate, and post-fixed for 15min in 1% Osmium in 0,1M cacodylatebuffer. Then washed in Milli-Q water and incubated in Ultrastain (uranyl) for 20min. Hereafter, cells were dehydrated with two runs of 50% ethanol for 5min, 70% ethanol for 5min, 90% ethanol for 5min and two runs of 100% ethanol for 5min, then incubated in propylene oxide for 10min and afterwards in an epon:propylene oxide-mix (1:1) for a minimum of 12 hours (h). Then, incubated for 1 h in epon at 37°C and put in molds to polymerize at 60°C. Ultrathin sections were cut at a Leica ultramicrotome and place on grids, which were first stained with first ultrastain solution and subsequently washed and stained with the second ultrastain solution. Then grids were washed in demi-water and dried on air. Microscopic imaging was performed on an Tecnai T12 (Thermo Fisher Fei Eindhoven) with Veleta and Xarosa camera using Radius software (EMSIS, Münster, Germany).

Supplementary Table S2 Description of the cytogenetic aberrations

Patient	Diagnostic test	Chromosome	Base pairs	Mb	Probes
1	SNP-array (Illumina Cyto-12 300x, Nexus 7.0) (hg18)	11q23.3q25	(119 956 891 -134 452 384) x1		
2	Karyotyping	46,XX, del(11Xq23.3q25) x1		15.5	
3	SNP-array; (GRCh37)	11q24.1q25 12q24.21q24.33	(122 893 213 -134 450 518) x1 (114 633 820 -133 779 217) x3	12 19.2	755 1,167
	Additional GTG FISH	46,XY,der(11)t(11;12) (q24.1;q24.21)			
4	SNP-array (CytoScan HD Array Affymetrix) (GRCh37)	11q24.1q25	(123 307 968 -134 938 470) x1	11.6	13,938
5	SNP-array; (GRCh37)	11q24.2q25	(126 728 342 -134 938 470) x1		
6	Whole genome Array, (Illumina HCS850k, Nexus); (hg19)	11q24.2q25	(124 552 720 -134 934 063) x1		
7	Micro-array, (Agilant 180K); (GRCh37) Micro-array, (BlueGnome Cytochip v1.1, Bac-Arr CGH); (GRCh37)	5q35.1q35.3 (RP11-20O22 à RP11-451H23) 11q24.2q25 (RP11-168K9 à RP11-469N6)	(171 063 816-180 099 598) x3 (126 456 874-126 971 201) x1	10 9	10 10
8	SNP-array (CytoScan HD Array Affymetrix); (GRCh37)	11q24.2q25	(126 908 896 -134 938 470) x1	8	9,498
9	Whole Genome Array (Illumina Cyto-12 300k, Nexus 7.0, 1Mb CNV analysis array); (hg18)	11q24.3q25	(127 727 172 -134 452 384) x1		
10	Karyotyping <1985	46,XX,t(6p;11q) del 11q25 x1			
11	Targeted array (Illumina HCS850k, Nexus); (hg19)	11q14.2q22.1	(79 729 111-98 48 532) x1		
	Additional FISH	46,XY,del(11)(q14.21q21-22) (RP11-13L14) x1			
12	SNP-array (Cytoscan HD Affymetrix, Thermo Fisher) (GRCh 37)	11q14.1q23.3	(84 558 527 -117 346 256) x1	32	34,144
13	Karyotyping	46,XX,+der(11).ish dup(11) (q21q23.3) x3			
	FISH with DNA probe WCP11 (paint 11) 2005	Dup(11)(q21-q23.3) Probes 3.16 (11q21), NCAM (11q23.2) and 4.13 (11q23.3) x2			
14	Karyotyping	47,XX,+der(22)t(11;22) (q23;q11) x3			

Abbreviations CNV = copy number variation, del = deletion, der = derived from translocation, dup = duplication, FISH = fluorescence in sity hybridization, ish = in situ hybridization, Mb = megabase, SNP = single nucleotid variant, t = translocation

Supplementary Table S3 Detailed overview of bleeding symptoms and comorbidities in 14 patients with 11q disorders

Patient number	**Age** years	**Bleeding** Score*	**Platelet count** Mean, (range)	**Bleeding symptoms**	**Hemostatic treatment Blood transfusions**	**Comorbidities**
Increased bleeding tendency according to bleeding score						
1	40	6	201 (180-219)	Easy bruising, large hematomas, prolonged bleeding after venipunctures, heavy menstrual bleeding	Iron supplementation. contraconceptive therapy. tranexamic acid. curettage once needed	Hypogammaglobulinemia (on IgRT); asymmetric aortic valve; surgery for bilateral ectropion and nystagmus; hearing aids for senso-neural hearing loss; lymph angiodysplasia lower legs; developmental delay
2	9	9	124 (52-183)	Easy bruising, large hematomas, postoperative bleeding	Tranexamic acid. prophylactic desmopressin platelet transfusions	Hypogammaglobulinemia (on IgRT) Surgery for craniosynostosis and bilateral ectropion; paronychia drainage; de novo IDDM type 1 at age 11 years; developmental delay
3	7	7	149 (136-175)	Easy bruising, large hematomas, prolonged bleeding after venipunctures, hematemesis during NGT-feeding, frequent postoperative bleeding and bleeding post-biopsy of esophagus; severe hematuria due to cystitis	Perioperative prophylactic platelet transfusions or tranexamic acid was not always sufficient; tranexamic acid for postoperative ptosis-bleeding; tranexamic acid and therapeutic platelet transfusion for post-biopsy bleeding	Hypogammaglobulinemia (on IgRT) Surgery for large VSD, bilateral ptosis, Nissen for GERD, circumcision and orchidopexy NGT for feeding difficulties Pathological fracture Developmental delay
4	1	4	80 (30-157)	Easy bruising, prolonged bleeding after venipunctures, prolonged bleeding after small wounds, postoperative bleeding, prolonged bleeding of umbilical cord		Surgery for multiple VSD and anal reposition ECMO post-surgery NGT for feeding difficulties Developmental delay

Supplementary Table S3 Detailed overview of bleeding symptoms and comorbidities in 14 patients with 11q disorders (*continued*)

Patient number	**Age** years	**Bleeding** Score*	**Platelet count** Mean, (range)	**Bleeding symptoms**	**Hemostatic treatment Blood transfusions**	**Comorbidities**
5	0	5	286 (226-332)	Easy bruising, major bleeding during craniosynostosis repairment surgery	Prophylactic platelet transfusions during craniosynostosis surgery, but extra plasma and platelet transfusions needed	Surgery for craniosynostosis normal development for age
7	15	11	91 (50-133)	Epistaxis, easy bruising, prolonged bleeding after venipunctures, prolonged bleeding after small wounds, postoperative bleeding, heavy menstrual bleeding	Prophylactic platelet transfusions and tranexamic acid, not always sufficient; hysterectomy needed after failure of contraconceptive therapy	Hypogammaglobulinemia (on IgRT) Surgery for large VSD, craniosynostosis and bilateral ectropion; Small for gestational age; Feeding difficulties and GERD Developmental delay, epilepsy Sudden death at age of 16 years
8	4	4	55 (30-80)	Epistaxis, easy bruising, large hematomas, prolonged bleeding small wounds		Normal development
9	8	5	117 (58-230)	Easy bruising, postoperative bleeding and heart tamponade after cardiac surgery	None	Surgery for variant HLHS with small aortic annular, overriding and small aorta, still sub- valvular aorta stenosis. Developmental delay, ADHD
10	22	6	94 (20-134)	Petechial rash, prolonged bleeding after dental extraction, heavy menstrual bleeding	Blood transfusions after car accident; contraconceptive therapy	Surgery for VSD; pulmonary hypertension Hearing aids for senso-neural hearing loss Developmental delay, fever-related convulsions
11	16	13	265 (216-479)	Epistaxis, prolonged bleeding after venipunctures, prolonged bleeding small wounds, period of rectal blood loss, postoperative bleeding after circumcision, muscular bleed after im vaccination	Tranexamic acid. Desmopressin. stitches of minor skin laceration and head wound	Hypogammaglobulinemia (on IgRT) Mild LPA stenosis Developmental delay

Supplementary Table S3 Detailed overview of bleeding symptoms and comorbidities in 14 patients with 11q disorders (*continued*)

Patient number	**Age** years	**Bleeding** Score*	**Platelet count** Mean, (range)	**Bleeding symptoms**	**Hemostatic treatment** **Blood transfusions**	**Comorbidities**
12	6	12	356 (235-849)	Easy bruising, prolonged bleeding small wounds, spontaneous vitreous bleeding needing retinal laser ablation, postoperative bleeding after ATE	Tranexamic acid; Desmopressin Platelet transfusions after ATE	Additional low VWF; mitral valve insufficiency Surgery for cleft palate; gastrostomy for feeding difficulties; horseshoe kidney Osteoporosis, needing bisphosphonates iv; Developmental delay, epilepsy as West syndrome
No increased bleeding tendency according to bleeding score						
6	4	2	94 (70-105)	Mild bleeding tendency	Sporadic epistaxis or easy bruising	Strabismus; benign hydrocephalus. Delayed speech at young age
13	16	0	240 (211-299)	Normal bleeding tendency	None	Developmental delay, fever-related convulsion

Abbreviations ADHD = attention deficit hyperactivity disorder, ATE = adenotonsillectomy, del = deletion, dupl = duplication, GERD = gastro-esophageal reflux disease, HLHS = hypoplastic left heart syndrome, IDDM = insulin dependent diabetes mellitus, im = intramuscular, ISTH-BAT = International Society of Thrombosis and Hematology – Bleeding Assessment Tool, IgRT = immunoglobulins replacement therapy, LPA = left pulmonary artery, NGT = nasal-gastro tube, PFA = Platelet function analyzer, qt = terminal part of chromosome 11q, corresponds with 11q25.0, TPO =thrombopoietin, VSD = Ventricular septum defect, VWF = von Willebrand factor

Legend *The bleeding score is the total score of the Bleeding Assessment Tool validated by the International Society of Thrombosis and Hemostasis. In children till 18 years of age a score ≥ 3 is increased, for males ≥18 years a score ≥4 and in females ≥18 years a score ≥6

I can only draw what I see

Claude Monet (1840 – 1926 AD)

6 Patients with chromosome 11q deletions are characterized by inborn errors of immunity involving both B- and T-lymphocytes

E.J. Huisman
A.R. Brooimans
S. Mayer
M. Joosten
L. de Bont
M. Dekker
E.L.M. Rammeloo
F.J. Smiers
P.M. van Hagen
C.M. Zwaan
M. de Haas
M.H. Cnossen
V.A.S.H. Dalm

Journal of Clinical Immunology 2022;42(7):1521-1534

ABSTRACT

Disorders of the long arm of chromosome 11 (11q) are rare and involve various chromosomal regions. Patients with 11q disorders, including Jacobsen syndrome, often present with a susceptibility for bacterial, prolonged viral and fungal infections partially explained by hypogammaglobulinemia. Additional T lymphocyte or granular neutrophil dysfunction may also be present.

In order to evaluate infectious burden and immunological function in patients with 11q disorders, we studied a cohort of 14 patients with 11q deletions and duplications. Clinically, 12 patients exhibited prolonged and repetitive respiratory tract infections, frequently requiring (prophylactic) antibiotic treatment (n=7), ear-tube placement (n=9) or use of inhalers (n=5). Complicated varicella infections (n=5), chronic eczema (n=6), warts and chronic fungal infections (n=4) were reported. Six patients were on immunoglobulin replacement therapy. We observed a high prevalence of low B lymphocyte counts (n=8), decreased T lymphocyte counts (n=5) and abnormal T lymphocyte function (n=12). Granulocyte function was abnormal in 29% without a clinical phenotype. Immunodeficiency was found in patients with terminal and interstitial 11q deletions and in one patient with terminal 11q duplication. Genetically, *FLI1* and *ETS1* are seen as causative for the immunodeficiency, but these genes were deleted nor duplicated in 4 of our 14 patients. Alternative candidate genes on 11q may have a role in immune dysregulation.

In conclusion, we present evidence that inborn errors of immunity are present in patients with 11q disorders leading to clinically relevant infections. Therefore, broad immunological screening and necessary treatment is of importance in this patient group.

Key words

Chromosome 11q, Jacobsen syndrome, primary immunodeficiency, inborn errors of immunity, B lymphocyte function, T lymphocyte function, granulocyte function, hypogammaglobulinemia, children

INTRODUCTION

Patients with 11q disorders are characterized by a partial deletion or partial duplication of the long arm of chromosome 11. Chromosomal alterations occur in various regions of 11q with deletion sizes ranging from <5-20 Mb(1), and can occur with alterations in other chromosomes.(2-4) Breakpoints arising within or distal to subband 11q23.3 and extending to the telomere, give rise to Jacobsen syndrome (JS), alternatively referred to as 11q terminal deletion disorder.(5, 6) The estimated incidence is 1:100.000 births with a male:female ratio of 1:2.(1) Clinical phenotype depends on the length, position and type of the chromosomal alteration and may include cognitive impairment, cardiac malformation, increased bleeding tendency and increased susceptibility for infections.(1) The latter is attributed to a humoral immunodeficiency with an abnormal B lymphocyte development and low memory B lymphocytes, resulting in hypogammaglobulinemia and an impaired response to immunization.(7-10) although immunoglobulin replacement therapy(IgRT) significantly decreases the infectious burden (7), complications still occur.(11) Some authors suggest that low T lymphocyte counts (7-9, 12) or T lymphocyte dysfunction (8, 9, 12) may be causative. We hypothesized that granular dysfunction of neutrophils may also play a role, because patients with JS are also known to suffer from increased bleeding tendency due to delta storage pool defects (13), or giant alpha granules in platelets leading to the Paris-Trousseau syndrome.(14) The combination of platelet en neutrophil dysfunction is seen in other syndromes, such as Hermansky-Pudlak type 2 and grey-platelet.(15, 16)

Genetically, focus is on the transcription factors ETS1 and FLI1, both located on 11q24.3. For *ETS1*, experimental evidence in mice with a homozygous deletion shows that proliferation of B and T lymphocytes is decreased,(17) and spontaneous apoptosis of T lymphocytes is increased.(18-20) Somatic loss of 11q23.3 has been found in B lymphocyte lymphoma (*MYC* negative), also indicating an effect on lymphocyte proliferation.(21) In humans, the role of *ETS1* was demonstrated in a recent case-report of a patient with a germline pathogenic frameshift mutation in *ETS1* in whom a decreased number of total and naïve B lymphocytes was found, as well as reduced presence of memory B lymphocytes. T lymphocyte number however was normal.(22)

FLI1 is a member of the E26 transformation specific (*ETS*) gene family that shares a DNA-binding domain called *ETS*-domain, which is responsible for sequence-specific DNA recognition of target promotors. This domain has a role in development of B and T lymphocytes.(23) Experimental evidence comes from *FLI1*-knockout mice, constructed with deletion of N-terminal region of *FLI1 (*FLI1ΔNT). FLI1ΔNT-mice exhibited thymic hypocellularity.(24) This defect was not associated with a specific subpopulation of thymocytes or apoptosis, implicating the role of *FLI1* in prethymic T lymphocyte progenitors.(24) The role of *FLI1* in B lymphocyte development has also been demonstrated by *FLI1*-knockout

mice lacking the CTA domain (FLI1ΔCTA).(23, 25) FLI1ΔCTA-homozygous mice show less splenic follicular B lymphocytes, and more transitional and marginal zone B lymphocytes. (23) Despite this fundamental evidence, clinically a variable phenotype-genotype is seen. In two families with an identical microdeletion in 11q24.2-11q24.3, one family demonstrated persistent lymphopenia and low IgG, but the other did not.(26)

In order to evaluate immunological dysfunction in patients with 11q disorders, we prospectively investigated 14 patients focusing on clinical features and tested both T lymphocyte and granulocyte function. We correlate our results with monosomy or trisomy of *ETS1* and *FLI1*.

METHODS

Patient population and study design

Patients were invited for this observational study by their treating physician or via the newsletter of the Dutch Chromosome 11 Network that represents patients with 11q deletions and 11q duplications. Institutional Review Board approval was obtained (MEC-2013-026) and the study was performed according to the Declaration of Helsinki. All patients were included after obtaining written informed consent according to local law and regulation. Clinical data included number and type of infections, antibiotic treatment and IgRT. Data was retrospectively derived from electronic patient files (EPF) after informed consent was given. Laboratory results for children were compared with age-dependent reference values for blood count,(27) lymphocyte subsets.(28) and IgGAM-values. (29, 30) Results were recorded as normal when test results were in p5-p95 interval of the normal references. Molecular diagnosis was extracted from the EPF if the diagnosis was made by karyotyping or array. Patients were followed up by their own referring physician.

Laboratory tests

For laboratory tests a minimum of 0.5 mL of EDTA-blood, 0.5 mL serum and 6 mL heparinized blood were drawn by venepuncture. Cell count and indices were analyzed on Sysmex XN-9100 (Sysmex®). Total IgG, IgA and IgM were investigated on Cobas 8000 Modular Analyzer Series (Roche Diagnostics®, Basel). Heparinized whole blood was used for flowcytometric measurement of lymphocyte count (FACSCanto II, BD®, USA).

Detection of antigen-specific CD4$^+$ T lymphocytes Antigen-specific CD4+ T lymphocytes were measured using a commercially available kit (Act-T4 Cell™, Cytognos, Spain) that is based on the 'OX40-assay'.(31) Heparinized whole blood was diluted 1:1 with RPMI-1640 (Gibco, Paisley, UK) culture medium supplemented with 10% fetal calf serum (Gibco), L-glutamine 2 mM (BioWhittaker), and penicillin and streptomycin (100 IU/mL; BioWhittaker). Then aliquoted into 500 µL volumes in sterile capped 5 mL polystyrene

flow cytometry tubes. The following conditions were used for each assay: no exogenous stimulation (negative control), PHA (2.5 µg/mL), Staphylococcal enterotoxin B (SEB; Sigma-Aldrich) (0.5 µg/mL), and activation with tuberculin PPD (4 µg/mL), Tetanus toxoid (1 µg/mL), Diphtheria toxoid (10 µg/mL), Candida lysate (4 µg/mL), CMV lysate (2 µg/mL), HSV1 lysate (1 µg/mL), HSV2 lysate (1 µg/mL), and VZV lysate (1 µg/mL). After 44-48 hour stimulation in a humidified atmosphere of 5% CO_2, samples were vortexed and 100 µL of each culture was stained with 20 µL of the 4-antibody mixture provided in the Act-T4 Cell™ kit (CD3-PerCP-Cy5.5, CD4-FITC, CD25-APC, and CD134-PE, Cytognos) and incubated for 15minutes at room temperature. The assay was protected from light. After incubation, 1 mL of erythrocyte lyse-non-wash solution provided in the Act-T4 Cell™ kit was added to each tube, mixed and incubated for 10 minutes at room temperature, still protected from light. After lysis 10,000 CD3 positive events were acquired on flow cytometer (FACSCanto II) and analyzed with Infinicyt software (Cytognos). Quantification of CD3+CD4+CD25+CD134+ T lymphocytes were calculated by setting gate coordinates on the negative control sample to equal 0.1% CD3+CD4+CD25+CD134+ events as a percentage of all CD3+CD4+ events. These gate coordinates were applied to subsequent antigen tubes to produce a percentage value of CD25+CD134+ double positive events. Normal reference values were determined in a cohort of 40 healthy controls.

Determination of granulocyte phagocytic activity–Phagotest The granulocyte phagocytic activity was measured in whole heparinized blood using a commercially available kit (Phagotest®, Glycotope Biotechnology, Germany) according to manufacturer's instructions. In short, FITC-labeled opsonized *E. coli* bacteria were added to whole blood and incubated for 10 min at 37°C (experimental tube) or 0°C (negative control tube). After incubation, the reaction was stopped, erythrocytes were lysed and the DNA staining solution was added. Fluorescence of samples was measured by flow cytometer (FACSCanto II) in <60 min after the last reagent had been added. Data was acquired by FACSDiva software (BD Biosciences) and analyzed by Infinicyte software. The Phagotest is performed with the involvement of fluorescein-stained *E. coli* bacteria which are phagocytized by the cells. The test determines the percentage of granulocytes and their phagocytic activity, i.e. the number of bacteria absorbed by a single cell in terms of mean fluorescence intensity (MFI).

Determination of oxidative burst activity of granulocytes–Phagoburst A respiratory burst-assay was performed using a commercially available kit (Phagoburst®, Glycotope Biotechnology, Germany) according to manufacturer's instructions. Briefly, opsonized *E. coli* bacteria (experimental tube), or phorbol 12-myristate 13-acetate (PMA, positive control tube) or washing solution (negative control tube) were added to whole blood and incubated for 10 min at 37°C. Following incubation, dihydrorhodamine (DHR 123) was added for 10 min, erythrocytes were lysed and DNA-staining solution was added. Dihydrorhodamine 123 becomes fluorescent when oxidized by reactive oxygen species, and

its fluorescence was measured in less than 30 min after the last reagent had been added by flow cytometry. The test determines the percentage of active cells, and the respiratory burst intensity within a single cell in terms of MFI.

Statistical analysis

We used descriptive statistics to summarize baseline characteristics of the study population. In case of a skewed distribution, data are presented as median and interquartile range (IQR). In case of a normal distribution, data are presented as mean and standard deviation (SD) or range. Categorical data are presented as numbers with percentages and range of minimum and maximum value.

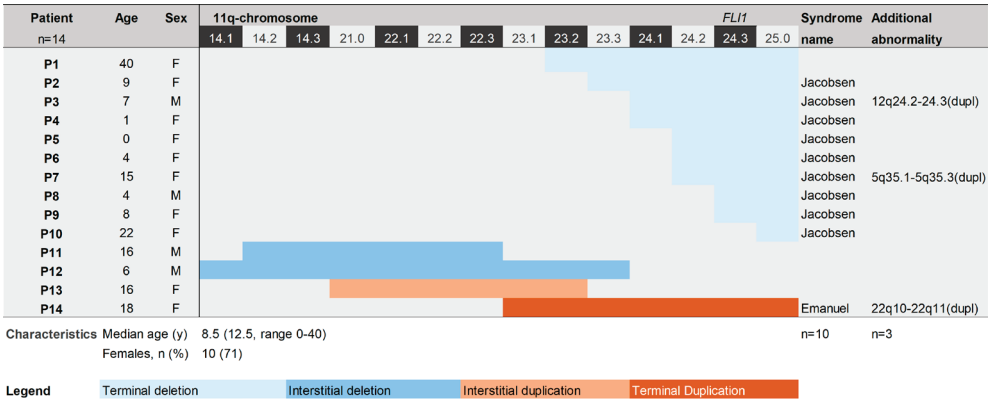
Reference values of healthy controls were available for the various antigens that were used as stimuli in the OX40 test. The results of the T lymphocyte function tests were considered abnormal when the results were <p5 of healthy controls. Also, for both granulocyte function tests obtained reference values of healthy controls were used. The phagocytic and oxidative burst capacity results were considered abnormal when the results were <p5 of healthy controls. Statistical data analyses were performed using SPSS version 21.0 (IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

Sixteen patients were identified, one patient declined participation and one passed away before study initiation. We included 14 patients (P), with a median age of 9.1 years (IQR 5.6-17.4 99; range 0-40), and four males (29%). Patients 1, 2, 7 and 11 of our cohort have been investigated in an earlier publication by Dalm et al. They correspond with the patients 2, 6, 5 and 4.(7) Twelve presented with an interstitial or terminal 11q deletion, two with 11q duplication, one interstitial and one terminal. The extent and location of the regions involved varied widely, and in three patients' additional chromosomal abnormalities were observed. During follow-up one patient (P7) passed away (Table 1).

Table 1 Patient characteristics of 14 patients with 11q disorders



Abbreviations F = female, IQR = interquartile range, M = male, n = number, P = patient number

Clinically, all patients were immunized according to recommendations of the Dutch National Institute for Public Health and the Environment (RIVM).(32) This does not involve varicella or rotavirus vaccines. Frequent infections were reported in the majority of patients, especially during childhood (12/14; 86%) and most often affecting upper and lower respiratory tract (RTI). These infections required frequent or prophylactic antibiotic treatment (7/12; 70%). Nine required ear-nose-throat (ENT) interventions such as ear tubes or adenotonsillectomy (9/14; 64%). Five required (5/14; 36%) therapy with inhaled β2 agonists, and three (3/14; 21%) were hospitalized for oxygen supplementation as supportive measures in case of infectious complications. Six patients (6/14; 43%) were on intravenous or subcutaneous IgRT because of hypogammaglobulinemia. All six patients on IgRT reported less infections and improved physical condition (Table 2).

Table 2 Immunological symptoms and comorbidities of 14 patients with 11q disorders

P	Age	IgRT	Viral infections	Bacterial infections	Eczema	Warts	Mycosis	Comorbidities
On Immunoglobulin Replacement Therapy (IgRT)								
1	40	Yes	Frequent prolonged viral URTI; frequent ENTI requiring tubes; VZV infection: caregiver does not recall	Frequent oral antibiotics; Paronychia requiring drainage	None	Multiple on hands and feet, needing Gardasil.	None	Increased bleeding tendency; asymmetric aortic valve; eyelid surgery; hearing aids; lymphangiodysplasia of lower legs; moderate developmental delay

Table 2 Immunological symptoms and comorbidities of 14 patients with 11q disorders (*continued*)

P	Age	IgRT	Viral infections	Bacterial infections	Eczema	Warts	Mycosis	Comorbidities
2	10	Since age of 6y	Frequent URTI; frequent ENTI requiring tubes; frequent rehydration by GI-infections; Severe VZV	Frequent oral antibiotics; paronychia requiring drainage; bacterial superinfection of skin during VZV requiring local and iv AB	None	None	Fungal nails and vaginal mycosis, chronic use of anti-mucosal treatment	Increased bleeding tendency; craniosynostosis repairment; eyelid surgery; type 1 diabetes mellitus (11y); moderate-severe developmental delay
3	7	Yes	Frequent URTI and ENTI; viral bronchitis and BHR requiring hospitalization for salbutamol and oxygen; mild-moderate VZV	Frequent oral antibiotics and prophylaxis till age of 6y	None	None	None	Increased bleeding tendency; large VSD; eyelid surgery; Nissen-surgery for GERD; moderate-severe developmental delay
7	15	Since age of 10y	Frequent URTI; frequent ENTI requiring tubes; severe H1N1 influenza in 2009, needing Tamiflu; recurrent hospitalization for dehydration due to GI-infections; moderate VZV	Frequent AB; hospitalization for sepsis* (occurred under IgRT)	None	None	None	Increased bleeding tendency; large VSD; craniosynostosis repairment; eye lid surgery; severe GERD; celiac disease; severe developmental delay. Epilepsy; sudden death (16y)
11	16	Since age of 5y	Frequent URTI and ENTI; severe RSV and parainfluenza infection requiring oxygen. BHR, needing salbutamol and ICS; hospitalization for dehydration due to Rotavirus; severe VZV	Frequent AB and prophylaxis; once iv for LRTI. Paronychia requiring topical AB. Balanitis, requiring circumcision; bacterial superinfection of the skin during VZV	Chronic, resolved under IgRT	None	Pityriasus rosacea, fungal nails needing oral terbinafin	Increased bleeding tendency; left pulmonary artery stenosis; mild developmental delay

Table 2 Immunological symptoms and comorbidities of 14 patients with 11q disorders (*continued*)

P	Age	IgRT	Viral infections	Bacterial infections	Eczema	Warts	Mycosis	Comorbidities
14	18	Since age of 14y	Frequent URTI; frequent ENTI requiring tubes; frequent oral aphthous stomatitis; severe VZV	Frequent AB; bacterial superinfection of the skin during VZV requiring AB	Chronic, resolved under IgRT	One persistent on toe	Topical ketoconazole for frequent diaper dermatitis	Increased bleeding tendency; congenital hip dysplasia; moderate-severe developmental delay
				Not on Immunoglobulin Replacement Therapy				
4	1	No	No prolonged or complicated viral infections	No infections	None	None	None	Increased bleeding tendency; multiple VSDs; feeding difficulties; mild developmental delay
5	0	No	No prolonged or complicated viral infections	No infections	None	None	None	Increased bleeding tendency; craniosynostosis repairment; normal development
6	4	No	Frequent URTI; dehydration due to GI-infection; severe VZV	Bacterial superinfection of the skin during VZV	Chronic in hair, axillary regions and umbilical area	None	Skin fungal dermatitis, needing miconazole.	Increased bleeding tendency; strabismus; benign hydrocephalus; mild speech delay
8	4	No	Frequent URTI; frequent ENTI requiring tubes; moderate-severe VZV	Frequent AB and prophylaxis	Moderate	None	Chronically affected, between toes, needing miconazole	Increased bleeding tendency; normal development
9	8	No	Frequent URTI; frequent ENTI requiring tubes; BHR, requiring salbutamol; once hospitalized for oxygen during viral LRTI; severe VZV	Frequent AB; bacterial superinfection of the skin during VZV requiring AB	Chronic	Chronically affected toe-nails	None	Normal bleeding tendency; variant HLHS; ADHD; mild developmental delay

Table 2 Immunological symptoms and comorbidities of 14 patients with 11q disorders (*continued*)

P	Age	IgRT	Viral infections	Bacterial infections	Eczema	Warts	Mycosis	Comorbidities
10	22	No	Frequent URTI and ENTI; BHR requiring salbutamol; once hospitalization for LRTI; moderate VZV	Frequent AB	Frequent seborrheic eczema around ears and in hair	None	None	Increased bleeding tendency; ASD, VSD, MS, ODB and pulmonary hypertension; hearing aids; fever-related convulsions; moderate developmental delay
12	6	No	Frequent URTI; frequent ENTI requiring tubes; BHR requiring salbutamol; severe pseudocroup requiring epinephrin; severe picornavirus and rhinovirus; No VZV	Frequent AB and AB prophylaxis	Yes	None	None	Increased bleeding tendency; Vision loss after vitreous hemorrhage; severe MI; cleft palate; feeding difficulties; retrognathia; horse shoe kidney; osteoporosis; epilepsy; moderate-severe developmental delay
13	16	No	Frequent URTI; frequent ENTI requiring tubes; no VZV	Sporadic antibiotics; frequent urinary tract infections, once complicated by pyelonephritis			Frequent diaper dermatitis, needing miconazole	No bleeding tendency; fever-related convulsions; moderate-severe developmental delay

Abbreviations AB = Antibiotics, ADHD = Attention deficit hyperactivity disorder, ASD = Atrial septum defect, BHR = Bronchial hyperreactivity, ENT = Ear-Nose-Throat, GERD = Gastro-esophageal reflux disease, GI = Gastro-intestinal, HLHS = Hypoplastic left heart syndrome, ICS = inhalation corticosteroids, IgRT = Immunoglobulin Replacement Therapy, iv = intravenous, LRTI = Lower respiratory tract infections, MI = mitral valve insufficiency, MS = Mitral valve stenosis, ODB = Open Duct of Botalli, P = patient number, RSV = Respiratory syncytial virus, URTI = Upper respiratory tract infections, VSD = Ventricular septum defect, VZV = Varicella-Zoster virus, VSD = Ventricular septum defect, y = year

Complicated viral infections leading to hospitalization were confirmed for respiratory syncytial virus (RSV), picornavirus, para-influenza virus and rhinovirus. Complicated and prolonged varicella zoster virus (VZV) infection occurred in 5/14 (36%). Four of 14 patients (29%) suffered from gastro-enteritis requiring hospitalization because of dehydration. Rotavirus was confirmed in two of these episodes. Fungal infections, most often in the diaper area or the nails were reported on 4/14 (29%), persistent warts by two patients

(2/14; 14%), and chronic eczema by six (43%), which resolved in patient P11 and 14 after IgRT was initiated.

Immunological laboratory results are presented in Table 3. Low leucocyte counts were seen in two patients (P2, 7): 2.8 and 3.2 x 10^9/L respectively (normal range 3.8-9.8x 10^9/L. In five (36%) absolute lymphopenia was observed (mean 0.78; absolute range 0.63-0.95 compared to age-dependent normal ranges). Total numbers of T lymphocytes and B lymphocytes were normal or elevated in four patients (4/14; 29%), all had IgG levels within the reference range. Total B lymphocyte counts were low in eight patients (57%, normal reference >0.20 x 10^9/L). In five (5/8, 63%) T lymphocyte numbers were also low. Remarkably, all eight patients with low B lymphocyte counts presented with clinically relevant and increased number of infections, regardless of IgG levels (P1-3, 7-10, 14). The absence of an association between total number of B lymphocytes and function is illustrated in P11, who had a hypogammaglobulinemia from infancy with normal but steadily decreasing B lymphocyte count up to the age of 18 years (from 1.68 x 10^9/L at age 1, to 0.21 x 10^9/L at age 18). Low IgG-levels were found in six patients (6/14; 43%), all corrected on IgRT. A concomitant low IgM (2/6) sometimes with combined low IgM and IgA (2/6) was seen in four (4/6, 67%). Only low IgM levels were seen in three patients (3/14; 21%), of whom two were <2 years of age at time of inclusion. One patient had a low combined IgA and IgM (P12).

Total T lymphocyte counts were low in five patients (5/14, 36%). In one patient both CD4+ and CD8+ T lymphocytes were decreased (1/5), in two only CD4+ and in two only CD8+ T lymphocytes were decreased (normal reference CD4+ >0.3 x 10^9/L, CD8+ >0.2 x 10^9/L), resulting in an abnormal CD4:CD8-ratio in four (P1, 6, 8, 9). T lymphocyte function was evaluated in 13 patients using an OX40-based test. (Table 4) In general, only two patients showed a normal response to all stimuli (P6, 13). T lymphocytes from ten patients showed at least one abnormal response to the various stimuli (83%). For the viral agonists, T lymphocytes of all patients on IgRT failed in CMV-response (5/5, 100%). Also, T lymphocytes of three patients (not on IgRT, but with a history of frequent viral upper airway and ENT infections) failed on CMV-response (P9-10, 12). As this assay is a functional test, diminished responses are either due to an intrinsic T lymphocyte defect, or due to the fact that a patient has not yet encountered CMV. Unfortunately, data of seroconversion were missing. Medical history was certain for 13 patients for VZV. Of these 13, three patients with a history of VZV infection, demonstrated an abnormal T lymphocyte response (P2, 3, 7; 3/12; 25%). Despite the fact that all patients were vaccinated, an impaired response to the conjugated vaccine against tetanus was observed in two patients (P-1 2; 2/12; 7%). All responded well to diphtheria and also to tuberculin. For the Toll-like receptor (TLR) agonists, five patients showed abnormal results after aspecific T cell stimulation with the superantigen SEB (through TLR2) or the mitogen PHA (through TLR4) P1, 3, 7, 9; 4/12, 33%).

Table 3 Immunological analysis of 14 patients with 11q-disorders

Patient	Leuco (10^9/L)	Neutro (10^9/L)	Lympho (10^9/L/%)	IgA (g/L)	IgG* (g/L)	IgM (g/L)	Tc Total, CD4+, CD8+ (10^9/L)			CD4/CD8 ratio	Bc Total (10^9/L)	NKc Total (10^9/L)	Granulocyte function		
													chemotaxis	phago cytosis	oxidative burst
On Immunoglobulin Replacement Therapy															
1	8,7	7,71	**0,68 (8%)**	1,93	9,1*	**0,22**	**0,58**	0,49	**0,09**	High	**0,04**	**0,09**	Normal	Normal	Normal
2	**2,8**	2,02	**0,63** (23%)	0,82	12,2*	**<0,05**	**0,55**	0,32	**0,23**	Normal	**0,04**	**0,03**	Normal	Normal	Inadequate
3	6,6	3,3	2,90 (44%)	**0,40**	**3,92***	**0,13**	**0,11**	**0,07**	**0,04**	Normal	**0,14**	**0,08**	-	-	-
7	**3,2**	1,94	**0,95** (30%)	0,91	17,9*	**0,24**	0,85	0,44	0,30	Normal	**0,09**	**0,13**	Normal	Normal	Inadequate
11	6,7	4,25	1,63 (24%)	**0,47**	**0,8*^**	**0,21**	1,14	0,74	0,38	Normal	0,21	0,40	Normal	Normal	Normal
14T†	17,5	13,2	2,08 **(12%)**	0,65	**5,2***	0,97	1,53	1,06	0,47	Normal	**0,11**	0,46	Normal	Normal	Normal
Not on Immunoglobulin Replacement Therapy															
4	6,2	2,9	2,71 (44%)	0,31	6,8	**0,20**	1,99	1,28	0,65	Normal	0,83	**0,17**	-	-	-
5	6,2	1,76	3,62 (58%)	0,26	3,8	**0,14**	1,71	1,17	0,50	Normal	0,50	0,29	-	-	-
6	4,9	2,77	1,76 (36%)	1,40	11,3	0,30	1,27	0,62	0,60	Low	0,46	**0,10**	Normal	Normal	Normal
8	6,1	4,8	**0,90 (15%)**	0,96	7,7	0,44	**0,58**	**0,22**	0,32	Low	**0,15**	**0,14**	Normal	Normal	Normal
9	6,0	5,0	**0,76 (13%)**	1,59	8,1	0,45	**0,63**	**0,27**	0,35	Low	**0,17**	**0,06**	Inadequate	Normal	Normal
10	5,5	3,5	1,63 (29%)	2,07	10,5	0,55	1,23	0,69	0,48	Normal	**0,17**	0,19	Normal	Normal	Normal
12	13,3‡	8,1	3,84 (28%)	**0,41**	8,0	**0,30**	3,12	2,27$^{\$}$	0,79	Normal	0,64	0,26	Normal	Normal	Normal
13T	5,8	2,8	2,16 (37%)	1,83	11,7	1,28	1.67	0,89	0,68	Normal	0,35	0,44	Inadequate	Normal	Normal
Mean	7,11	4,58	1,88	1,00	8,12$^{\#}$	0,42	1,21	0,64	0,42		0,28	0,20			
SD	3,88	3,17	1,07	0,64	2,69	0,34	0,77	0,38	0,22		0,24	0,14			

Abbreviations Bc = B lymphocyte (cell), CD = Cluster of Differentation, chemotax = chemotaxin, Ig = Immunoglobulin, IgRT = Immunoglobulin Replacement Therapy, Leuco = leucocyte, Lympho = lymphocyte, Neutro = neutrophil granulocyte, NKc = natural killer cell, SD = Standard Deviation, T = trisomy, Tc = T lymphocyte (cell)

Legend Bold = <p5 age-adapted reference values, + AB = Specific antibody against polysaccharide vaccine against *S. pneumoniae*, *= IgG during immunoglobulin replacement therapy. If pre-substitution IgG was know, this is mentioned. Otherwise the IgG at inclusion time is mentioned; ^= at 3 months of age IgG total was 0,8, this increased till 3,1 at age of 3 years. Patient started IgG-substitution in 2004 † T = 11q trisomy, ‡ patient had a viral bronchitis at time of inclusion, but retrospectively total leucocyte count was always higher than the age-adjusted reference values, # mean calculated on >3 values pre- or non IgRT

Table 4 T lymphocyte Function Assay analysis of 13 patients with 11q-disorders

Patient	CD4+ Tc#	Sample control	PHA	SEB	CMV	Candida	TT	Tuberculin	Diphtheria	HSV 1	HSV 2	VZV%	Interpretation
On Immunoglobulin replacement therapy													
1	0,49	0,20	**1,43**[%]	**8,60**	**0,31**	0,18	0,19	0,19	0,25	0,39	0,32	***0,25***[&]	Inadequate response
2	0,32	0,09	12,95	**1,59**	**0,07**	0,18	0,20	0,15	0,12	0,15	0,11	***0,13***[^]	Inadequate response
3	0,37	0,23	22,74	**3,83**	**0,14**	0,65	1,86	0,16	0,20	0,16	0,27	***0,39***	Inadequate response
7	0,44	0,13	**4,38**	**7,15**	**0,13**	0,63	0,97	0,24	0,64	0,11	0,07	***0,15***[^]	Inadequate response
11	0,74	0,06	54,36	16,36	**0,06**	3,83	8,20	1,59	2,38	11,06	6,84	*3,55*	Inadequate response to CMV
14T	1,06	0,09	26,21	15,01	**0,12**	7,20	3,50	2,38	4,10	0,20	0,13	*2,07*[^]	Inadequate response to CMV and HSV1
Not on Immunoglobulin replacement therapy													
4	0,65	0,03	33,79	17,63	1,67	0,13	0,24	0,29	**0,02**	**0,09**	**0,07**	**0,15**	Inadequate response
6	0,58	0,22	13,14	17,36	1,44	0,64	3,10	0,45	2,41	**0,13**	0,39	*1,24*[^]	Inadequate response to HSV1
8	**0,22**	0,06	15,25	**9,43**	**0,69**	0,30	0,21	1,58	0,18	1,81	0,74	***0,60***	Inadequate response
9	**0,27**	0,07	**3,03**	16,2	1,39	0,09	0,27	0,14	0,64	**0,12**	0,26	***0,63***[^]	Inadequate response
10	0,69	0,34	29,71	18,22	**0,16**	0,96	1,10	0,29	0,65	**0,35**	0,51	1,29	Inadequate response to CMV and HSV1
12	2,27	0,07	10,13	18,74	**0,15**	0,82	3,04	0,69	2,20	**0,10**	0,15	**0,16**	Inadequate response
13T	0,89	0,35	23,91	19,96	8,94	12,02	2,34	9,82	3,91	13,99	8,93	3,68	Normal response
Healthy Controls	**P5**		8,69	11,77	1,35	0,08	0,13	0,06	0,04	1,70	0,10	0,99	
n=40	**Mean**		27,60	20,67	6,69	1,05	2,81	1,45	0,68	6,96	2,50	2,22	
	P95		61,17	33,78	12,29	9,14	8,47	5,85	3,70	15,52	5,88	7,73	

Abbreviations PHA = Phytohemagglutinin, SEB = Staphylococcal enterotoxin B, CMV = Cytomegalovirus, n = number, Tc = T lymphocyte (cell), TT = Tetanus toxoid, HSV = Herpes Simplex Virus, VZV = Varicella-Zoster virus

Legend [#] CD4+ Tc-number in sample taken for functional T lymphocyte test. This can slightly differ from table 2 due to physiological variation in blood cell numbers; [%] Bold = <p5 reference values based on 40 healthy controls (HC); [^] underscored italic numbers in VZV column demonstrated patients that have encountered VZV based on medical history; [&] Caregiver does not recall if patient has had VZV

Neutrophil granulocyte counts were normal in all 14 patients. Neutrophil granulocyte function was abnormal in four patients (P2, 7, 9, 13; 4/14, 29%). Two patients (P2, 7), who responded with a lower than normal oxidative burst, presented with a history of frequent infections and antibiotic usage, but none of the patients had opportunistic infections with microbes that are pathognomonic for neutrophil dysfunction, e.g. invasive *S. aureus, Aspergillus, Nocardia, Burkholderia* or *Serratia*. Both patients are currently on IgRT. The two other patients (P9, 13) showed abnormal responses in chemotaxis, of whom patient 13 (P13) with a trisomy only reported chronic (diaper) dermatitis and ENT infections in infancy, and patient 9 (P9) reported an increased infectious burden in ENT and respiratory tract but is not on IgRT due to normal IgG levels. *ETS1* and *FLI1* involvement did not correlate to patients with immunodeficiency of B nor T lymphocyte dysfunction in our cohort. See Table 5.

Table 5 ETS1 and FLI1-involvement in 14 patients with 11q-disorders

Patient	Age	Infectious burden	Bc count	Tc count	CD4: CD8 ratio	IgG	Tc function	FLI1	ETS1
On Immunoglobulin Replacement Therapy									
1	40	↑↑	↓	↓	↑	↓	Inadequate	Deleted	Deleted
2	10	↑↑	↓	↓	Normal	↓	Inadequate	Deleted	Deleted
3	7	↑↑	↓	↓	Normal	↓	Inadequate	Deleted	Deleted
7	15	↑↑	↓	Normal	Normal	↓	Inadequate	Deleted	Deleted
11	16	↑↑	Normal	Normal	Normal	↓	Inadequate	Normal	Normal
14	18	↑↑	↓	Normal	Normal	↓	Inadequate	Duplicated	Duplicated
Not on Immunoglobulin Replacement Therapy									
4	1	Normal	Normal	Normal	Normal	Normal	Inadequate	Deleted	Deleted
5	0	Normal	Normal	Normal	Normal	Normal	Inadequate	Deleted	Deleted
6	4	↑↑	Normal	Normal	↓	Normal	Inadequate	Deleted	Deleted
8	4	↑↑	↓	↓	↓	Normal	Inadequate	Deleted	Deleted
9	8	↑↑	↓	↓	↓	Normal	Inadequate	Deleted	Deleted
10	22	↑↑	↓	Normal	Normal	Normal	Inadequate	Normal	Normal
12	6	↑↑	Normal	Normal	Normal	Normal	Inadequate	Normal	Normal
13	16	↑	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Abbreviations Bc = B lymphocyte, CD = Cluster of Differentiation, Ig = Immunoglobulin, P = Patient number, Tc = T lymphocyte

DISCUSSION

We present 14 patients with a variety of 11q disorders. We demonstrated an increased vulnerability to infections in all, but with varying clinical impact. Infections started in infancy with recurrent and/or chronic ENT and airway infections, leading to high consumption of antibiotics, but also of inhalation therapy, hospitalization and the performance of ENT surgery. Vulnerability for both viral and bacterial infections is illustrated by the finding that almost half of patients suffered from VZV infections with multiple skin lesions leading to secondary bacterial skin infections. Also, other skin manifestations, including eczema, dermatitis, fungal skin and nail infections, and warts, were encountered regularly.

We confirm low B lymphocyte numbers in 57% (8/14), as well as B lymphocyte dysfunction resulting in hypogammaglobulinemia in 64% (9/14) of patients in infancy or at older age, necessitating IgRT in 6/9.(7, 8) We underline the relevant finding that low IgG is not always accompanied by low total B lymphocyte counts and vice versa, as demonstrated in patient 11 (P11).(7, 12)

We demonstrate that T lymphocyte dysfunction occurs in the majority of patients with 11q disorders. We showed that decreased T lymphocyte counts or abnormal CD4/CD8-ratio occur in 43% of our cohort and abnormal in vitro T lymphocyte activation in 92% (12/13). Especially, the abnormal responses to stimuli such as PHA, SEB, Diphtheria, CMV and VZV are clinically relevant. Unfortunately, we were not able to test seroconversion for CMV. The abnormal response may be due to naivety towards CMV, HSV1 or VZV in four patients, still giving an abnormal test result in 8. Qualitative T lymphocyte defects have been reported in cases before.(8, 12, 33), but none of the applied stimulation tests in these studies were identical to the assays we used, which makes comparisons between results difficult. We conclude that inborn errors of T lymphocyte immunity regularly occur, which was suggested in previous case-series.(7, 8 34-37)

Finally, although we demonstrate a disturbed granulocyte function *in vitro*, these findings may be interpreted as mild or irrelevant as none of the patients experienced typical infections with e.g. *Aspergillus* or invasive *S. aureus*. It therefore remains unclear whether the need for antibiotics due to a bacterial superinfection after VZV (P2, 6, 9, 11, 14; 56%) is due to hypogammaglobulinemia only or combined with a potential granular dysfunction (only P2, 11, 14 are on IgRT). In a single patient on IgRT hospitalization for possible septicemia was documented. In this patient, an isolated abnormal response in the Phagoburst assay was found in granulocyte function analysis (P7). Our current results do not clearly support nor rule out the hypothesis that granulocyte dysfunction is responsible for the high incidence of infections, more research in this area is indicated. Novel diagnostic tools as electro-microscopy (EM) may be promising.(16)

ETS1 and *FLI1*-involvement did not correspond with abnormal B or T lymphocyte number or function. While monosomy was seen in 9 patients (P1-9) and trisomy in 1 (P14), an abnormal B or T lymphocyte number was only seen in 7. On the other hand, low T lymphocyte numbers (P10) and abnormal function was seen in 3 patients (P10-12) in which *ETS1* and *FLI1* copies were normal (Table 5). This variable phenotype-genotype may be due to incomplete penetrance, as is suggested in congenital microdeletions in two families with an identical microdeletion in 11q24.2-11q24.3. One family demonstrated persistent lymphopenia and low IgG, the other did not.(26) But, this variability may also be due to other genes or gene clusters that are located on 11q and are involved in cell proliferation and differentiation. Genes of interest are *ATM, CD3, CBL* and *THYN1*. *ATM* is located on 11q22.3 (OMIM #607585). Homozygous or compound heterozygous mutations cause ataxia-telangiectasia (AT).(38) AT is characterized by cerebellar ataxia, telangiectasia, and immune defects, such as reduced antibody levels, lymphopenia and abnormalities in T lymphocyte maturation. Also, variant-AT with residual ATM protein expression and kinase-activity causes a milder phenotype. In variant-AT only one truncating mutation is found, together with a compound missense, splice variant or leaky mutation.(39, 40) The cluster of *CD3EAP, CD3D, CD3E, CD3G, CD3Z* are all located on 11q23.2, and regulate the synthesis of T lymphocyte antigen receptor chains: Tγ, Tδ, Tε, Tζ.(41) During development, the CD3 complex plays an important role in the transition of thymocytes from immature precursors to the final mature CD4+ or CD8+ T lymphocytes.(42) Pathogenic variants in any of these genes may cause blockage at the stage of CD4+/CD8+ lymphocytes, resulting in reduced Tγδ lymphocytes.(43) *CBL*, located on 11q23.3, encodes for an E3 ubiquitin ligase acting as a regulator in the tyrosine kinase signaling pathway.(43) *CBL* plays a role in lymphocyte development and activation by regulating development of the thymocyte, and by regulating receptor signaling thresholds for B lymphocyte maturation.(44, 45) Besides single nucleotide variants, Hanson et al. describe this entity as the result of uniparental isodisomy of 11q23, underlining the possible influence of this gene for the clinical phenotype of 11q disorders. (46) *THYN1*, located on 11q25, encodes for thymocyte nuclear protein 1, which is expressed in the thymus.(47) This gene may be involved in the induction of apoptosis or T lymphocyte regulation.(48, 49) As yet, we are not aware of diseases in humans caused by mutations in this gene.

Our study has several limitations. Firstly, we describe only a small cohort in whom the genetic defects vary considerably and concurrent chromosomal abnormalities occur. This reflects medical practice as 11q disorders are a rare contiguous gene syndrome, but extrapolation of results to other 11q disorders is challenging. In our cohort, seven patients had the 'classical' Jacobsen syndrome (11q23.3-terminal deletion syndrome). Two patients had an interstitial 11q deletion. We have chosen to include these 2 patients as well, because both show the same vulnerability to infections and bleeding as terminal 11q patients exhibit. It is unknown if this is due to other candidate genes or due to downstream loss of function resembling terminal 11q deletion patients. There are hardly

any data available on this rare entity, and with our results we like to raise awareness that inborn errors of immunity may be seen in patients with interstitial 11q disorders. The same accounts for the two 11q duplication patients. Three patients had additional chromosomal abnormalities, of whom P14 had a combination also referred to as Emanuel syndrome. No clear inborn errors of immunity have yet been reported to occur in Emanuel syndrome, but frequent ENT infections are reported in cases and are mentioned by the unique patient organization to occur "in a minority".(50, 51) The trisomy of 11q23.3 in our patient may give rise to hypogammaglobulinemia and T lymphocyte dysfunction, but the 22q11 region can also cause T lymphocyte dysfunctions or T lymphocytopenia as is seen in other chromosome 22q abnormalities and in one case-report of a child with a 22q11.2 microduplication.(52) No hypogammaglobulinemia was seen in this child. (53, 54) We therefore feel that it is unclear whether the hypogammaglobulinemia and abnormal T lymphocyte function in our patient are only due to terminal 11q duplication. We decided to include this patient in our cohort as the effect of 11q cannot be ruled out and it is essential to be aware that 11q trisomy patients may be prone to inborn errors of immunity as well. More research in this specific patient group is needed.

Also, although a study design with a standardized protocol was followed, we were not able to avoid missing values with regard to clinical data and laboratory test results. Still, we think that study results are applicable to other 11q patients as specific clinical symptoms and immunological laboratory results were more frequently observed than expected in the general population.

Lastly, we were not able to organize serological testing for CMV infections retrospectively due to ongoing IgRT or lack of stored plasma. Therefore, it is more difficult to draw definite conclusions on the results of the OX40-based T lymphocyte function test. We recommend treating physicians to test EBV, CMV seroconversion before T lymphocyte function is tested or before IgRT is started to enable more clear conclusions in the near future.

Overall, we conclude that patients with partial 11q deletion or 11q duplication regardless of involvement of genes such as *ETS1* or *FLI1*, have a risk of inborn errors of immunity, consisting of both quantitative and qualitative defects. We therefore recommend regular immunological screening by testing IgG, IgA and IgM, response to immunization and B and T lymphocyte counts in all patients with 11q disorders. It is important to realize that lymphocyte dysfunction does not always correlate with B- and T lymphocyte count. When newborn screening by T lymphocyte receptor excision circle (TREC) is performed, T lymphocytopenia should promptly lead to further investigation involving array-analysis, copy number variation or comparative genomic hybridization.(55, 56) Immunological abnormalities may develop over time and need repetitive testing. In case of humoral immunodeficiency, prophylactic antibiotics and/or IgRT should be considered based on number and severity of infectious complications and potential end-organ damage.

In case of chronic fungal infections, especially affecting skin or nails, local treatment or systemic prophylaxis is indicated. Physicians should be aware that fungal infections and prolonged viral infections may still occur while on IgRT therapy.

As many questions remain in this intriguing immunological field, future research should focus on pathophysiological understanding of this combined immunodeficiency in patients with 11q disorders which can be seen as a model to identify modifiers of immunological function in various disorders. Focus should be on other candidate genes that can influence the immunological dysfunctions seen in patients with 11q disorders.

REFERENCES

1. Grossfeld, P.D., et al., The 11q terminal deletion disorder: a prospective study of 110 cases. Am J Med Genet A, 2004. 129A(1): p. 51-61.
2. Hagen, A., et al., Combined partial trisomy 11q and partial monosomy 10p in a 19-year-old female patient: phenotypic and genotypic findings. Am J Med Genet A, 2011. 155A(12): p. 3075-81.
3. Maruyama, K., T. Koizumi, and H. Ikeda, Partial monosomy 10q with partial trisomy 11q due to paternal balanced translocation. J Paediatr Child Health, 2001. 37(2): p. 198-200.
4. Emanuel, B.S., E.H. Zackai, and L. Medne, Emanuel Syndrome. 1993.
5. Penny, L.A., et al., Clinical and molecular characterization of patients with distal 11q deletions. Am J Hum Genet, 1995. 56(3): p. 676-83.
6. Su, J., et al., de novo interstitial deletions at the 11q23.3-q24.2 region. Mol Cytogenet, 2016. 9: p. 39.
7. Dalm, V.A., et al., The 11q Terminal Deletion Disorder Jacobsen Syndrome is a Syndromic Primary Immunodeficiency. J Clin Immunol, 2015. 35(8): p. 761-8.
8. Seppänen, M., et al., Terminal Deletion of 11q with Significant Late-Onset Combined Immune Deficiency. Journal of Clinical Immunology, 2013. 34(1): p. 114-118.
9. Miller, C.D.E., A.R. Atkinson, and C.M. Roifman, Immunodeficiency in Jacobsen syndrome. Journal of Allergy and Clinical Immunology, 2003. 111(2): p. S232.
10. Puglisi, G., et al., 11q terminal deletion disorder and common variable immunodeficiency. Ann Allergy Asthma Immunol, 2009. 103(3): p. 267-8.
11. Quinti, I., et al., Effectiveness of immunoglobulin replacement therapy on clinical outcome in patients with primary antibody deficiencies: results from a multicenter prospective cohort study. J Clin Immunol, 2011. 31(3): p. 315-22.
12. Blazina, S., et al., 11q terminal deletion and combined immunodeficiency (Jacobsen syndrome): Case report and literature review on immunodeficiency in Jacobsen syndrome. Am J Med Genet A, 2016. 170(12): p. 3237-3240.
13. Mattina, T., C.S. Perrotta, and P. Grossfeld, Jacobsen syndrome. Orphanet J Rare Dis, 2009. 4: p. 9.
14. Breton-Gorius, J., et al., A new congenital dysmegakaryopoietic thrombocytopenia (Paris-Trousseau) associated with giant platelet alpha-granules and chromosome 11 deletion at 11q23 [see comments]. Blood, 1995. 85(7): p. 1805-1814.
15. Wenham, M., et al., Two patients with Hermansky Pudlak syndrome type 2 and novel mutations in AP3B1. Haematologica, 2010. 95(2): p. 333-7.
16. Aarts, C.E.M., et al., Neutrophil specific granule and NETosis defects in gray platelet syndrome. Blood Adv, 2021. 5(2): p. 549-564.
17. Bories JC, Willerford DM, Grévin D, Davidson L, Camus A, Marti P et al: Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene, Nature 1995;377(6550):635-8.
18. Muthusamy N, Barton K, Leiden JM. Defective activation and survival of T cells lacking the ETS-1 transcription factor. Nature 1995:377:639-642
19. Russell, L. and L.A. Garrett-Sinha, Transcription factor Ets-1 in cytokine and chemokine gene regulation. Cytokine, 2010. 51(3): p. 217-26.
20. Garret-Sinha LA. Review of ETS1 structure, function, and roles in immunity. Cell Mol Life Sci 2013;70(18):3375-90
21. Salaverria, I., et al., A recurrent 11q aberration pattern characterizes a subset of MYC-negative high-grade B-cell lymphomas resembling Burkitt lymphoma. Blood, 2014. 123(8): p. 1187-98.

22. Tootleman, E., et al., Partial Jacobsen syndrome phenotype in a patient with a de novo frame-shift mutation in the ETS1 transcription factor. Cold Spring Harb Mol Case Stud, 2019. 5(3).
23. Zhang, X.K., et al., The transcription factor Fli-1 modulates marginal zone and follicular B cell development in mice. J Immunol, 2008. 181(3): p. 1644-54.
24. Melet, F., et al., Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukemia. Mol Cell Biol, 1996. 16(6): p. 2708-18.
25. Moussa, O., et al., Thrombocytopenia in mice lacking the carboxy-terminal regulatory domain of the Ets transcription factor Fli1. Mol Cell Biol, 2010. 30(21): p. 5194-206.
26. Conrad, S., et al., 11q24.2q24.3 microdeletion in two families presenting features of Jacobsen syndrome, without intellectual disability: Role of FLI1, ETS1, and SENCR long noncoding RNA. Am J Med Genet A, 2019. 179(6): p. 993-1000.
27. Li, K., et al., Age-dependent changes of total and differential white blood cell counts in children. Chin Med J (Engl), 2020. 133(16): p. 1900-1907.
28. Tosato, F., et al., Lymphocytes subsets reference values in childhood. Cytometry A, 2015. 87(1): p. 81-5.
29. https://www.nvkc.nl/zoek-een-test/?id=237
30. https://www.star-shl.nl/zorgverlener/labbepalingen/immunoglobuline-iga-igg-igm/
31. Zaunders, J.J., et al., High levels of human antigen-specific CD4+ T cells in peripheral blood revealed by stimulated coexpression of CD25 and CD134 (OX40). J Immunol, 2009. 183(4): p. 2827-36.
32. https://rijksvaccinatieprogramma.nl/vaccinaties/vaccinatieschema
33. Lotz, D.R. and A.P. Knutsen, Janus kinase 3 missense mutation in a child with jacobsen syndrome. Annals of Allergy, Asthma & Immunology, 2010. 104(6): p. 536-537.
34. Õunap, K., et al., Girl with combined cellular immunodeficiency, pancytopenia, malformations, deletion 11q23.3 qter, and trisomy 8q24.3 qter. American Journal of Medical Genetics, 2002. 108(4): p. 322-326.
35. Fernández-San José, C., et al., HYPOGAMMAGLOBULINEMIA IN A 12-YEAR-OLD PATIENT WITH JACOBSEN SYNDROME. Journal of Paediatrics and Child Health, 2011. 47(7): p. 485-486.
36. Puglisi, G., et al., 11q TERMINAL DELETION DISORDER AND COMMON VARIABLE IMMUNODEFICIENCY. Annals of Allergy, Asthma & Immunology, 2009. 103(3): p. 267-268.
37. Blazina, Š., et al., 11q terminal deletion and combined immunodeficiency (Jacobsen syndrome): Case report and literature review on immunodeficiency in Jacobsen syndrome. American Journal of Medical Genetics Part A, 2016. 170(12): p. 3237-3240.
38. https://omim.org/entry/607585
39. Saviozzi, S., et al., A late onset variant of ataxia-telangiectasia with a compound heterozygous genotype, A8030G/7481insA. J Med Genet, 2002. 39(1): p. 57-61.
40. Verhagen, M.M., et al., Presence of ATM protein and residual kinase activity correlates with the phenotype in ataxia-telangiectasia: a genotype-phenotype study. Hum Mutat, 2012. 33(3): p. 561-71.
41. https://omim.org
42. de Saint Basile, G., et al., Severe combined immunodeficiency caused by deficiency in either the delta or the epsilon subunit of CD3. J Clin Invest, 2004. 114(10): p. 1512-7.
43. https://omim.org/entry/165360
44. Huang, F. and H. Gu, Negative regulation of lymphocyte development and function by the Cbl family of proteins. Immunol Rev, 2008. 224: p. 229-38.
45. Rudd, C.E. and H. Schneider, Lymphocyte signaling: Cbl sets the threshold for autoimmunity. Curr Biol, 2000. 10(9): p. R344-7.

46. Hanson, H.L., et al., Germline CBL mutation associated with a noonan-like syndrome with primary lymphedema and teratoma associated with acquired uniparental isodisomy of chromosome 11q23. Am J Med Genet A, 2014. 164A(4): p. 1003-9.
47. https://omim.org/entry/613739
48. Jiang, X.Z., et al., Anti-IgM-induced down-regulation of nuclear Thy28 protein expression in Ramos B lymphoma cells. Apoptosis, 2003. 8(5): p. 509-19.
49. Ji T, Wu Y, Wang H. et al. Diagnosis and fine mapping of a deletion in distal 11q in two Chinese patients with developmental delay. J Hum Genet 2010;55:486–489
50. https://www.rarechromo.org/media/information/Chromosome%2011/Emanuel%20syndrome%20FTNW.pdf
51. Carter, M.T., et al., Phenotypic delineation of Emanuel syndrome (supernumerary derivative 22 syndrome): Clinical features of 63 individuals. Am J Med Genet A, 2009. 149A(8): p. 1712-21.
52. Traynor, R., et al., Immunodeficiency in a Child with 22q11.2 Microduplication Syndrome. J Clin Immunol, 2016. 36(5): p. 418-9.
53. Bjork, A.H., et al., Antibody deficiency in adults with 22q11.2 deletion syndrome. Am J Med Genet A, 2012. 158A(8): p. 1934-40.
54. Colarusso, G., et al., Evans syndrome and antibody deficiency: an atypical presentation of chromosome 22q11.2 deletion syndrome. Pediatr Rep, 2010. 2(2): p. e13.
55. Puck, J.M., Newborn screening for severe combined immunodeficiency and T-cell lymphopenia. Immunol Rev, 2019. 287(1): p. 241-252.
56. Dosanjh A: The Use of TREC Analysis as a Newborn Screening Test for Jacobsen Syndrome and T cell Deficiency. Int J Rare Dis Orphan Drugs 2(2): 1007.

Supplementary Table 1 Description of the cytogenetic alterations

Patient	Diagnostic test	Chromosome	Base pares	MB	Probes
1	Array (Illumina Cyto-12 300x, Nexus 7.0) (GRCh18)	11q23.3q25	(119,956,891 -134,452,384) x1		
2	Karyotyping	46,XX, del(11Xq23.3q25)		15.5	
3	Array (GRCh37)	11q24.1q25 12q24.21q24.33	(122,893,213 -134,450,518) x1 (114,633,820 -133,779,217) x3	12 19.2	755 1,167
	Additional GTG FISH	46,XY,der(11)t(11;12) (q24.1;q24.21) mat			
4	SNP-array (CytoScan HD Array Affymetrix) (GRCh37)	11q24.1q25	(123,307,968 -134,938,470) x1	11.6	13,938
5	Array (GRCh37)	11q24.2q25	(126,728,343-134,938,470) x 1		
6	Whole genome Array (Illumina HCS850k, Nexus); (GRCh19)	11q24.2q25	(124,552,720 -134,934,063) x1		
7	Micro-array, (Agilant 180K); (GRCh37) Micro-array, (BlueGnome Cytochip v1,1, Bac-Arr CGH); (GRCh37)	5q35.1q35.3 (RP11-20O22 à RP11-451H23) x3 11q24.2q25 (RP11-168K9àRP11-469N6) x1	(171,063,816-180,099,598) x3 (126,456,874-126,971,201) x1	10 9	10 10
8	SNP-array (CytoScan HD Array Affymetrix); (GRCh37)	11q24.2q25	(126,908,896 -134,938,470) x1	8	9,498
9	Whole Genome Array (Illumina Cyto-12 300k, Nexus 7.0, 1Mb CNV analysis array) (GRCh18)	11q24.3q25	(127,727,172 -134,452,384) x1		
10	Karyotyping <1985	46,XX,t(6p;11q) deletion 11q25			
11	Targeted array (Illumina HCS850k, Nexus); (GRCh19)	11q14.1q22.1	(79,729,111) -(98,148,532) x1		
	Additional FISH	46,XY,del(11)(q14-21q21-22) (RP11-13L14) x1			
12	SNP-array (Cytoscan HD Affymetrix, Thermo Fisher) (GRCh 37)	11q14.1q23.3	(84,558,527 -117,346,256) x1	32	34,144
13	Karyotyping	46,XX,+der(11).ish dup(11)(q21q23.3) x3			
	FISH with DNA probe WCP11 (paint 11) 2005	Dup(11)(q21-q23.3) Probes 3.16 (11q21), NCAM (11q23.2) and 4.13 (11q23.3) x2			
14	Karyotyping	47,XX,+der(22)t(11;22) (q23;q11) x3, mat			

Somewhere, something incredible is waiting to be known

Carl Sagan (1939 – 1996 AD)

7

Heterozygous nonsense *FLI1* p.Met100* variant results in thrombocytopenia and increased erythroid gene expression

H. Zhang
E.J. Huisman
R. van Rhijn
A.F. Svendsen
J. Janssen
N.N. van der Wel
C. van der Zwaan
N. Gillemans
W.F.J. van Ijcken
M. Hansen
M.H. Cnossen
S. Philipsen
A.J. Hoogendijk
E. van den Akker

Research and Practice in Thrombosis and Haemostasis 2025; Online, ahead of publication

ABSTRACT

Background

Megakaryocytes and platelets arise from hematopoietic stem- and progenitor cells through a tightly regulated process involving various transcription factors, including Erythroblast Transformation Specific (ETS) family member FLI1. Pathogenic variants in *FLI1*, particularly those affecting the ETS DNA-binding domain, have been implicated in inherited platelet disorder and are associated with abnormal giant alpha granules, as seen in Paris-Trousseau Syndrome. However, the precise pathophysiological mechanisms remain unclear.

Objectives

Here, we describe a patient with a de novo heterozygous nonsense mutation, *FLI1* p.Met100* (c.297del), and investigate its effects on megakaryopoiesis. We hypothesized that this variant generates a truncated protein that exerts a dominant-negative effect on megakaryocyte maturation.

Methods

Wild-type (WT) FLI1 and FLI1 p.Met100* were expressed in Meg01 cells. Bulk RNA sequencing, proteomics, and flow cytometry were performed to assess their functional consequences.

Results

Western blot analysis confirmed truncated FLI1 p.Met100* protein expression in Meg01 cells. Gene set enrichment analysis revealed that WT FLI1 enhanced megakaryocytic phenotypes while suppressing erythroid phenotypes, whereas FLI1 p.Met100* upregulated both. Flow cytometry confirmed that erythroid markers CD235a and KLF1 were downregulated by WT FLI1 but elevated by FLI1 p.Met100*. Additionally, WT FLI1 promoted megakaryocyte maturation and adhesion, as evidenced by increased expression of the megakaryocyte marker CD61, and adhesion markers CD34 and CD44. Moreover, we demonstrated through immunoprecipitation that both WT FLI1 and FLI1 p.Met100* interact with shared cofactors.

Conclusion

Collectively, our findings suggest that FLI1 p.Met100* impairs megakaryocyte maturation through a dominant-negative manner, potentially contributing to thrombocytopenia by promoting erythroid features at the expense of proper megakaryocytic development.

Key words

FLI1; blood platelet disorder; thrombocytopenia; thrombopoiesis

Essentials

- Platelets are made from stem cells with control from genes such as FLI1.
- We studied a patient with a rare mutation in the FLI1 gene.
- The altered FLI1 protein caused defective platelets with red blood cell traits.
- This work improves understanding of platelet development in health and disease.

INTRODUCTION

Inherited platelet disorders (IPDs) are characterized by a reduced platelet count (<150 x 10^9/L) and/or abnormalities in platelet size and function. Advances in clinical diagnostics and next generation sequencing have led to the identification of ~ 70 genes associated with different forms of IPDs, including transcription factors such as *FLI1*, *ETV6*, *GFI1B*, *GATA1*, *RUNX1*, *IKZF5*, *EVI1*, *NFE2*, and *HOXA11*.(1) However, the precise molecular mechanisms by which these genetic variants impair megakaryopoiesis are still poorly understood.

The ETS family transcription factors play an important role in hematopoiesis. These factors share a conserved C-terminal DNA-binding domain, while some, including FLI1, ERG, and ETV6, also possess an N-terminal pointed domain mediating protein-protein interactions. FLI1 was discovered alongside SPI1/PU.(1) as one of the two integration sites for Friend-virus induced erythroleukemia, leading to their abnormal expression in erythroid cells and subsequent transformation.(2-4) One of our recent studies demonstrated that *FLI1* is highly expressed in the hemogenic endothelium and early hematopoietic progenitors derived from induced pluripotent stem cells (iPSCs).(5) Furthermore, it has been demonstrated by others that FLI1 is essential for hematopoietic stem cell (HSC) maintenance and differentiation into downstream hematopoietic lineages.(6, 7)

Most of the transcription factors that regulate early hematopoietic stem and progenitors, such as FLI1, are also critical for megakaryocyte development. The direct differentiation of megakaryocytes from HSCs may explain the close regulatory relationship between the two.(8-10) In addition, megakaryocytes arise from megakaryocyte-erythrocyte progenitors (MEPs), a process that is thought to be controlled by the balance of FLI1 and KLF1.(11) Overexpression of FLI1 in cell lines and primary erythroblasts suppresses erythroid development,(4, 12, 13) while while combined ectopic expression of GATA1, FLI1, and TAL1 in hematopoietic progenitors promotes megakaryocyte expansion.(14)

The involvement of FLI1 in platelet disorder was first noted in Jacobsen syndrome, which is caused by a terminal 11q deletion (~3-20 Mb) that includes *FLI1* and its close family member *ETS1*, both located at 11q24.3. Some of these patients present with thrombocytopenia and/or platelet dysfunction, sometimes combined with lymphopenia and/or lymphocyte dysfunction.(15) However, in Jacobsen syndrome, it remains unclear whether the observed phenotypes result from *FLI1* haploinsufficiency alone or from the concurrent deletion of *ETS1*. Pathogenic variants in *FLI1* have since been identified in individuals with inherited platelet disorders (IPDs), typically characterized by normal or mildly reduced platelet counts, enlarged a-granules, and impaired dense granule secretion, occasionally accompanied by alopecia, eczema, psoriasis, and recurrent viral infections.(16-19) Most mutations occur within the ETS DNA binding domain and follow an auto-

somal dominance inheritance pattern, though homozygous cases have been reported.(16-18, 20) While the exact mechanisms underlying FLI1-related platelet disorder remain unclear, certain mutations have been shown to disrupt DNA binding and cause cytoplasmic retention of FLI1.(15-17) Multiple studies have suggested MYH10 as a potential marker for FLI1-associated platelet disorder.(16-21) MYH10 must be downregulated during proplatelet formation but remains elevated in patients with *FLI1*, *RUNX1*, and *GATA1* mutations.(22) In this study, we describe a patient with a novel pathogenic *FLI1* variant in the N-terminal region, presenting clinically with neonatal thrombocytopenia. The mutation introduces a premature stop codon at amino acid 100 (p. Met100*), suggesting a potential role for the FLI1 N-terminal domain in disease progression. We hypothesized that this variant, resulting in a putative truncated protein, has a dominant negative effect on megakaryocyte maturation. To investigate this, we expressed FLI1 p.Met100* in megakaryocytic cells and examined its molecular impact on megakaryopoiesis.

MATERIALS AND METHODS

Patient data and study design

This study was conducted in accordance with the Medical Research Involving Human Subjects Act and was approved by the Medical Ethics Committee of Erasmus University Medical Center (MEC-2016-218). The patient was included after providing written informed consent, in compliance with national laws and regulations. Clinical data were derived from electronic patient files and included medical history, bleeding symptoms, and laboratory results for blood counts and hemostatic tests performed prior to inclusion. Bleeding symptoms were scored on the day of inclusion using the Bleeding Assessment Tool of the International Society on Thrombosis and Haemostasis (ISTH-BAT), which has been validated for platelet disorders.(23) The tool evaluates 13 predefined bleeding items, each scored from 0 (absent) to 1–4 according to severity. The total score is the sum of all items, with values ≥3 considered abnormal in children under 18 years.(24) Laboratory tests for hemostatic function, molecular diagnostics, and electron microscopy of patient platelets are detailed in the Supplementary Methods.

Cell culture

Meg01 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) supplemented with 10% Fetal Calf serum (FCS, Bodinco) and 1% Penicillin/Streptomycin (P/S, Sigma). For differentiation experiments, Meg01 cells were treated with 10 nM PMA (Thermo Fisher Scientific) for 3 days. HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) with 10% FCS and 1% P/S. Cells were incubated at 37°C with 5% CO_2. For virus production, HEK293T cells were cultured in Meg01 culturing medium. Images of Meg01 cells were captured using a Bresser LCD Evos microscope. Additional experimental procedures, including the

generation of *in vitro*-transcribed single guide RNAs (IVT-gRNAs), nucleofection, PCR, and Sanger sequencing, are described in the Supplementary Methods.

Transduction

Lentivirus was produced by transfecting HEK293T cells with either the empty vector (EV) or the FLI1 lentiviral construct (Supplementary Methods), along with the psPAX2 (Addgene #12260) and VSV.G (Addgene #14888) packaging plasmids, using Polyethylenimine (PEI, Sigma). Viral supernatant was collected at 48- and 72-hours posttransfection and directly applied to Meg01 cells.

Flow cytometry

Meg01 cells were stained in FACS buffer (PBS + 0.1% BSA + 0.5mM EDTA) at 4°C for 30 min using the following antibodies: CD31 (1:800, APC-eF780, eBioscience, clone WM-59), CD34 (1:300, BV-510, Biolegend, clone 581), CD41a (1:100, Pacific Blue, BioLegend, clone HIP8), CD42b (1:50, APC, eBioscience, clone p3.6.2.8.1), CD44 (1:2000, APC, eBioscience, clone IM7), CD117 (1:50, FITC, Life Technologies, clone 104D2), CD235a (1:2500, PE, Origene, clone JC159), CD36 (1:100, APC, BD Biosciences, clone CB38 (NL07)), CD61 (1:50, PerCp-Cy5.5, BD Biosciences, clone VI-PL2). After staining, cells were washed with FACS buffer and analyzed on a FACSymphony™ A5 (BD Bioscience). LentiV2GFP-EV-transduced Meg01 cells were single-cell sorted into a 96-well plate using a BD FACSAria III (BD Bioscience). Flow cytometry data were analyzed using FlowJo software version 10 (BD Biosciences).

Western blot and immunoprecipitation

Meg01 cells were washed with PBS and lysed in CARIN buffer supplemented with orthovanadate (BioLabs),(25) phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich), protease inhibitor (Merck), okadaic acid (Calbiochem), and benzonase (Sigma-Aldrich).

For western blotting, lysates were heated in sample buffer (Invitrogen) at 95°C for 5 min and loaded onto NuPAGE 4-12% Bis-Tris gels (Invitrogen). Proteins were transferred using the iBlot-NC system (Thermo Fisher Scientific) and probed with the following primary antibodies: rabbit anti-FLI1-N-terminus (Invitrogen, PA5-96374), rabbit anti-FLI1-C-terminus (Abcam, AB180902), mouse anti-V5-HRP (Novex, R96125), mouse anti-GAPDH (Merck, MAB374), and mouse anti-RhoGDI (Thermo Fisher Scientific, MA5-17032). Goat anti-mouse Ig HRP (Dako, p0447) and goat anti-rabbit Ig HRP (Dako, p0448) were used as secondary antibodies.

For immunoprecipitation, lysates were first incubated with the indicated antibodies and the antigen/antibody complexes were precipitated using Dynabeads Protein G (Thermo Fisher Scientific). Beads were washed five times with PBS before being processed for mass spectrometry analysis (Supplementary Methods). For western blotting, beads were dried

and heated in sample buffer at 95°C for 5 min. TidyBlot-HRP (BIO-RAD) was used to reduce the inference of denatured IgG.

Bulk RNA sequencing analysis

Parental Meg01 cells were transduced with EV, WT FLI1, or FLI1 p.Met100*. RNA library was prepared as previously described.(26) Sequencing was conducted on the Illumina HiSeq 2500 system. RNA reads were mapped to the GRCh38 (hg38) reference genome using STAR. Low-quality reads were filtered, and differential gene expression analysis was performed using the glmLRT function in edgeR, applying a fold-change threshold at 1.5 and a false discovery rate at 0.01.

Gene enrichment analysis

Differentially expressed genes between EV and WT FLI1/FLI1 p.Met100* transduced Meg01 cells were ranked based on $\log_2$ fold change. This ranked gene list was used as input for gene set enrichment analysis (GSEA) using the 'clusterProfiler' R package with the C5: ontology gene sets and C8: cell type signature gene sets from the Molecular Signatures Database (MSigDB). For overlapping genes identified in RNA sequencing and mass spectrometry, clustering and gene ontology analysis were performed using the 'ClusterGVis' R package.

Statistics

Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software). Group comparisons were conducted using either the Mann-Whitney test or one-way/two-way ANOVA, followed by Tukey's or Šídák's multiple comparisons test where applicable. A p-value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

We present a 17-year-old girl with congenital thrombocytopenia. She is the first child of non-consanguineous parents. The pregnancy was uneventful after six previous miscarriages. She was born at 39 weeks of gestation with a normal birth weight. At birth, she was diagnosed with bilateral clubfeet, which required plaster casting and correction during the first year of life. Additionally, she presented with generalized alopecia from an early age and was diagnosed with hypermetropia at the age of 4, which was corrected with glasses. At the same age, a progressive swelling in the neck was observed. MRI showed a large, right-sided thoracic benign lipoma measuring 13 x 3 x 10 cm in the mediastinal space, extending from the cranial level of C5 to the caudal level at the origin of the superior vena cava. The lipoma encircled the brachiocephalic trunk and innominate vein, displacing the right carotid artery, internal jugular vein, and right subclavian

artery. Histopathological analysis of a biopsy confirmed the diagnosis of benign lipoma. Follow-up MRIs at ages 10 and 17 years showed only minimal progression of the lesion. MRI showed a structurally normal heart.

Thrombocytopenia was identified incidentally. Laboratory tests revealed severe macrothrombocytopenia (~30 x 10^9/L) during the first year of life, which ameliorated over time to a mean platelet count of 138 x 10^9/L (Table 1). This was accompanied by an increased mean platelet volume (MPV) and a mildly elevated immature platelet fraction (IPF) (Table 1). Platelet function screening showed normal results on the Platelet Function Analyzer (PFA) but clearly abnormal findings on light transmission aggregometry (LTA), performed with platelet rich plasma (PRP) count of 342 x 10^9/L (Table 1; Supplemental Fig. 1). Clinically, this resulted in an increased bleeding tendency, with a bleeding score of 8 at the age of 17 (normal score <3 in children <18 years of age), primarily due to recurrent epistaxis requiring coagulant treatment, hematomas, and heavy menstrual bleeding requiring treatment with oral contraceptives and tranexamic acid.

Targeted next-generation sequencing of a 73-gene panel related to platelet biogenesis and function identified a novel heterozygous frameshift variant in *FLI1*, designated NM_002017.4 (FLI1):c.297del, p.(Met100*) (Fig. 1A). To assess the potential pathogenicity of the FLI1 variant, we used the Combined Annotation Dependent Depletion (CADD) tool. (29) The variant received a CADD-PHRED score of 28, which places it among the top ~1% of all possible human single nucleotide variants in terms of predicted deleteriousness. Such a high score supports a likely functional impact of this variant.

Given the reported association of FLI1 variants with enlarged α-granules,(16, 17, 19) we performed electron microscopy on the patient's platelets. The analysis revealed large α-granules. Additionally, an unidentified crystalloid structure was observed (Fig. 1B).

Table 1 Laboratory results of hemostatic tests

Parameters	Results
Blood count and platelet indices	
Platelet count, mean (range; x 10^9/L)	138 (30-198; N 150-370)
MPV, mean (range; L/L)	12.7 (12.4-13.0; N 8-11.9)
IPF, mean (range; %)	11 (10-12; N 1-5)
Hemoglobin, mean (range; g/dL)	13.4 (12.8-14.1; N 12.0-15.2)
Leucocyte count, mean (range; x 10^9/L)	5.3 (3.4-7.0; N 3.5-10.0)

Table 1 Laboratory results of hemostatic tests (*continued*)

Parameters	Results
Other platelet related tests	
TPO, mean (range; U/mL)	39 (33-51; N 4-31)
PFA-EPI (sec)	115 (N<150)
LTA, descriptive	Too weak response on ADP (N>50%)
	Reversible response on Collagen (N>70%)
	Too weak response on U46619 (N>70%)
	Inconclusive response on high concentrations ristocetin

Abbreviations ADP = adenosine diphosphate, IPF = immature platelet fraction, LTA = light transmission aggregometry, MPV = mean platelet volume, N = normal (age-dependent reference), PFA-EPI = Platelet function analyzer 200© - epinephrin, TPO = thrombopoietin, U = unit

Figure 1 Identification of a novel pathogenic *FLI1* variant**,** *FLI1* p.Met100*

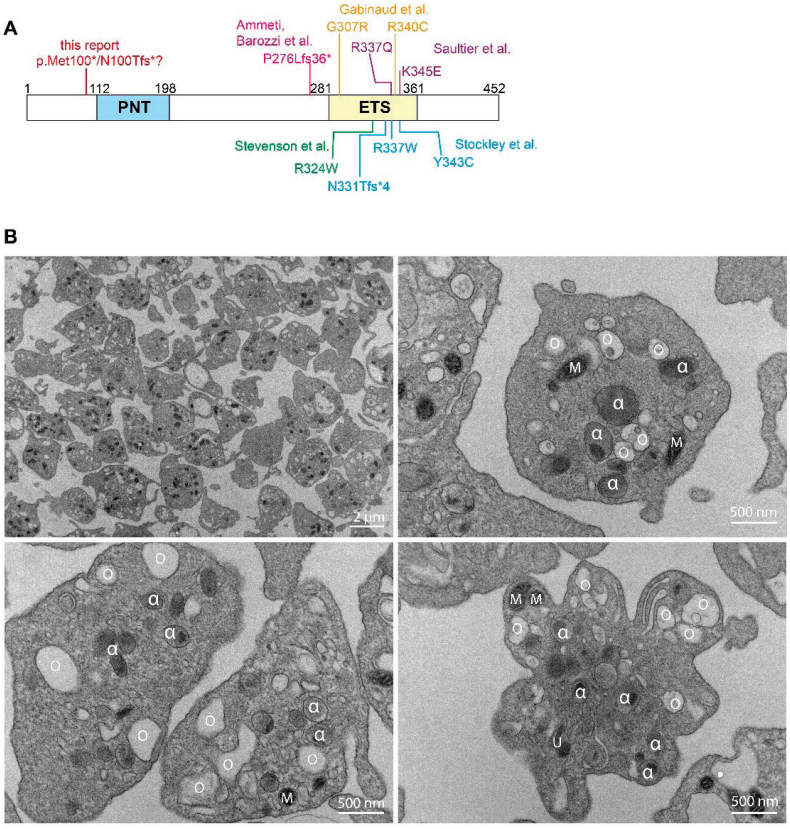


Fig. 1

Abbreviations a = alpha granule, M = mitochondrion, O = open canicular system, U = unidentified, crystalloid structure

Legend (A) Overview of documented pathogenic variants associated with FLI1-related platelet disorders (B) Electron microscopy images of platelets from a patient carrying the heterozygous *FLI1* p.Met100* variant

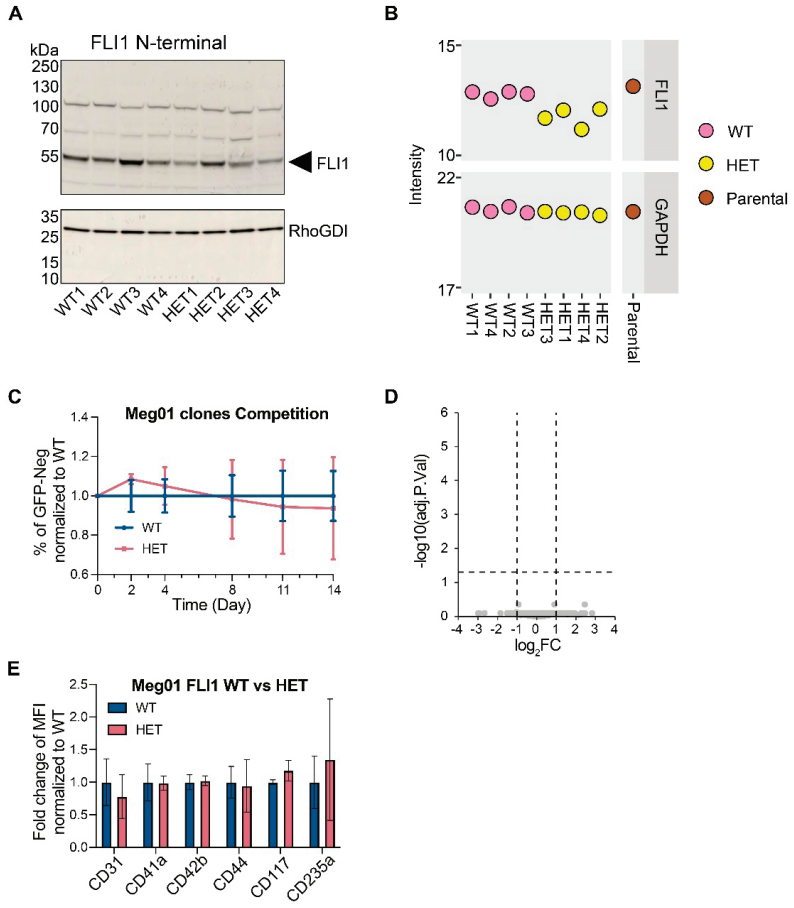
Reduced FLI1 expression in Meg01 cells does not manifest altered phenotype and growth disadvantage

First, we aimed to generate *FLI1* knockout clones using Meg01 cells to establish a cellular model for studying the role of FLI1 p.Met100* in the presence or absence of wild-type FLI1 (parental lines). This cell line carries three copies of chromosome 11, where the *FLI1* gene is located.(27) Ribonucleoproteins (RNPs) composed of in-house-produced Cas9 and guide RNAs (gRNAs) targeting either exon 3 (g-exon3) or exon 4 (g-exon4#1, g-exon4#2, and g-exon4#3) of FLI1 were generated and delivered in a double RNP-approach,(28) (Supplemental Fig. 2A&B). PCR using primers flanking the RNP target sites confirmed successful gene editing. Expected deletion bands of 573bp, 581bp, and 699bp were observed when g-exon3 was combined with g-exon4#1, g-exon4#2, or g-exon4#3 (Supplemental Fig. 2B; lanes 1-3). The wild-type band (5363bp) was not detected in control samples, presumably due to its large size (Supplemental Fig. 2B; lane 6). When using the more closely spaced gRNA pairs (g-exon4#3/g-exon4#1 or g-exon4#3/g-exon4#2), smaller PCR products of 814bp and 817bp were observed, migrating just below the non-edited wild-type PCR product of 935bp (Supplemental Fig. 2B; lane 4, 5 and 7, where lane 7 is a non-CRISPR-edited control). We expanded single-cell clones from three RNP conditions: g-exon3+g-exon4#2, g-exon3+g-exon4#3, and g-exon4#2+g-exon4#3. Four clones (HET1-4) derived from the g-exon3/g-exon4#2 condition showed a 581bp deletion (Supplemental Fig. 2C). Interestingly, PCR analysis using primer pairs spanning exon 3 or exon 4 also detected wild-type bands (973bp for exon 3 and 891bp for exon4) (Supplemental Fig. 2C). These 4 clones (HET1-4) were confirmed to be *FLI1*-disrupted by Sanger sequencing and western blot, where 2 of the 3 *FLI1* alleles were mutated and FLI1 protein expression was reduced (Fig. 2A; Supplemental Fig. 2C&D & 3). For comparison, we selected 4 unmodified clones for further study (Fig. 2A&B; Supplemental Fig. 2C&D & 3). To assess whether reduced FLI1 expression affected cell proliferation, we conducted a competition assay. A Meg01 clone in which a constitutive GFP reporter was lentivirally introduced, was mixed in equal proportions with either unmodified or *FLI1*-disrupted clones. The percentage of GFP-positive cells was recorded over the course of 2 weeks, revealing no significant differences in proliferation between unmodified and *FLI1*-disrupted clones (Fig. 2C; Supplemental Fig. 4). Proteomic analysis indicated that partial *FLI1* deletion did not result in statistically significant changes in protein expression compared to unmodified clones. (Fig. 2D; Supplemental Table 2). Flow cytometry further confirmed that *FLI1* disruption had no significant impact on the expression of megakaryocytic markers (CD41a, CD42b), the erythroid marker (CD235a), or hematopoietic progenitor markers (CD31, CD44, CD117) (Fig. 2E; Supplemental Fig. 5). Overall, our results suggest that a complete *FLI1* knockout in Meg01 cells could not be achieved using these gRNAs, possibly due to FLI1 being indispensable. Furthermore, partial FLI1 loss in Meg01 cells did not impair cell growth, induce major phenotypic changes, or significantly alter protein/RNA expression.

Ectopic expression of WT FLI1 in Meg01 cells downregulates erythroid features, while FLI1 p.Met100* upregulates these

To investigate how FLI1 p.Met100* impairs megakaryopoiesis, we generated lentiviral constructs expressing either WT *FLI1* (lenti_FLI1-WT) or *FLI1* p.Met100* (lenti_FLI1-Met100*) with a GFP reporter. These constructs were transduced into parental Meg01 cells (Fig. 3A; Supplemental Fig. 6A). Western blot confirmed the presence of lenti_FLI1-WT or lenti_FLI1-Met100* (Fig 3A). RNA sequencing followed by multidimensional scaling (MDS) analysis revealed that 61% of the variance was explained by dim1 and 9% by dim2, effectively clustering the different conditions. Notably, lenti_FLI1-WT transduced Meg01 cells separated distinctly from lenti_FLI1-Met100*-expressing and control (lenti_EV) cells (Supplemental Fig. 6B). Compared to control Meg01 cells, lenti_FLI1-WT

Figure 2 Generation and functional characterization of *FLI1*-disrupted Meg01 clones



Abbreviations GFP = green fluorescence protein, LFQ = Log_2-transformed Label-free quantitation, MFI = Mean fluorescence intensity, WT = wild-type

Legend (A) Western blot of FLI1 expression in unmodified (WT1-4) and *FLI1*-disrupted (HET1-4) clones, probed with an N-terminal FLI1 antibody. The arrow indicates the expected position of FLI1 (51 kDa). RhoGDI was used as a loading control. (B) LFQ alues of FLI1 in unmodified and *FLI1*-disrupted Meg01 clones and the parental line (GAPDH as endogenous control). (C) Competition assay between a GFP^+ Meg01 clone and either unmodified or *FLI1*-disrupted clones over 2 weeks. The X-axis represents time (days), and the Y-axis shows the fold change in the percentage of GFP-negative cells, normalized to unmodified clones at each time point. Statistical test: two-way ANOVA, followed by Šídák's multiple comparisons test. (D) Volcano plot of differentially expressed proteins between unmodified and *FLI1*-disrupted clones. Thresholds were set at $\log_2$ fold change (FC) >1 and p-value <0.05. (E) MFI comparison of CD31, CD41a, CD42b, CD44, CD117, and CD235a between unmodified and *FLI1*-disrupted clones (N=3; mean +/- standard deviation; analyzed via Mann-Whitney test)

overexpression resulted in 693 upregulated and 801 downregulated genes, whereas expression of lenti_FLI1-Met100* led to 167 upregulated and 226 downregulated genes (Supplemental Table 3A&B). As expected, lentivirally expressed *FLI1* was among the top differentially expressed genes (Supplemental Fig. 6C; Supplemental Table 3A&B). Gene set enrichment analysis (GSEA) revealed an upregulation of megakaryocytic signatures in both lenti_FLI1-WT and lenti_FLI1-Met100* transduced Meg01 cells (Supplemental Table 3C&D). However, key erythroid genes, including *KLF1* and *CD235a*, were repressed by lenti_FLI1-WT yet activated by lenti_FLI1-Met100* (Fig. 3B; Supplemental Fig. 6D; Supplemental Table 3C&D). *CD36*, which is shared by both megakaryocytic and erythroid programs, was similarly activated in the lenti_FLI1-Met100* condition (Supplemental Fig. 6D; Supplemental Table 3C&D). GSEA also revealed enrichment of cell division-related processes in lenti_FLI1-Met100*-transduced Meg01 cells compared with lenti_FLI1-WT (Supplemental Fig. 6C, E; Supplemental Table 3E). PCA analysis of mass spectrometry data from the same samples showed a clustering pattern similar to that observed in the RNA sequencing results (Supplemental Fig. 7A). The presence of ectopically expressed FLI1 was confirmed by mass spectrometry (Supplemental Fig. 7B and C; Supplemental Table 3F). When we compared differentially expressed genes with corresponding proteins detected by mass spectrometry, we found that gene expression patterns (Fig. 3C) closely mirrored proteomics data (Fig. 3D). Notably, CD36 and KLF1 followed the same trends (Supplemental Fig. 7D; Supplemental Table 3F). Unsupervised clustering of differentially expressed genes resulted in 5 distinct clusters (Fig. 3C; Supplemental Table 3G). Among them, clusters C3 and C5 were downregulated by lenti_FLI1-WT and upregulated by lenti_FLI1-Met100*, while cluster C2 showed the opposite trend. Gene ontology (GO) analysis of C3 and C5 highlighted enrichment in erythroid cell types (Supplemental Fig. 8; Supplemental Table 3H), reinforcing the GSEA findings (Fig. 3B). Genes in C2 were associated with cellular components such as the mitochondrial matrix and secretary granules (Supplemental Fig. 9; Supplemental Table 3I), as well as sulphur and glutathione metabolism pathways (Supplemental Fig. 10; Supplemental Table 3J). In conclusion, we identified a subset of genes dysregulated by lenti_FLI1-Met100*, particularly those involved in erythroid differentiation, which were suppressed by lenti_FLI1-WT but upregulated by

lenti_FLI1-Met100*. The data suggest that FLI1 p.Met100* interferes with the regular FLI1 transcriptional program.

Ectopic expression of WT FLI1 in Meg01 cells increases cellular adhesion and megakaryocyte differentiation

By overlaying RNA sequencing and mass spectrometry data, we identified a distinct gene cluster (C1) that was exclusively upregulated by lenti_FLI1-WT (Fig. 3C&D) and significantly enriched in endothelial and megakaryocytic cell types (Supplemental Fig. 8; Supplemental Table 3G&H). This cluster included genes such as ETV6, IL1R1, IL6R, ITGB3 (CD61), SELP, and CD44. Flow cytometry confirmed these findings, showing increased fold changes in CD61+ and CD34+ cell percentages and CD44 mean fluorescence intensity (MFI) in lenti_FLI1-WT expressing cells, but not in lenti_FLI1-Met100*-transduced cells (Fig. 4A; Supplemental Fig. 11). Consistent with our previous findings, flow cytometry also revealed a decrease in CD235a+ cells in lenti_FLI1-WT transduced cells, whereas lenti_FLI1-Met100* expression displayed a trend toward increased $CD235a^+$ levels, though this difference was not statistically significant (Fig. 4A; Supplemental Fig. 11). Gene ontology analysis further revealed enrichment in pathways related to platelet activation and cell adhesion (Supplemental Fig. 9-10; Supplemental Table 3G, I, J). Additionally, a cell adhesion strength assay performed three days post-transduction confirmed enhanced cellular attachment upon lenti_FLI1-WT overexpression, whereas no such effect was observed in untransduced or lenti_FLI1-Met100* conditions (Fig. 4B). Next, we examined the effect of lenti_FLI1-WT and lenti_FLI1-Met100* on PMA-induced Meg01 differentiation. The percentage of $CD41^+CD61^+$ megakaryocytes increased markedly in both transduced conditions after PMA treatment (Fig. 4C; Supplemental Fig. 12). Lenti_FLI1-WT cells consistently exhibited the highest $CD41^+CD61^+$ expression, both at baseline and following PMA stimulation (Fig. 4A, C, D; Supplemental Fig. 12). In contrast, the percentage of $CD235a^+$ cells decreased across all conditions, with the greatest reduction in lenti_FLI1-WT and the smallest in lenti_FLI1-Met100* (Fig. 4C, E; Supplemental Fig. 12). Interestingly, the expression of CD36 increased after PMA treatment in all groups, with the strongest induction observed in lenti_FLI1-WT (Fig. 4C, E; Supplemental Fig. 12). In summary, overexpression of wild-type FLI1 in Meg01 cells enhances cellular adhesion.

FLI1 p.Met100* does not interact with endogenous or overexpressed FLI1

Figure 3 Lentiviral expression of WT FLI1 suppresses erythroid features, while lenti_FLI1-Met100* enhances them

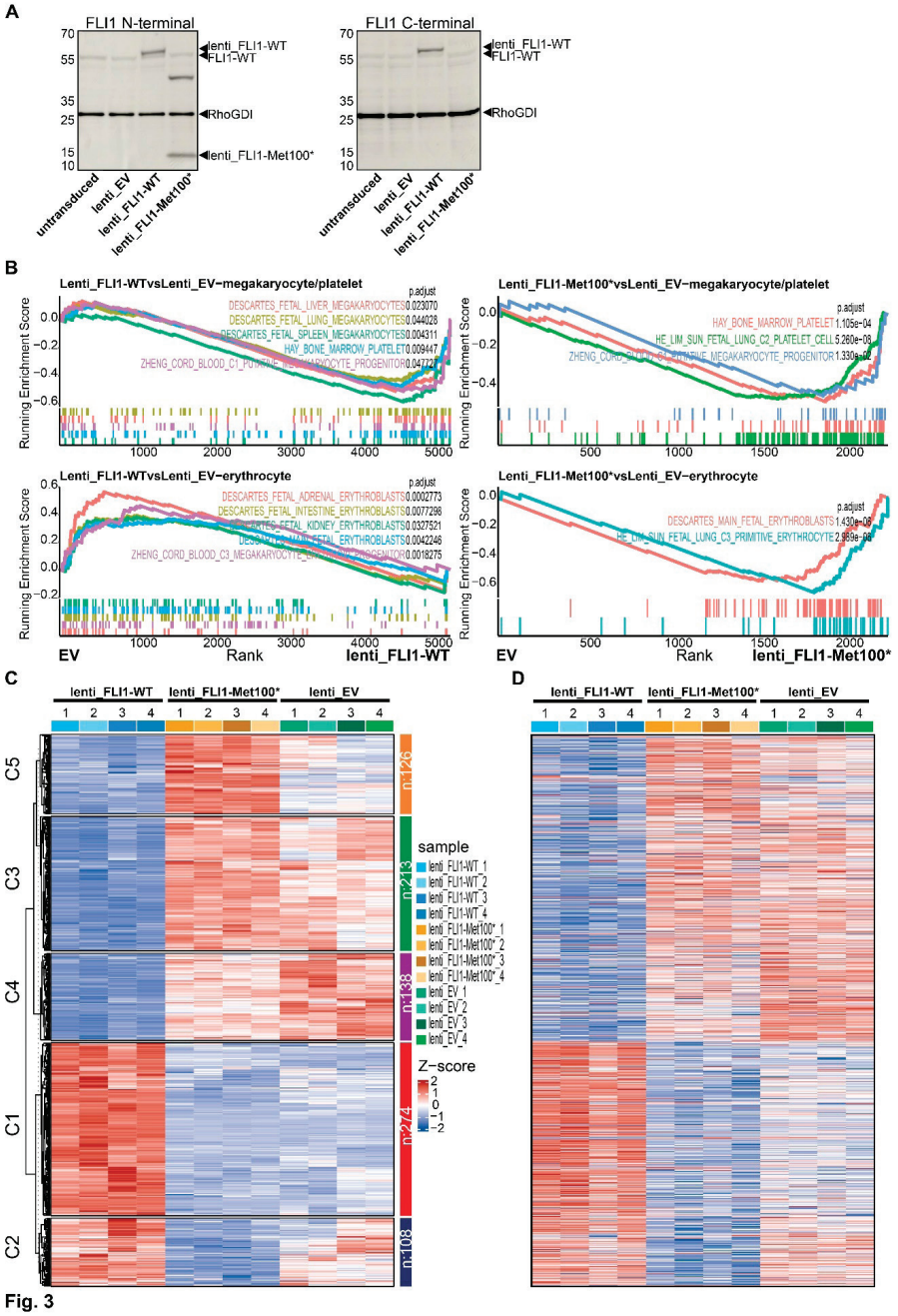


Fig. 3

Abbreviations n = number of genes in each cluster, vs = versus, WT = wild-type

Legend (A) Western blot of FLI1 expression in Meg01 cells five days after transduction with lenti_FLI1-WT or lenti_FLI1-Met100*. The left blot was probed with an N-terminal antibody, the right blot with a C-terminal antibody. Arrows indicate the expected position of endogenous FLI1 (51kDa), lenti_FLI1-WT (52.84kDa) or lenti_FLI1-Met100* (10.64kDa). RhoGDI was used as a loading control. The extra ~45 kDa band in the lenti_FLI1-Met100* condition likely reflects incomplete cleavage of FLI1 p.Met100* and GFP at the P2A site generating a fusion protein. (B) Gene set enrichment analysis (GSEA). Left: lenti_FLI1-WT upregulates megakaryocyte signatures while downregulating erythroid-associated genes. Right: lenti_FLI1-Met100* upregulates both megakaryocyte and erythroid markers. (C) Unsupervised clustering (mfuzz method) of differentially expressed genes from RNA-sequencing in lenti_FLI1-WT and lenti_FLI1-Met100* compared with the empty vector (lenti_EV) control. *n* indicates the number of genes in each cluster. (D) Corresponding protein expression for the genes in panel C, detected by proteomics. No clustering was applied to the protein data; proteins are displayed in the same order as the genes in panel C

Next, we aimed to provide mechanistic insights into how the mutant interferes with the function of FLI1 by investigating whether FLI1 p.Met100* interacts with wild-type FLI1 or other cofactors. To test this, we co-transfected lenti_FLI1-WT and lenti_FLI1-Met100* into HEK293T cells, which do not express FLI1 endogenously. Immunoprecipitation of WT FLI1 using a C-terminal antibody did not co-immunoprecipitate FLI1 p.Met100* (Supplemental Fig. 13, of note this C-terminal epitope is not present in FLI1 p.Met100*). In addition, we lentivirally transduced Meg01 cells with lenti_V5-FLI1-WT or lenti_V5-FLI1-Met100*, and successfully immunoprecipitated the V5-tagged FLI1 proteins using an anti-V5 antibody (Fig. 5A). To examine a potential interaction with endogenous FLI1, we stained the blot with an antibody targeting the N-terminus of FLI1. However, no interaction between endogenous FLI1 and either V5-FLI1-WT or V5-FLI1-Met100* was observed (Fig. 5A). Furthermore, when we immunoprecipitated endogenous FLI1 using a C-terminal antibody in Meg01 cells expressing V5-FLI1-Met100*, no co-immunoprecipitation of FLI1 p.Met100* was observed (Fig. 5B). Mass spectrometry analysis revealed that the transcription factor ETV6, E3 ubiquitin ligases UBR4, USP9X, and UCHL5, as well as the histone acetyltransferase HAT1, specifically co-immunoprecipitated with V5-FLI1-WT (Fig. 5C; Supplemental Table 4). Interestingly, several proteins were found to interact with both V5-FLI1-WT and V5-FLI1-Met100*, including the transcription factor GTF2I, previously linked to bleeding and platelet dysfunction,(29) although this interaction was not statistically significant for the mutant form (Fig. 5C; Supplemental Table 4). In conclusion, our findings suggest that FLI1 p.Met100* exerts a possible dominant-negative function likely through interaction with cofactors rather than through dimerization with wild-type FLI1.

Figure 4 Ectopic expression of lenti_FLI1-WT enhances cellular adhesion

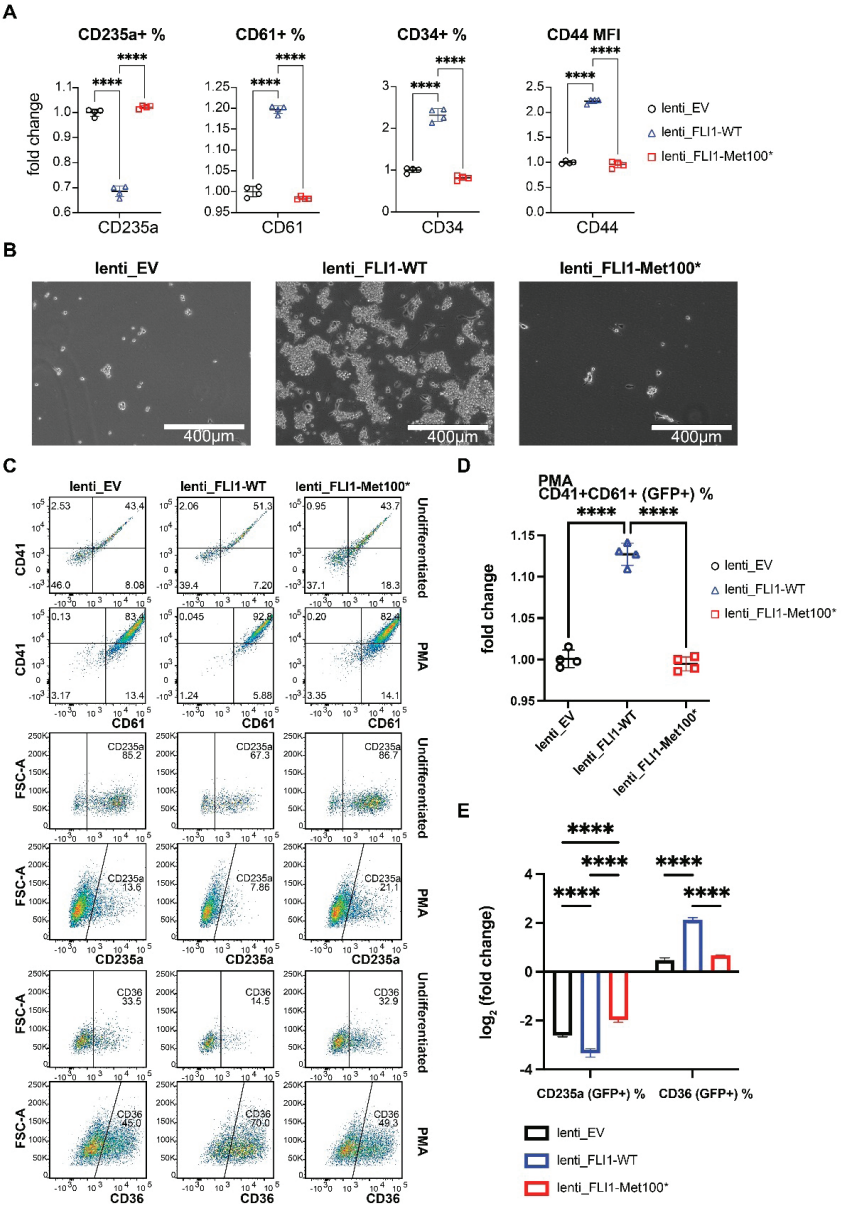


Fig. 4

Abbreviations CD = cluster of differentiation, FSC = forward scatter area, WT = wild-type

Legend (A) Fold change of mean fluorescence intensity (MFI) for CD44 and percentages of CD235a+, CD61+, and CD34+ cells in lenti_FLI1-WT and lenti_FLI1-Met100* conditions, comparing to lenti_EV (N=4; mean +/- standard deviation). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons tests. (B) Representative images illustrating the cellular adhesion of Meg01 cells. Scale bar: 400 µm

Figure 5 Immunoprecipitation (IP) of transduced V5-FLI1-WT/V5-FLI1-Met100* and endogenous FLI1

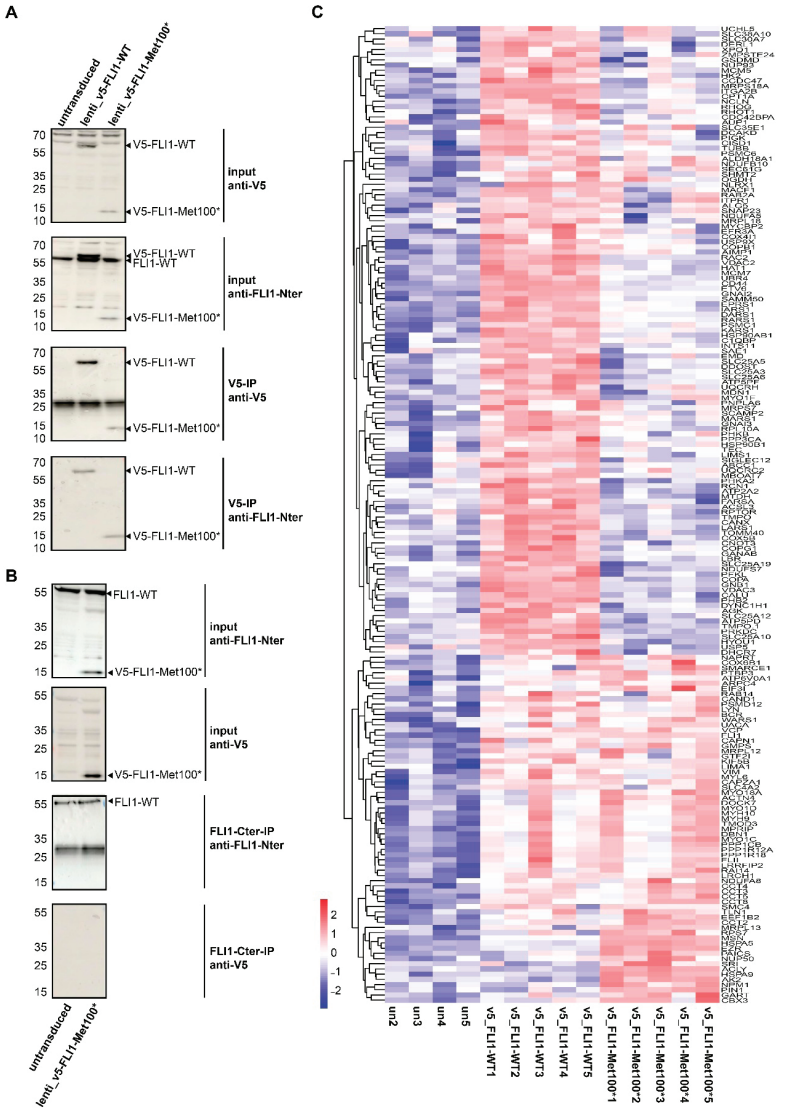


Fig. 5

Abbreviations IP = immunoprecipitation, MS = mass-spectrometry, un = untransduced, vs = versus, WT = wild-type

Legend (A) IP of V5-FLI1-WT/V5-FLI1-Met100* using an anti-V5 antibody. Both input and IP samples were analyzed via western blot using anti-V5 and anti-FLI1-N-terminus (anti-FLI1-Nter) antibodies. (B) IP of endogenous FLI1 using an anti-FLI1-C-terminus (anti-FLI1-Cter) antibody, analyzed by western blot with anti-V5 and anti-FLI1-Nter antibodies. (C) IP-Mass spectrometry (IP-MS) analysis of samples from panel A

DISCUSSION

ETS family transcription factors, such as FLI1, ERG and ETV6, play crucial roles at various stages of hematopoiesis, including the development of hematopoietic stem cells from hemogenic endothelium and differentiation into functional blood lineages.

Complete deletion of *Fli1* in mice results in embryonic lethality (E11.5-12.5) due to severe thrombocytopenia and defective vasculature development.(30) Additionally, hematopoietic specific deletion of *Fli1* in mice leads to reduced peripheral blood counts and an inability of HSCs to engraft. Beyond hematopoiesis, FLI1 is implicated in cellular proliferation across various cancers, and its overexpression in cell lines inhibits apoptosis.(31) Consequently, FLI1 inhibition has emerged as a therapeutic strategy, as its suppression can induce cell death in cancer cells.(32) Given FLI1's essential role in cell survival and megakaryopoiesis, we speculate that we did not observe any Meg01 clones (of 64 screened) with a homozygous deletion of *FLI1*, as this would be lethal. Western blot and mass spectrometry demonstrated half-normal FLI1 levels in *FLI1*-disrupted clones, with no significant change in overall protein expression. FLI1 expression dynamics vary during megakaryocyte differentiation, with mono-allelic expression in early megakaryocyte progenitors and bi-allelic expression in late-stage megakaryocytes.(33) Although Meg01 cells harbor three copies of *FLI1* due to +1 Chr11, they represent early megakaryocytes and thus possibly require less FLI1 expression.(27) This may explain the phenotypic similarities between the parental Meg01s (3 copies of *FLI1*) and the *FLI1*-disrupted Meg01s.

FLI1 functions both as a transcription activator and repressor, regulating both megakaryocyte and erythrocyte differentiation.(32) Overexpression of FLI1 in human pluripotent stem cells enhances megakaryopoiesis.(14, 34) It activates megakaryocytic genes,(35) including *MPL*, *ITGA2B*, *GPIBA*, *GP6*, *GP9*, and *CXCL4*.(36-38) Our findings provide more resolution on the timepoint where FLI1 may be operational during megakaryopoiesis, showing and confirming that FLI1 overexpression enhances megakaryocytic features. Additionally, we observed increased cellular adhesion, that is similar to the effects seen when inducing Meg01 differentiation with phorbol 12-myristate 13-acetate (PMA).(38) FLI1 and KLF1 have been shown to antagonize each other during megakaryocyte-erythroid differentiation.(11) In mice, *Klf1* deletion increases platelet production and retains platelet markers on committed erythroid cells, whereas *Fli1* deletion enhances erythroid differentiation.(39) Overexpression of FLI1 and KLF1 in cell lines suppresses erythroid and megakaryocytic development, respectively.(40) Single cell proteomics revealed co-expression of FLI1 and KLF1 in human erythroid-biased megakaryocyte-erythroid progenitors (e-MEPs), followed by a decrease in FLI1 expression during erythroid differentiation.(41) The same study demonstrated that FLI1 overexpression in e-MEPs shifts differentiation toward the megakaryocytic trajectory.(41) Consistently, we found that

ectopic FLI1 expression in Meg01 cells not only upregulates megakaryocytic features but also suppresses erythroid genes, including KLF1, at both the mRNA and protein levels.

So far, almost all documented *FLI1* mutations occur within the ETS DNA-binding domain. We have reported the de novo variant *FLI1* p.Met100*, which introduces a premature stop codon upstream of the pointed domain. When ectopically expressed in Meg01 cells, the truncated peptide was detected by western blot and mass spectrometry. MYH10, previously described as a marker of FLI1-related platelet disorders, was not upregulated in Meg01 cells carrying the variant, likely because this cell line corresponds to an early stage of megakaryocyte differentiation. In contrast, the patient's platelets showed elevated MYH10, consistent with a defect in its normal downregulation during megakaryocyte differentiation.(1) Transcriptomic and proteomic analyses revealed that FLI1 p.Met100* overexpression led to an upregulation of erythroid signatures, contrasting with WT FLI1. Furthermore, we identified a subset of genes upregulated by WT FLI1 but downregulated by FLI1 p.Met100*. Gene ontology analysis of this subset indicated enrichment in mitochondrial matrix and sulphur/glutathione metabolism, processes linked to reactive oxygen species (ROS) production. Given that ROS play crucial roles in megakaryopoiesis, further investigation is required to establish this connection.(42) Physiologically, megakaryocytes undergo endomitosis to become polyploid, which is reflected in the reduced cell division signatures observed in WT FLI1 transduced cells. In contrast, the upregulation of cell division programs in FLI1 p.Met100* may point to a disruption of this process, potentially contributing to impaired megakaryocyte maturation. Overall, the gene dysregulation caused by FLI1 p.Met100* supports a dominant-negative function, disrupting FLI1's regulatory role in megakaryopoiesis.

FLI1 p.Met100* lacks the DNA-binding domain but may still interact with and sequester cofactors from WT FLI1, potentially disrupting its function. Structural analyses have predicted and identified a potential dimerization region within the DNA-binding domain.(43) Although dimerization of the EWS-FLI1 fusion protein via the FLI1 C-terminal domain has been reported in Ewing Sarcoma, germline FLI1 homotypic interaction was not observed using fluorescence resonance energy transfer (FRET) microscopy.(44) In line with this, we did not observe FLI1 dimerization or interaction between FLI1 p.Met100* and WT FLI1 using Meg01 and HEK293T cells. It remains to be determined whether FLI1 dimerization is stage-dependent during megakaryopoiesis. The hematopoietic transcription factor ETV6 was co-immunoprecipitated exclusively by WT FLI1, consistent with previously reported interactions between these two proteins. FLI1 has been shown to contain a sumoylation site at Lys and to interact with PIASxα/ARIP3 for repression.(45) Although we did not observe this, immunoprecipitation-mass spectrometry (IP-MS) revealed that the E3 ubiquitin ligases UBR4, USP9X, and UCHL5 co-immunoprecipitated with WT FLI1 but not with the truncated FLI1 p.Met100*. Notably, USP9X has been shown to bind the ETS domain of EWS-FLI1, which may explain its absence in the FLI1^{Met100} complex.(46)

Given their role in protein degradation, loss of E3 ligase interaction could affect the stability of the mutant protein. FLI1 is acetylated at multiple sites within its ETS domain by histone acetyltransferases such as p300 and PCAF, which modulate its DNA binding and transcriptional activity.(47, 48) Although p300 and PCAF were not detected by IP-MS, the specific co-immunoprecipitation of HAT1 with WT FLI1 suggests it may similarly contribute to FLI1 regulation. The data here suggest that the first 100 amino acids of FLI1 are not sufficient for these interactions. However, several proteins were co-immunoprecipitated with both WT FLI1 and FLI1 p.Met100*, including the transcription factor GTF2I, which is hemizygously deleted in Williams-Beuren Syndrome (WBS) and associated with bleeding. (49-51) *Gtf2i* deletion in mice leads to vascular defects and megakaryocyte reduction. (29, 51) Additionally, GTF2I represses beta-globin gene expression in K562 and MEL cells. (29) Given FLI1's role in vascular and megakaryocyte development, as well as its involvement in beta-globin transcription repression in MEL cells(11, 32), competitive sequestering of GTF2I may contribute to the upregulation of erythroid gene expression in FLI1 p.Met100*-expressing Meg01 cells and the thrombocytopenia observed in the patient with *FLI1* p.Met100* mutation.

In summary, we identified a novel *FLI1* variant (*FLI1* p.Met100*), without the ETS DNA-binding domain, which is associated with a congenital platelet disorder leading to thrombocytopenia and increased bleeding. Overexpression of FLI1 p.Met100* in Meg01 cells produced a truncated peptide. While WT FLI1 enhanced megakaryocytic gene expression and suppressed erythroid genes, FLI1 p.Met100* upregulated erythroid markers, potentially via a dominant-negative mechanism. The molecular interactions underlying this effect remain to be elucidated.

ACKNOWLEDGMENTS

This study was supported by the Dutch Scientific Research organization (NWO) in the framework of the NWA-ORC Call grant agreement NWA.1160.18.038 (SYMPHONY consortium),(52) and by the ZonMw TRACER consortium as part of the PSIDER program (grant number 10250022110001).

DATA AVAILABILITY

Sequencing data are available at GEO under accession number GSE291026. Proteomic data are available via ProteomeXchange with identifier PXD063984. Original data will be made available upon request.

REFERENCES

1. Kreft IC, Huisman EJ, Cnossen MH, van Alphen FPJ, van der Zwaan C, van Leeuwen K, et al. Proteomic landscapes of inherited platelet disorders with different etiologies. J Thromb Haemost. 2023;21(2):359-72 e3.
2. Bendavid Y, Giddens EB, Letwin K, Bernstein A. Erythroleukemia Induction by Friend Murine Leukemia-Virus - Insertional Activation of a New Member of the Ets Gene Family, Fli-1, Closely Linked to C-Ets-1. Gene Dev. 1991;5(6):908-18.
3. Quang CT, Wessely O, Pironin M, Beug H, Ghysdael J. Cooperation of Spi-1/PU.1 with an activated erythropoietin receptor inhibits apoptosis and Epo-dependent differentiation in primary erythroblasts and induces their Kit ligand-dependent proliferation. Embo J. 1997;16(18):5639-53.
4. Ano S, Pereira R, Pironin M, Lesault I, Milley C, Lebigot I, et al. Erythroblast transformation by FLI-1 depends upon its specific DNA binding and transcriptional activation properties. J Biol Chem. 2004;279(4):2993-3002.
5. Zhang H, Hansen M, di Summa F, von Lindern M, Gillemans N, Ijcken WFJV, et al. LSD1/KDM1A and GFI1B repress endothelial fate and induce hematopoietic fate in induced pluripotent stem cell-derived hemogenic endothelium. Haematologica. 2024;109(12):3975-88.
6. Badwe C, Duran JGB, Kunar B, Lis R, Rafii S. Fli1 Is Essential for the Maintenance of Hematopoietic Stem Cell Homeostasis and Function. Exp Hematol. 2017;53:S131-S.
7. Ben-David Y, Gajendran B, Sample KM, Zacksenhaus E. Current insights into the role of Fli-1 in hematopoiesis and malignant transformation. Cell Mol Life Sci Springer Science and Business Media Deutschland GmbH; 2022; 79: 163.
8. Psaila B, Mead AJ. Single-cell approaches reveal novel cellular pathways for megakaryocyte and erythroid differentiation. Blood. 2019;133(13):1427-35.
9. Sanjuan-Pla A, Macaulay IC, Jensen CT, Woll PS, Luis TC, Mead A, et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature. 2013;502(7470):232-+.
10. Carrelha J, Meng YR, Kettyle LM, Luis TC, Norfo R, Alcolea V, et al. Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. Nature. 2018;554(7690):106-+.
11. Starck J, Cohet N, Gonnet C, Sarrazin S, Doubeikovskaia Z, Doubeikovski A, et al. Functional cross-antagonism between transcription factors FLI-1 and EKLF. Molecular and Cellular Biology. 2003;23(4):1390-402.
12. Pereira R, Quang CT, Lesault I, Dolznig H, Beug H, Ghysdael J. FLI-1 inhibits differentiation and induces proliferation of primary erythroblasts. Oncogene. 1999;18(8):1597-608.
13. Tamir A, Howard J, Higgins RR, Li YJ, Berger L, Zacksenhaus E, et al. Fli-1, an Ets-related transcription factor, regulates erythropoietin-induced erythroid proliferation and differentiation: Evidence for direct transcriptional repression of the gene during differentiation. Molecular and Cellular Biology. 1999;19(6):4452-64.
14. Moreau T, Evans AL, Vasquez L, Tijssen MR, Yan Y, Trotter MW, et al. Large-scale production of megakaryocytes from human pluripotent stem cells by chemically defined forward programming. Nat Commun. 2016;7.
15. Huisman EJ, Brooimans AR, Mayer S, Joosten M, de Bont L, Dekker M, et al. Patients with Chromosome 11q Deletions Are Characterized by Inborn Errors of Immunity Involving both B and T Lymphocytes. Journal of Clinical Immunology. 2022;42(7):1521-34.
16. Stockley J, Morgan NV, Bem D, Lowe GC, Lordkipanidze M, Dawood B, et al. Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects. Blood. 2013;122(25):4090-3.

17. Saultier P, Vidal L, Canault M, Bernot D, Falaise C, Pouymayou C, et al. Macrothrombocytopenia and dense granule deficiency associated with FLI1 variants: ultrastructural and pathogenic features. Haematologica. 2017;102(6):1006-16.
18. Gabinaud E, Hannouche L, Veneziano-Broccia M, Van Agthoven J, Suffit J, Maurizio J, et al. FLI1 and GATA1 govern transcription: new insights into FLI1-related platelet disorders. Haematologica. 2025;110(7):1584-95.
19. Ammeti D, Barozzi S, Pecci A, Zanchetta ME, Cesnik E, Ferlini A, et al. Characterization of a novel FLI1 mutation in a family with thrombocytopenia and other congenital malformations. Haematologica. 2025;110(11):2792-8.
20. Stevenson WS, Rabbolini DJ, Beutler L, Chen Q, Gabrielli S, Mackay JP, et al. Paris-Trousseau thrombocytopenia is phenocopied by the autosomal recessive inheritance of a DNA-binding domain mutation in FLI1. Blood. 2015;126(17):2027-30.
21. Antony-Debré I, Bluteau D, Itzykson R, Baccini V, Renneville A, Boehlen F, et al. MYH10 protein expression in platelets as a biomarker of and alterations. Blood. 2012;120(13):2719-22.
22. Lordier L, Bluteau D, Jalil A, Legrand C, Pan JJ, Rameau P, et al. RUNX1-induced silencing of non-muscle myosin heavy chain IIB contributes to megakaryocyte polyploidization. Nat Commun. 2012;3.
23. Adler M, Kaufmann J, Alberio L, Nagler M. Diagnostic utility of the ISTH bleeding assessment tool in patients with suspected platelet function disorders. J Thromb Haemost. 2019;17(7):1104-12.
24. Elbatarny M, Mollah S, Grabell J, Bae S, Deforest M, Tuttle A, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. Haemophilia. 2014;20(6):831-5.
25. Iacono G, Abay A, Murillo JSG, Aglialoro F, Yagci N, Varga E, et al. Differentiating erythroblasts adapt to mechanical stimulation by upregulation of cholesterol biosynthesis via S1P/SREBP-induced HMGCR expression. Sci Rep-Uk. 2024;14(1).
26. Heshusius S, Grech L, Gillemans N, Brouwer RWW, den Dekker XT, van IWFJ, et al. Epigenomic analysis of KLF1 haploinsufficiency in primary human erythroblasts. Sci Rep. 2022;12(1):336.
27. Ogura M, Morishima Y, Ohno R, Kato Y, Hirabayashi N, Nagura H, Saito H. Establishment of a Novel Human Megakaryoblastic Leukemia-Cell Line, Meg-01, with Positive Philadelphia-Chromosome. Blood. 1985;66(6):1384-92.
28. Verhagen HJMP, Kuijk C, Rutgers L, Kokke AM, van der Meulen SA, van Mierlo G, et al. Optimized Guide RNA Selection Improves Cas9 Gene Editing of Human Hematopoietic Stem and Progenitor Cells. Crispr J. 2022;5(5):702-16.
29. Gurumurthy A, Wu Q, Nar R, Paulsen K, Trumbull A, Fishman RC, et al. TFII-I/Gtf2i and Erythro-Megakaryopoiesis. Front Physiol. 2020;11.
30. Hart A, Melet F, Grossfeld P, Chien K, Jones C, Tunnacliffe A, et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. Immunity. 2000;13(2):167-77.
31. Yi HK, Fujimura Y, Ouchida M, Prasad DDK, Rao VN, Reddy ESP. Inhibition of apoptosis by normal and aberrant Fli-1 and erg proteins involved in human solid tumors and leukemias. Oncogene. 1997;14(11):1259-68.
32. Fuchs O. Importance and Presentation of Transcription Factor Fli-1 in Hematopoiesis and in Hematological and Other Malignancies. ARC Journal of Hematology ARC Journals; 2017; 2: 23–37.
33. Raslova H, Komura E, Le Couedic JP, Larbret F, Debili N, Feunteun J, et al. FLI1 monoallelic expression combined with its hemizygous loss underlies Paris-Trousseau/Jacobsen thrombopenia. J Clin Invest. 2004;114(1):77-84.

34. Vo KK, Jarocha DJ, Lyde RB, Hayes V, Thom CS, Sullivan SK, et al. FLI1 level during megakaryopoiesis affects thrombopoiesis and platelet biology. Blood. 2017;129(26):3486-94.
35. Deveaux S, Filipe A, Lemarchandel V, Ghysdael J, Roméo PH, Mignotte V. Analysis of the Thrombopoietin Receptor (MPL) Promoter Implicates GATA and Ets Proteins in the Coregulation of Megakaryocytic-Specific Genes. Blood Content Repository Only; 1996; 87: 4678–85.
36. Bastian LS, Kwiatkowski BA, Breininger J, Danner S, Roth G. Regulation of the megakaryocytic glycoprotein IX promoter by the oncogenic Ets transcription factor Fli-1. Blood. 1999;93(8):2637-44.
37. Jackers P, Szalai G, Moussa O, Watson DK. Ets-dependent regulation of target gene expression during megakaryopoiesis. J Biol Chem. 2004;279(50):52183-90.
38. Ogura M, Morishima Y, Okumura M, Hotta T, Takamoto S, Ohno R, et al. Functional and morphological differentiation induction of a human megakaryoblastic leukemia cell line (MEG-01s) by phorbol diesters. Blood. 1988;72(1):49-60.
39. Starck J, Weiss-Gayet M, Gonnet C, Guyot B, Vicat JM, Morlé F. Inducible gene deletion in adult mice modifies several myeloid lineage commitment decisions and accelerates proliferation arrest and terminal erythrocytic differentiation. Blood. 2010;116(23):4795-805.
40. Frontelo P, Manwani D, Galdass M, Karsunky H, Lohmann F, Gallagher PG, Bieker JJ. Novel role for EKLF in megakaryocyte lineage commitment. Blood. 2007;110(12):3871-80.
41. Palii CG, Cheng Q, Gillespie MA, Shannon P, Mazurczyk M, Napolitani G, et al. Single-Cell Proteomics Reveal that Quantitative Changes in Co-expressed Lineage-Specific Transcription Factors Determine Cell Fate. Cell Stem Cell. 2019;24(5):812-+.
42. Chen S, Su Y, Wang J. ROS-mediated platelet generation: a microenvironment-dependent manner for megakaryocyte proliferation, differentiation, and maturation. Cell Death Dis. 2013;4.
43. Hou CX, Tsodikov OV. Structural Basis for Dimerization and DNA Binding of Transcription Factor FLI1. Biochemistry-Us. 2015;54(50):7365-74.
44. Spahn L, Siligan C, Bachmaier R, Schmid JA, Aryee DNT, Kovar H. Homotypic and heterotypic interactions of EWS, FLI1 and their oncogenic fusion protein. Oncogene. 2003;22(44):6819-29.
45. van den Akker E, Ano S, Shih HM, Wang LC, Pironin M, Palvimo JJ, et al. FLI-1 functionally interacts with PIASxα, a member of the PIAS E3 SUMO ligase family. J Biol Chem. 2005;280(45):38035-46.
46. Wang S, Huo XF, Yang YP, Mo YX, Kollipara RK, Kittler R. Ablation of EWS-FLI1 by USP9X inhibition suppresses cancer cell growth in Ewing sarcoma. Cancer Lett. 2023;552.
47. Asano Y, Czuwara J, Trojanowska M. Transforming growth factor-β regulates DNA binding activity of transcription factor fli1 by p300/CREB-binding protein-associated factor-dependent acetylation. J Biol Chem. 2007;282(48):34672-83.
48. Schlottmann S, Erkizan HV, Barber-Rotenberg JS, Knights C, Cheema A, Uren A, et al. Acetylation Increases EWS-FLI1 DNA Binding and Transcriptional Activity. Front Oncol. 2012;2:107.
49. Alghobaishi A, Mousa AH, Almonaye HS, Maghrebi TK, Amin A, Al Sharif FA. A case report of interventricular hemorrhage in William-Beuren syndrome. Ann Med Surg (Lond). 2022;80:104305.
50. Kalbhenn T, Neumann LM, Lanksch WR, Haberl H. Spontaneous intracerebral hemorrhage and multiple infarction in Williams-Beuren syndrome. Pediatr Neurosurg. 2003;39(6):335-8.
51. Enkhmandakh B, Makeyev AV, Erdenechimeg L, Ruddle FH, Chimge NO, Tussie-Luna MI, et al. Essential functions of the Williams-Beuren syndrome-associated TFII-I genes in embryonic development. Proc Natl Acad Sci U S A. 2009;106(1):181-6.

52. Cnossen MH, van Moort I, Reitsma SH, de Maat MPM, Schutgens REG, Urbanus RT, et al. SYMPHONY consortium: Orchestrating personalized treatment for patients with bleeding disorders. J Thromb Haemost. 2022;20(9):2001-11.

SUPPLEMENTARY METHODS

Laboratory tests for hemostatic function

At inclusion, venepuncture was performed to collect a total of 26.2 mL of whole blood, distributed into the following tubes: 3.0 mL into EDTA tubes (for cell counts, cell indices, and molecular analyses), 0.5 mL into serum tubes (for thrombopoietin [TPO] measurement, and 22.7 mL into sodium-citrate tubes (for platelet function and coagulation assays). Cell count and indices were analyzed using the Sysmex XN-9100 (Sysmex, Netherlands), complemented by a manually performed blood smear. TPO levels were measured in serum using an in-house ELISA assay (Sanquin, Netherlands). Citrated plasma was used for the following tests: activated partial thromboplastin time (aPTT; Actin FS), prothrombin time (PT; Thromborel S), and fibrinogen (Thrombin Reagent), all performed on the Sysmex CS5100 (Siemens Healthcare, Germany), as well as the PFA-200 collagen-epinephrine assay (PFA-EPI; Siemens Healthcare, Germany). A PFA-EPI result was considered normal if the closure time was <150 seconds. Light transmission aggregometry (LTA) was performed on a Chrono-Log aggregometer 490 (Stago Benelux, Netherlands) using the following agonists: collagen (2 µg/mL), U46619 (4 µM), thrombin (40 U/mL), TRAP (30 µM), ADP (5 and 10 µM), epinephrin (5 µM), ristocetin (0.31 and 1.25 mg/mL), and arachidonic acid (1 µM). A normal response was defined as a final amplitude ≥50% for weak agonists (ADP and epinephrin) and ≥70% for strong agonists (collagen, TRAP6, high ristocetin, arachidonic acid, U46199), with no second wave, no reversibility, and a response to low dose ristocetin <10%.(1)

Molecular diagnosis

DNA was isolated according to the manufacturer's instructions using either the Gentra Puregene kit or the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). An in-house developed Ampliseq targeted next-generation sequencing (tNGS) panel (Thermo Fisher Scientific, Waltham, Mass) was used to sequence the coding regions of a pre-set panel of 73 genes involved in platelet production and function.(2) Library preparation and sequencing were performed following the manufacturer's protocols on an S5 system (Thermo Fisher Scientific). Ion Reporter software (Thermo Fisher Scientific) was used to analyze the genetic sequence data. Variants were assessed and classified according to the current ACMG guidelines.(3)

Electron microscopy

For electron microscopy (EM), Epon resin was prepared by mixing 24g (±21 mL) of Agar 100 resin, 13g (±13 mL) of *Dodecenyl Succinic Anhydride* (EM grade), 13g (±12 mL) of Methylnadic anhydride (EM grade), and 1 mL of *Benzyldimethylamine* (added in fume-hood). Before embedding, cells were washed and fixed with 2% paraformaldehyde and 2.5% gluteraldehyde in 0.1M cacodylate buffer, followed by post-fixation in 1% osmium in 0.1M cacodylate buffer for 15 min. The cells were then washed with Milli-Q water

and incubated in Ultrostain I (Uranyl Acetate Solution, Leica) for 20 min. Next, the cells underwent a dehydration process consisting of two washes in 50% ethanol for 5 min, followed by sequential washes in 70% ethanol (5 min), 90% ethanol (5 min), and two final washes in 100% ethanol (5 min each). The samples were then incubated in propylene oxide for 10 min, followed by infiltration with an epon:propylene oxide mixture (1:1) for a minimum of 12 hours. Afterwards, the cells were incubated in pure epon for 1 hour at 37°C before being placed in molds and polymerized at 60°C. Ultrathin sections were cut using a Leica ultramicrotome and mounted on grids. The grids were first stained with Ultrostain I, washed, and then stained with Ultrostain II (Lead Citrate Solution, Leica). After a final wash in demi-water, they were air-dried. Microscopic imaging was performed using a Tecnai T12 (Thermo Fisher Fei, Eindhoven) equipped with Veleta and Xarosa cameras, with image analysis conducted using Radius software (EMSIS, Münster, Germany).

IVTsgRNA production and nucleofection

In vitro transcribed single guide RNAs (IVTsgRNAs) were generated according to previously described method.(4). Briefly, DNA oligos containing the T7 promoter, CRISPR RNA (crRNA, Table 1), and an overhang complementary to the tracrRNA template were amplified using two-step PCR (98°C for 10 seconds, 68°C for 10 seconds) for 35 cycles. IVTsgRNAs were transcribed by incubating 5 µL of the PCR product in a T7 transcription mix (Hi-SCribe T7, NEB) at 37°C for 20 hours. DNA contamination was removed with DNase I (NEB) treatment at 37°C for 15 minutes, followed by inactivation at 80°C for 15 minutes. IVTsgRNAs were purified using the NEBs purification kit (NEB, T2040), and RNA concentrations were measured by Nanodrop. RNA was diluted to 0.5 µg/µL with ultrapure water and stored at -80°C. For nucleofection, ~2 x 10^5 Meg01 cells were mixed with the RNP complex (3 µg Cas9 + 1 µg IVTsgRNA) and enhancer (1:50, IDT) in homemade nucleofection buffer.(5) Nucleofection was performed using the 4D-Nucleofector (LONZA, program EN-102). Afterward, cells were resuspended in pre-warmed RPMI media (10% FCS, 1% P/S) and cultured in 48-well plates.

Polymerase Chain Reaction (PCR) and Sanger sequencing

For PCR, 5-50 ng of DNA was amplified using primers (Table 1) and the Invitrogen Taq DNA Polymerase recombinant kit (Thermo Fisher Scientific) following the manufacturer's instructions. Reactions were performed on a Veriti 96-Well Thermal Cycler with an initial denaturation at 94°C for 3 min, followed by 35-40 cycles of 94°C for 45 seconds, 60-65°C for 30 seconds, and 72°C for 60-90 seconds, concluding with a final elongation at 72°C for 10 min. For Sanger sequencing, PCR products were either enzymatically cleaned using Exonuclease I (NEB) and Shrimp Alkaline Phosphatase (NEB) or purified after gel electrophoresis using the NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL). Sequencing was performed using the BigDye™ Terminator (BDT) Cycle Sequencing Kit (Thermo Fisher Scientific) on an Applied Biosystems 3730 DNA Analyzer.

Generation of *FLI1* lentiviral construct

A DNA template containing either the WT FLI1 or FLI1 p.Met100* coding sequence, preceded by a Kozak sequence and an XbaI restriction site and followed by an HpaI restriction site, was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). After bacterial transformation and inoculation, plasmids were extracted, digested with XbaI and HpaI, and the WT FLI1 or FLI1 p.Met100* fragments were purified from an agarose gel. These fragments were then inserted into a previously established LentiV2GFP empty vector construct (55), digested with the same restriction enzymes. To introduce a V5 tag at the N-terminus of FLI1, PCR was performed using the WT FLI1 or FLI1 p.Met100* lentiviral plasmids as templates. The forward primer contained a sequence matching the first 30 coding nucleotides of FLI1, along with an overhanging V5 tag, a Kozak sequence, and an XbaI restriction site. The reverse primer matched the last 31 coding nucleotides of WT FLI1 or FLI1 p.Met100* with an HpaI restriction site. PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL), digested with XbaI and HpaI, and inserted to the LentiV2GFP backbone.

Mass spectrometry

The IP samples were prepared with an on-bead digestion:

Beads were resuspended in 50 µL 1M Urea, 100 mM Tris-HCl pH 8 (Invitrogen). Proteins were reduced using 10 mM dithiothreitol (Thermo Fisher Scientific) for 20 minutes at 25°C, whereafter the reduced cysteine residues were alkylated with 50 mM iodoacetamide (Thermo Fisher Scientific) for 10 minutes in the dark at 25 °C. Proteins were digested on beads using 375 ng MS-grade Trypsin-LysC (Thermo Fisher Scientific) for 2 hours at 25°C. The supernatant fraction was collected after digestion, whereas beads were incubated with 50 µL 1M Urea, 10 mM DTT in 100 mM Tris-HCl (pH=8) for 5 minutes at 25°C. Supernatants from both fractions were combined, and proteins were further digested for 18h at 25°C using 150 ng Trypsin-LysC. The enzymatic reaction was quenched by adding 12 µL of 10% (v/v) trifluoroacetic acid (Thermo Fisher Scientific). 75 µL was loaded on Evotip pure, according to manufacturer's instructions.

The total cell lysate was processed in solution:

Cell pellets were lysed in 1% sodium deoxycholate (SDC, BioWORLD), 10 mM Tris-(2-carboxyethyl) phosphine (Thermo Fisher Scientific), 40 mM Chloroacetamide (Sigma-Aldrich), 100 mM Tris-HCl pH 8, (Life Technologies) and heated for 5 minutes at 95°C. After cooling to room temperature, the samples were sonicated in a sonication water bath (Branson Ultrasonics) for 10 minutes. Total protein was measured using a BCA assay (Thermo Fisher Scientific). 10 µg of protein was diluted in 50 mM Tris pH 8 and digested for 18h at 25 °C with Trypsin Gold (Promega) in a 1:50 enzyme-to-protein ratio. After adding trifluoroacetic acid (Thermo Fisher Scientific) to a final concentration of 1%, samples were centrifuged for 5 minutes at 10,000×*g* to pellet the precipitated SDC. Supernatant

was transferred to a new vial, and 500 ng of peptides was loaded on Evotip pure, according to manufacturer's instructions.

Peptides were separated on an Evosep one (Evosep) with the preset 60 samples-per-day method on an 8 cm Evosep Performance Column (EV-1109, 150 µm I.D., 1.5 µm particle size). Acquisition was performed on a timsToF-HT (Bruker Daltonics) mass spectrometer operated in dia-PASEF mode. Ion mobility accumulation and ramp time were set to 100 ms. Further, 16 DIA windows were set per cycle, ranging from 0.7–1.5 $1/k_0$ to 421–1594 m/z, and the size of DIA windows was set based on precursor density. Collision energy was set as a linear function of the ion mobility (0.6 $1/k_0$ = 20 eV, 1.60 $1/k_0$ = 59 eV). Raw files were processed in DIA-NN 1.8.1.; proteins and peptides were detected by querying the filtered human Swissprot database (release 2021.22.04). Standard settings were used, using a generated library-based spectra search. Maximum number of variable modifications was set to 2. Protein Interference used was "Protein names (from FASTA)" and quantification strategy "Robust LC (high precision)". Data were analyzed using R 4.4.3/RStudio (2024.12.1). Detected proteins were filtered for proteotypic and ≥2 unique peptides per protein, and proteins were quantified in 75% of samples in at least one condition.

Label free quantification (LFQ) values were transformed in log2 scale. Missing values were imputed by normal distribution (width = 0.3, shift = 1.8), assuming these proteins were close to the detection limit. statistical analyses were performed using LIMMA, a Benjamini Hochberg-adjusted $p < 0.05$ was considered statistically significant.

REFERENCES

1. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. Journal of Thrombosis and Haemostasis. 2013;11(6):1183-9.
2. Kreft IC, Huisman EJ, Cnossen MH, van Alphen FPJ, van der Zwaan C, van Leeuwen K, et al. Proteomic landscapes of inherited platelet disorders with different etiologies. J Thromb Haemost. 2023;21(2):359-72 e3.
3. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405-24.
4. Verhagen HJMP, Kuijk C, Rutgers L, Kokke AM, van der Meulen SA, van Mierlo G, et al. Optimized Guide RNA Selection Improves Cas9 Gene Editing of Human Hematopoietic Stem and Progenitor Cells. Crispr J. 2022;5(5):702-16.
5. Zhang H, Hansen M, Di Summa F, Von Lindern M, Gillemans N, Van IWFJ, et al. LSD1/KDM1A and GFI1B repress endothelial fate and induce hematopoietic fate in induced pluripotent stem cell-derived hemogenic endothelium. Haematologica. 2024;109(12):3975-88.

Supplementary Table S1 gRNAs and primers used in this study

Gene	gRNAs	Description	Sequence
FLI1	g-exon3	gRNA – sense	TCACTTTAGGGAGTCTCCGG|TGG
	g-exon4#1	gRNA – sense	ACTGTGTAAAATGAACAAGG|AGG
	g-exon4#2	gRNA – sense	GGAACTGTGTAAAATGAACA|AGG
	g-exon4#3	gRNA – sense	CCTCTGCAGACCCCACACTG|TGG
	Primers		
	ex3-F#1	PCR/sequencing primer - forward intron 2	GGCAGCATGGAGGATAGAAA
	ex3-F#2	PCR primer - forward intron 2	CTGCTCACCTCCTGTAAGAATAC
	ex3-R	PCR primer - reverse intron 3	CTGGGAATATAAGCGTCCACTAC
	ex4-F#1	PCR primer - forward intron 3	GAGGGATCCGTGTTCTTTCAT
	ex4-F#2	PCR/sequencing primer - forward intron 3	CCAGAGAGAAAGAGAAGGGAAAG
	ex4-R#1	PCR primer - reverse intron 4	GCACCACCCATCTCTATCTATTT
	ex4-R#2	PCR primer - reverse intron 4	TCTTGGCTGTACCAGAAGAAAG

Supplementary Table S2 Proteins detected in wild-type and heterozygous FLI1 Meg01 clones

Online only

Legend Log_2-transformed LFQ (Labor-free quantification) values of proteins detected in wild-type and heterozygous FLI1 Meg01 clones

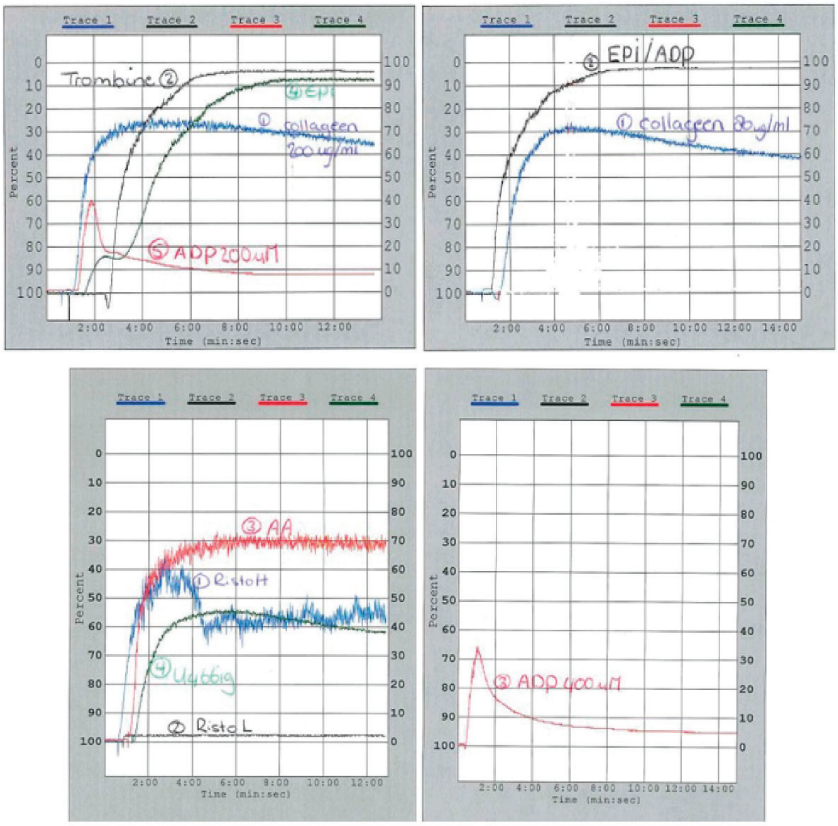
Supplementary Table S3 Online only

Legend (A). Differentially expressed genes: lenti_FLI1-WT vs. lenti_EV transduced Meg01 cells. (B). Differentially expressed genes: lenti_FLI1-Met100* vs. lenti_EV transduced Meg01 cells. (C). Gene set enrichment analysis (GSEA) using MSigDB C8 cell type gene sets for differentially expressed genes between lenti_EV and lenti_FLI1-WT. (D). GSEA using MSigDB C8 cell type gene sets for differentially expressed genes between lenti_EV and lenti_FLI1-Met100*. (E). GSEA using MSigDB C5 ontology gene sets for differentially expressed genes between lenti_FLI1-WT and lenti_FLI1-Met100*. (C-D). NES refers to the Normalized Enrichment Score. (F). Log_2-transformed LFQ values of proteins detected in lenti_EV, lenti_FLI1-WT and lenti_FLI1-Met100* transduced Meg01 cells. (G). Clusters of differentially expressed genes with corresponding proteins detected by mass spectrometry. (H). Cell type enrichment analysis for differentially expressed genes (lenti_FLI1-WT/lenti_FLI1-Met100* vs. lenti_EV) using the Descartes Cell Types and Tissues database. (I). Gene ontology (GO) cellular component enrichment analysis for differentially expressed genes (lenti_FLI1-WT/lenti_FLI1-Met100* vs. lenti_EV). (J). KEGG pathway enrichment analysis for differentially expressed genes (lenti_FLI1-WT/lenti_FLI1-Met100* vs. lenti_EV).

Supplementary Table S4 Log_2 fold-change (LogFC) of LFQ (Label-free quantification) values for proteins co-immunoprecipitated with WT FLI1 or FLI1 p.Met100*

Online only

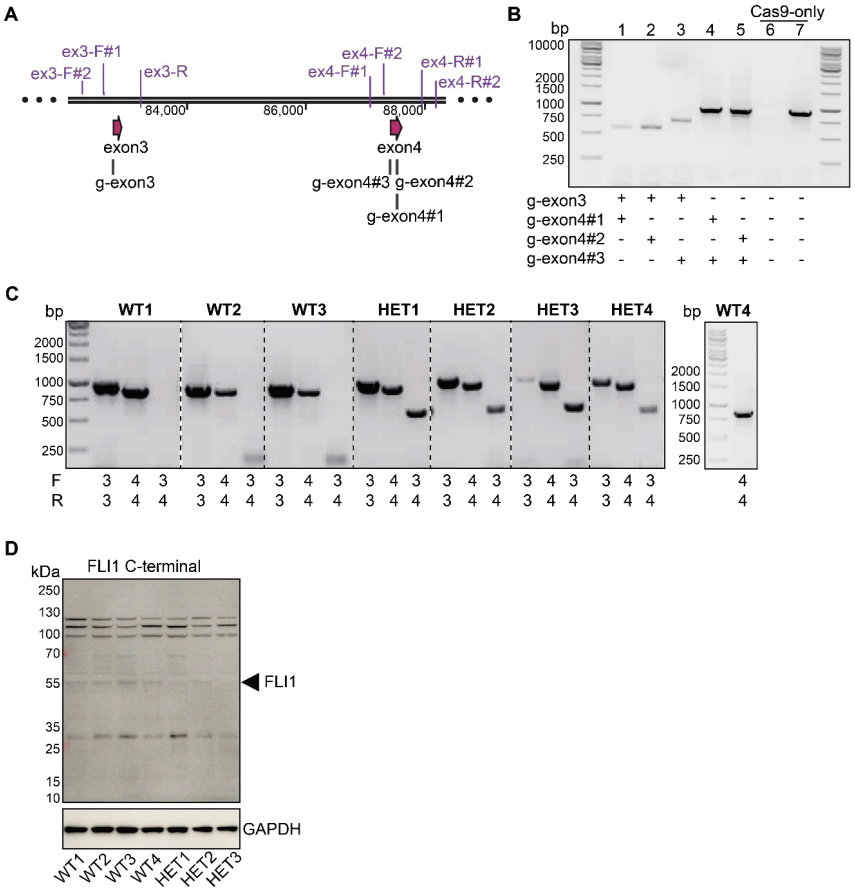
Supplementary Figure S1 Light transmission aggregometry



Abbreviations AA = arachidonic acid, EPI = epinephrin, Risto = ristocetin

Legend Light transmission aggregometry (LTA) results show abnormal platelet aggregation in response to ADP, collagen, and U46619, with an inconclusive response to high-dose ristocetin. Abbreviations: AA = arachidonic acid; ADP = adenosine diphosphate; Epi = epinephrine; Risto = ristocetin. Panel descriptions: Top left: blue = collagen, black = thrombin, green = epinephrine, red = ADP; Top right: blue = collagen, black = epinephrine/ADP; Bottom left: blue = high-dose ristocetin (Risto H), black = low-dose ristocetin (Risto L), red = arachidonic acid, green = U46619; Bottom right: ADP response shown

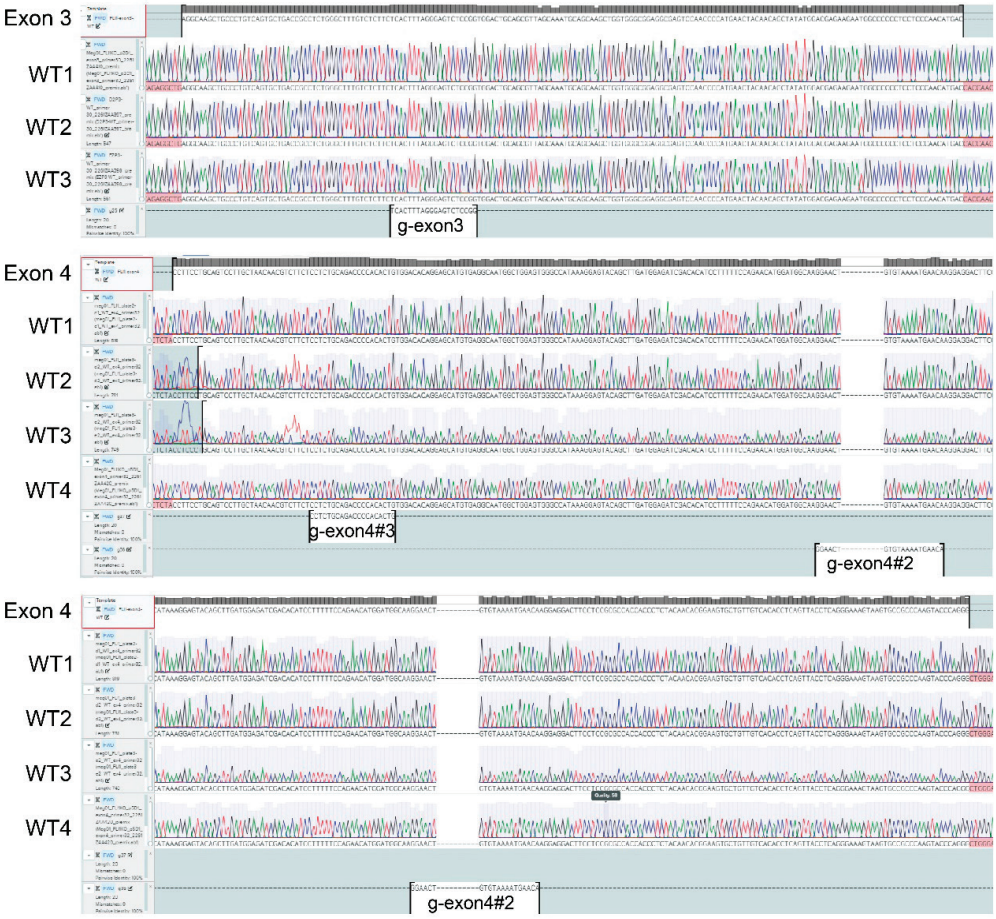
Supplementary Figure S2 Generation of *FLI1*-disrupted Meg01 clones



Abbreviations Da = Dalton, WT = wild-type

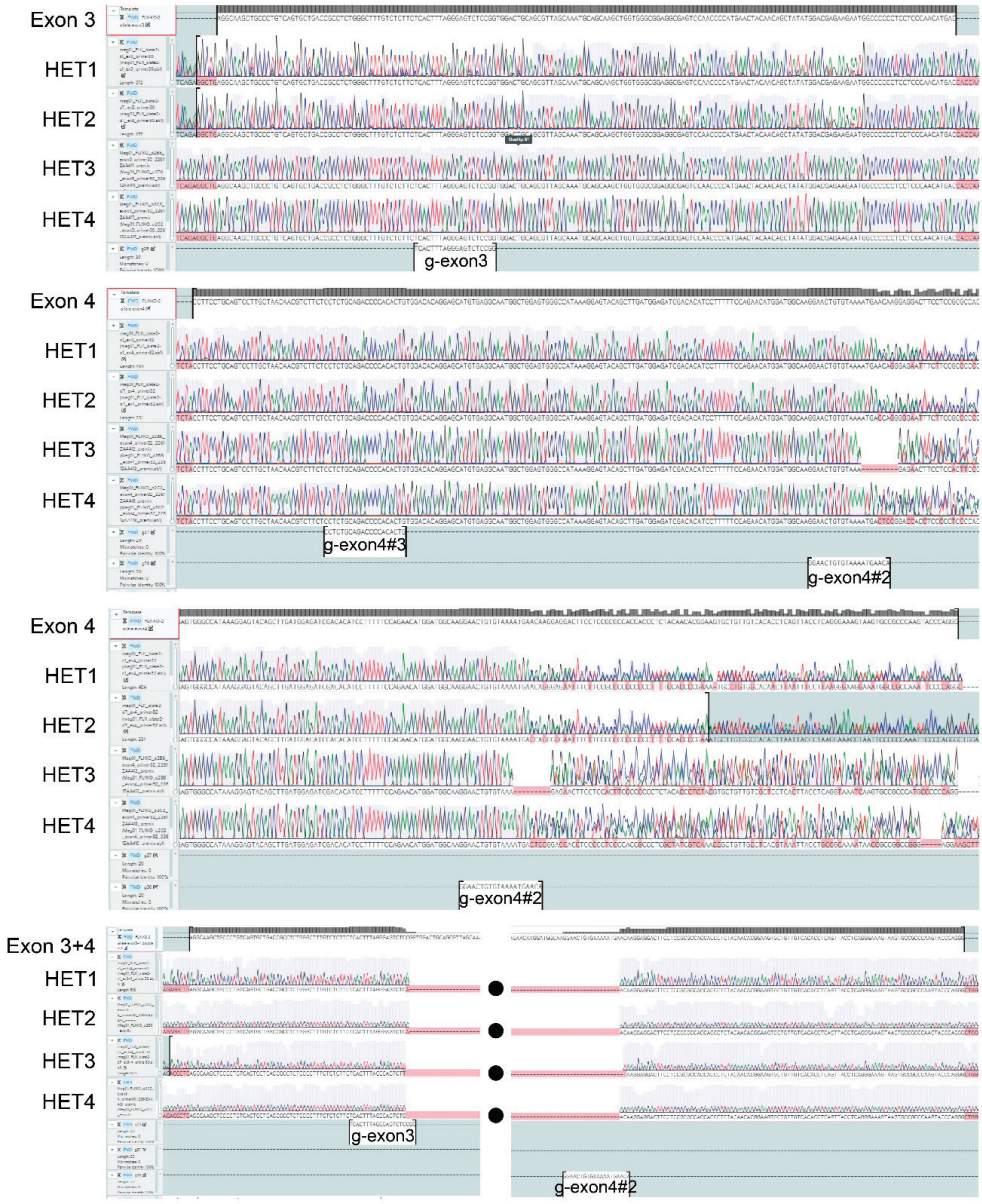
Legend (A). Schematic of the FLI1 knockout strategy, exon 3 and 4 of the FLI1 gene are depicted. (B). PCR validation of FLI1 deletion. For lanes 1-3 (g-exon3 paired with g-exon4 gRNA), primers ex3-F#1 and ex4-R#1 were used. For lanes 4-5 (g-exon4 gRNAs combined), primers ex4-F#1 and ex4-R#1were used. Lane 6 (ex3-F#1 and ex4-R#1) and lane 7 (ex4-F#1 and ex4-R#1) served as Cas9-only controls. (C). PCR analysis of exon 3 and/or exon 4 in unmodified (WT) and FLI1-disrupted (HET) clones. Primer pairs ex3-F#2/ex3-R and ex4-F#2/ex4-R#2 targeted individual exons, while primers ex3-F#1/ex4-R#1 spanned both exons. (D). Western blot of FLI1 expression in WT1-4 and HET1-3 clones, probed with a C-terminal FLI1 antibody. The arrow indicates the expected position of FLI1 (51 kDa). GAPDH was used as a loading control

Supplementary Figure S3 Sanger sequencing and alignment



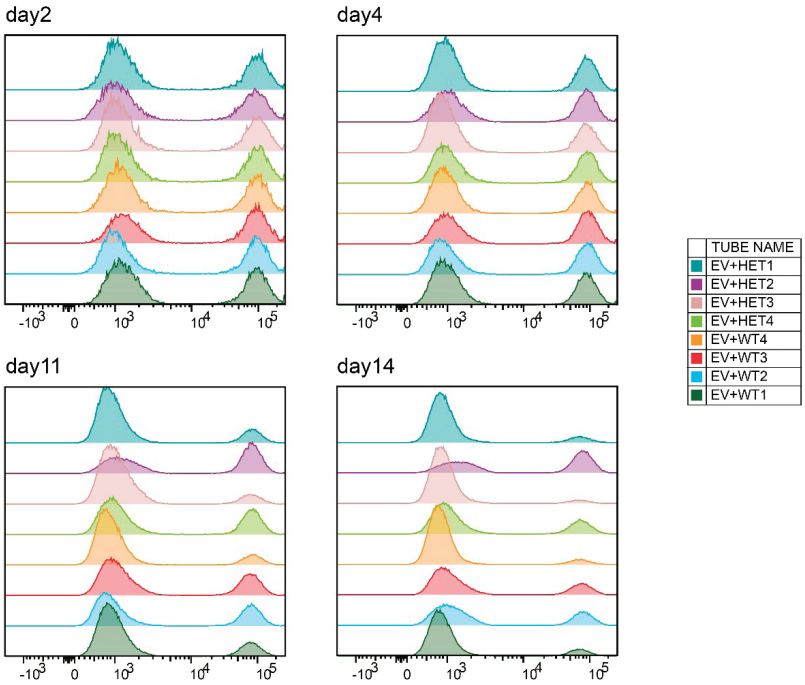
Legend Sanger sequencing and alignment of gel-purified PCR fragments spanning *FLI1* exon 3 and/or exon 4 from wild-type and heterozygous Meg01 clones. Guide RNA target sites (g-exon3, g-exon4#2, g-exon4#3) are indicated

Supplementary Figure S3 Sanger sequencing and alignment (*continued*)



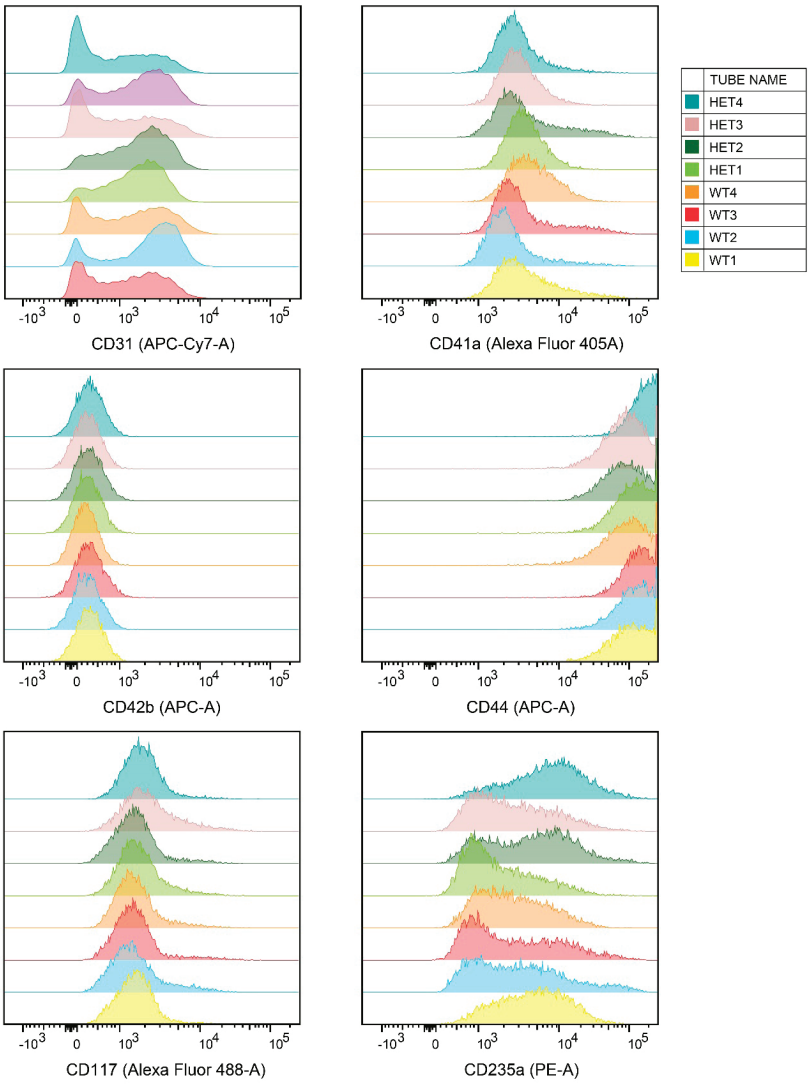
Legend Sanger sequencing and alignment of gel-purified PCR fragments spanning *FLI1* exon 3 and/or exon 4 from wild-type and heterozygous Meg01 clones. Guide RNA target sites (g-exon3, g-exon4#2, g-exon4#3) are indicated

Supplementary Figure S4 Competition assay between wild-type/heterozygous FLI1 clones and a lenti_EV GFP clone



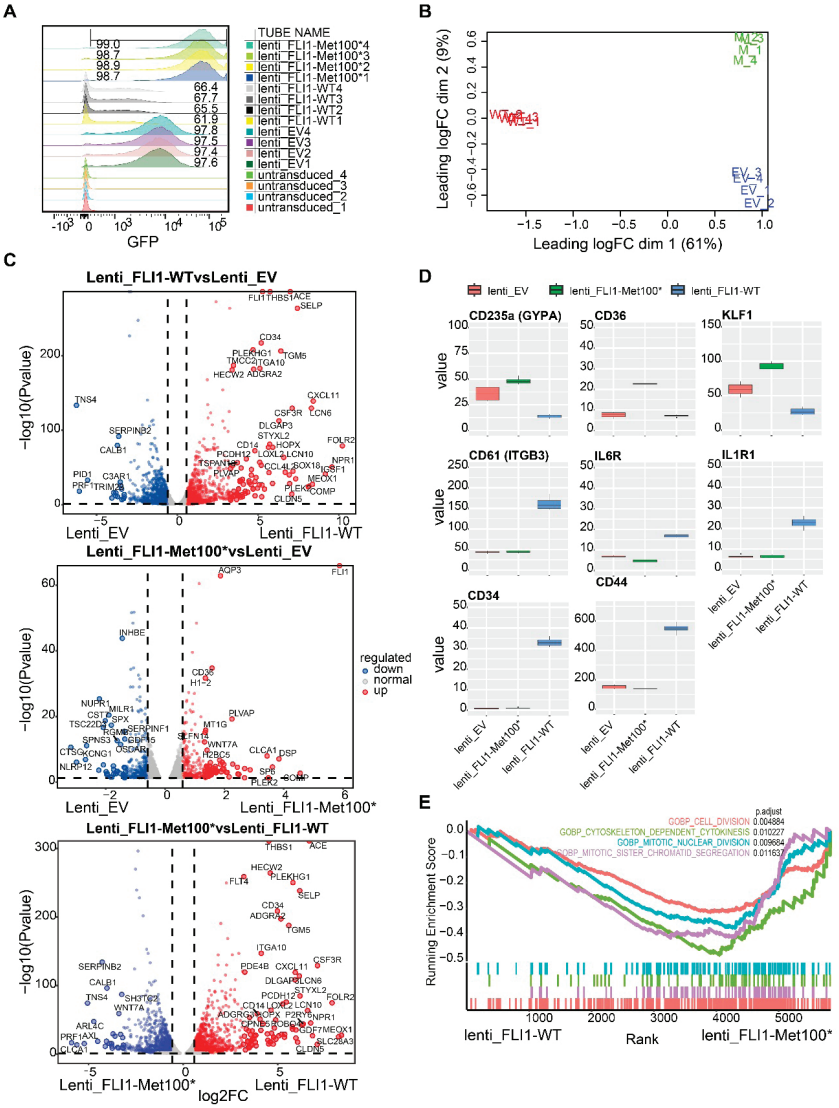
Legend Cells were mixed equally, and GFP+ cells were tracked via flow cytometry on days 2, 4, 11, and 14

Supplementary Figure S5 Flow cytometry plots



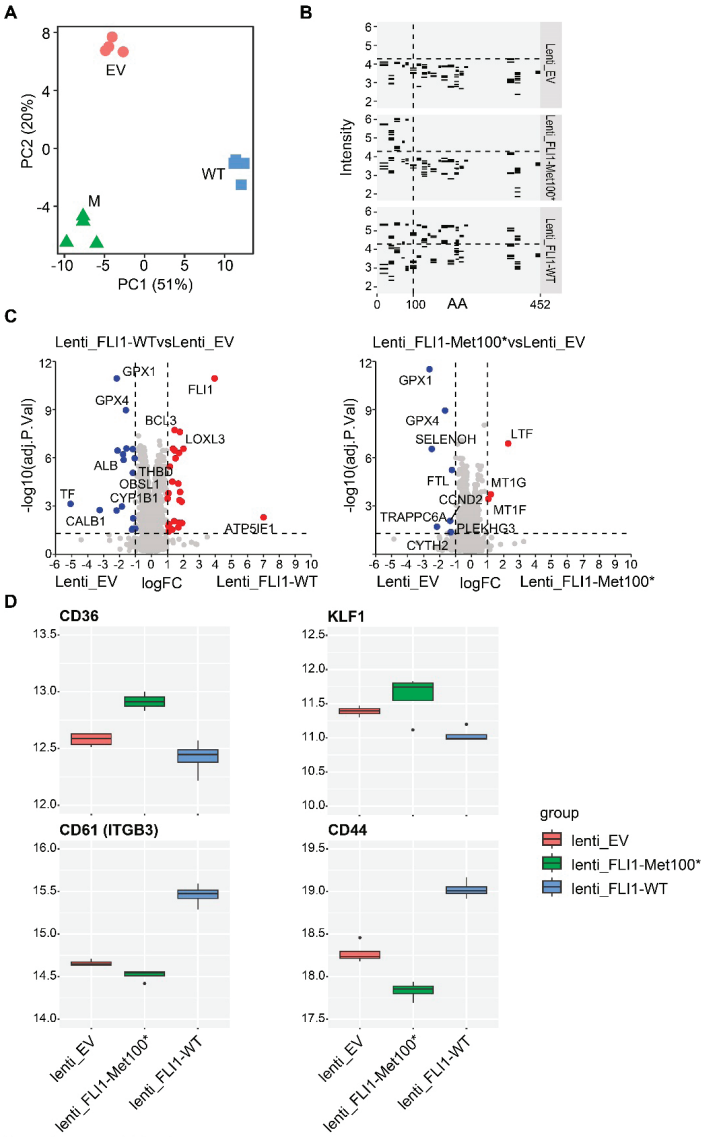
Legend Plots showing CD31, CD41a, CD42b, CD44, CD117, and CD235a expression in wild-type and heterozygous FLI1 clones

Supplementary Figure S6 RNA sequencing of lenti_FLI1-WT/lenti_FLI1-Met100* transduced Meg01 cells



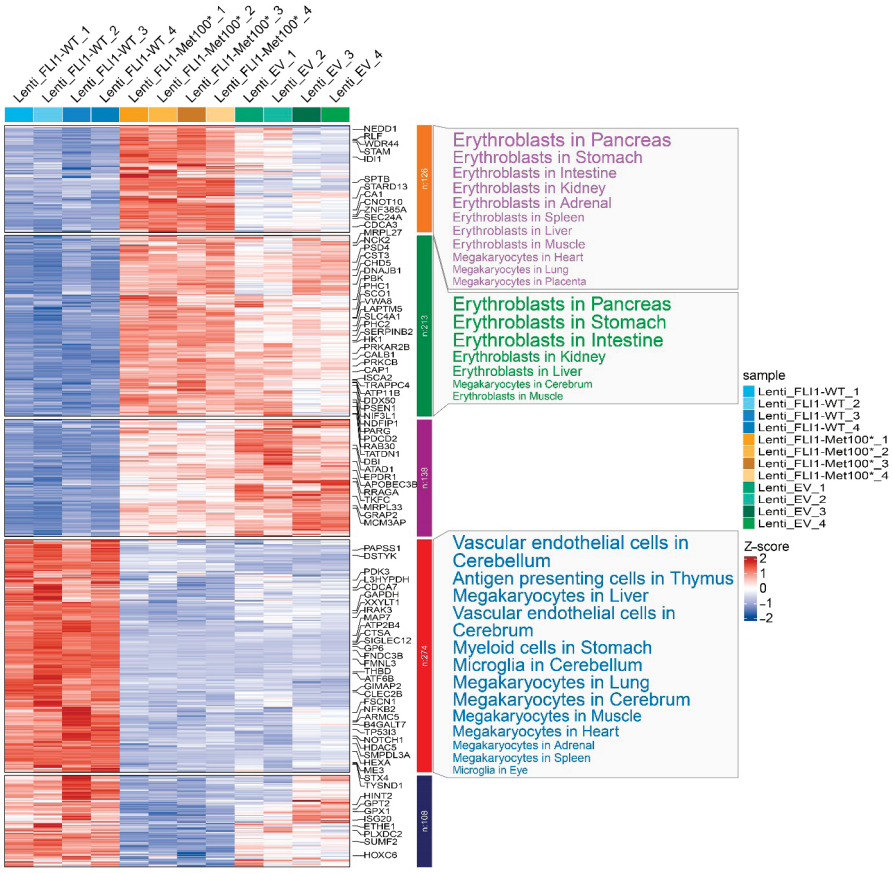
Legend (A) Transduction efficiency (GFP+ cells) at day 3 post-transduction. (B) Principal component analysis (PCA) of RNA sequencing data from day 3 lenti_EV (EV)/lenti_FLI1-WT (WT)/lenti_FLI1-Met100* (M) transduced cells. (C) Volcano plot of differentially expressed genes for all pairwise comparisons between lenti_FLI1-WT, lenti_FLI1-Met100*, and lenti_EV (fold change > 1.5, $p < 0.05$). Upregulated genes (red), downregulated genes (blue). (D) Normalized CPM (count per million) values of CD235a (GYPA), CD36, KLF1, CD61 (ITGB3), IL6R, IL1R1, CD34, and CD44. (E) GSEA using MSigDB C5 ontology gene sets for differentially expressed genes between lenti_FLI1-WT and lenti_FLI1-Met100*

Supplementary Figure S7 Proteomic analysis of lenti_FLI1-WT/lenti_FLI1-Met100* transduced Meg01 cells



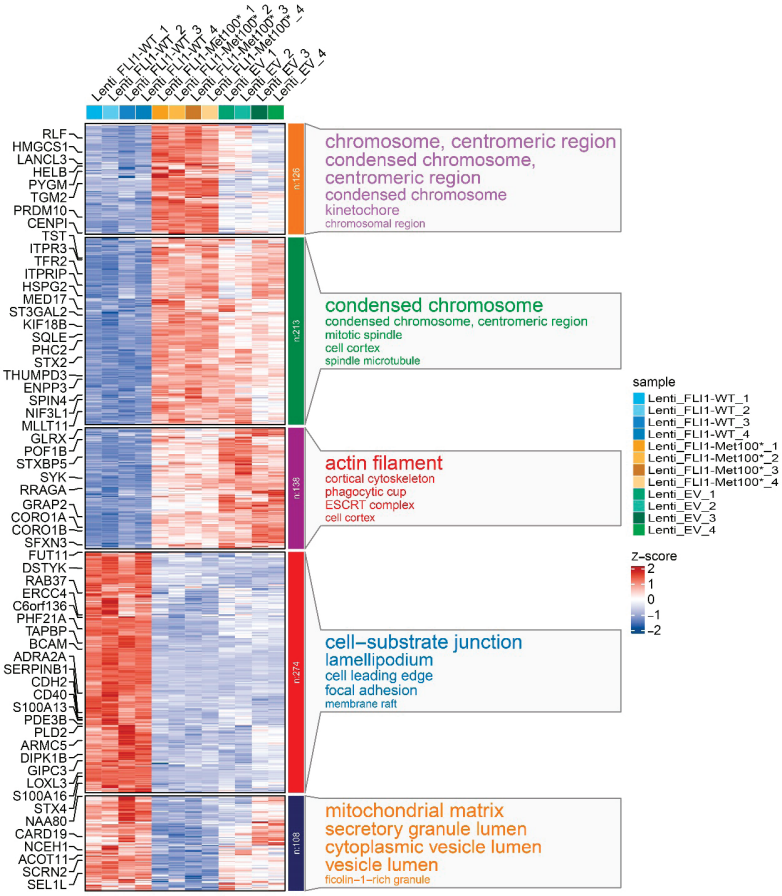
Legend (A) Principal component analysis (PCA) of mass spectrometry data from day 3 lenti_EV (EV)/lenti_FLI1-WT (WT)/lenti_FLI1-Met100* (M) transduced Meg01 cells. (B) Log_2-transformed LFQ (Label-free quantification) values of mapped FLI1 peptides in the lenti_EV/lenti_FLI1-WT/lenti_FLI1-Met100* transduced Meg01 cells. (C) Volcano plot of differentially expressed proteins (lenti_FLI1-WT/lenti_FLI1-Met100* vs. lenti_EV, fold change > 2, $p < 0.05$). Upregulated proteins (red), down-regulated (blue). (D) Log_2-transformed LFQ values of CD36, KLF1, CD61 (ITGB3), and CD44

Supplementary Figure S8 Cell type enrichment analysis



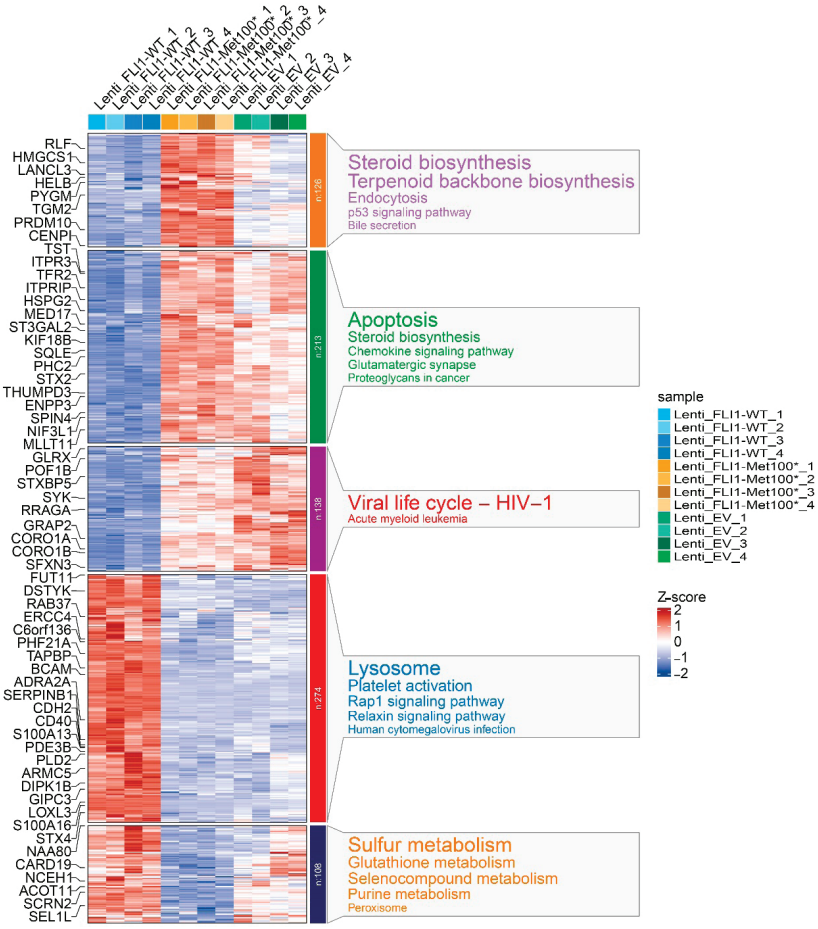
Legend Enrichment analysis for differentially expressed genes (lenti_FLI1-WT/lenti_FLI1-Met100* vs. lenti_EV) using the Descartes Cell Types and Tissues database

Supplementary Figure S9 Gene ontology cellular component enrichment analysis



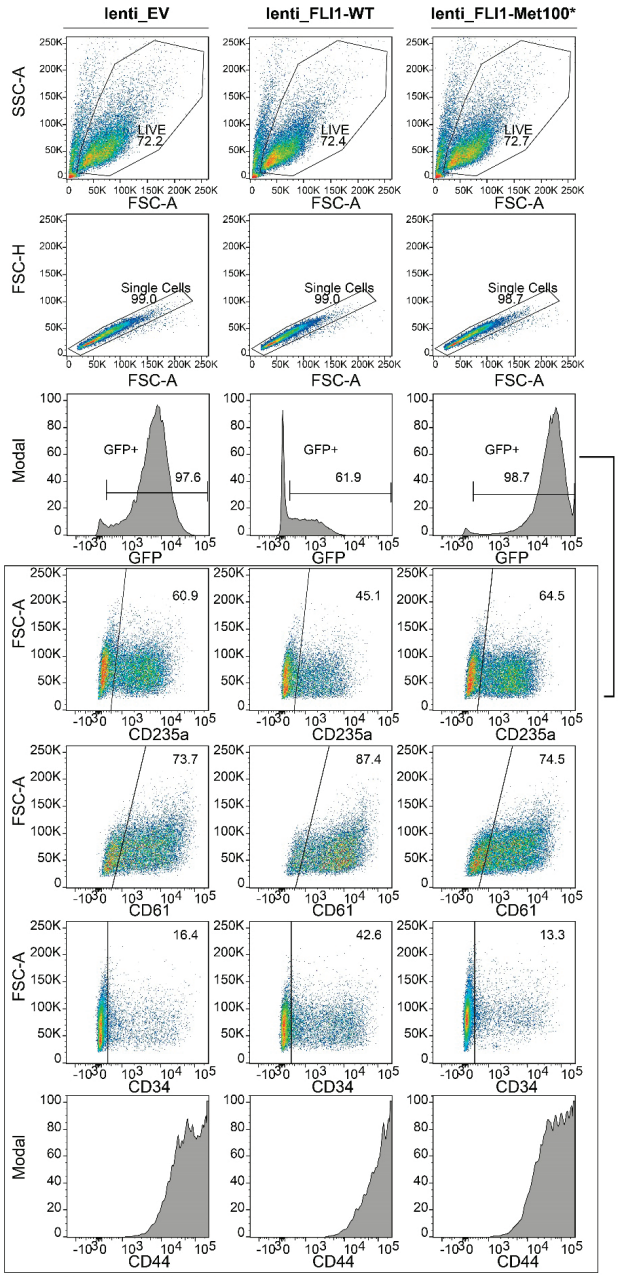
Legend Gene ontology cellular component enrichment analysis for differentially expressed genes (lenti_FLI1-WT/lenti_FLI1-Met100* vs. lenti_EV)

Supplementary Figure S10 KEGG pathway enrichment analysis



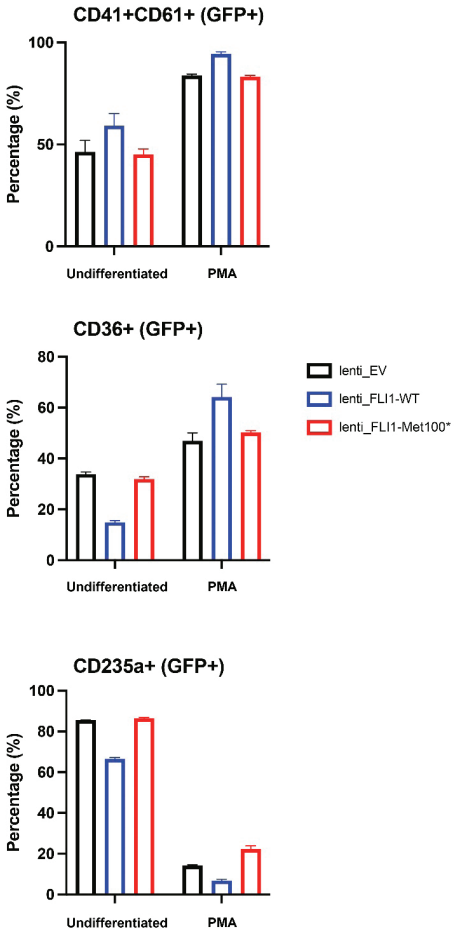
Legend KEGG pathway enrichment analysis for differentially expressed genes (lenti_FLI1-WT/lenti_FLI1-Met100* vs. lenti_EV)

Supplementary Figure S11 Flow cytometry plots in Meg01 cells



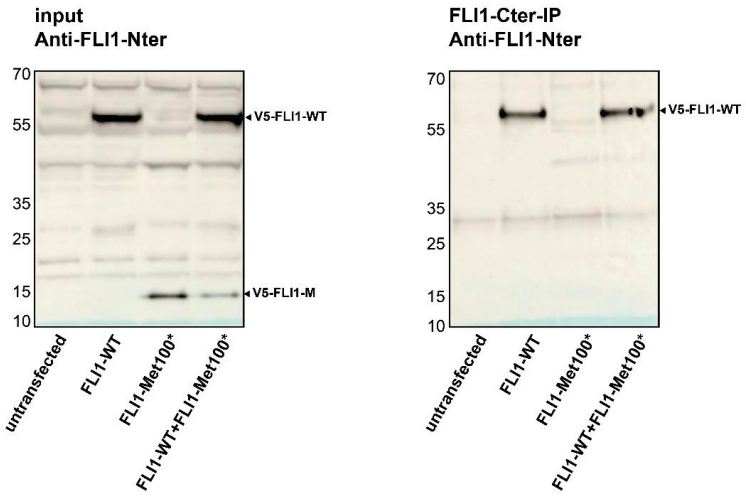
Legend Representative flow cytometry plots showing CD235a, CD61, CD34, and CD44 expression in Meg01 cells three days after lenti_FLI1-WT/lenti_FLI1-Met100* transduction. Gating was performed on live cells (FSC-A vs. SSC-A) followed by singlet selection (FSC-A vs. FSC-H)

Supplementary Figure S12 Flow cytometry plots among GFP$^+$ cells



Legend Percentages of CD41$^+$CD61$^+$, CD235a$^+$, and CD36$^+$ cells among GFP$^+$ lenti_EV, lenti_FLI1-WT, and lenti_FLI1-Met100* transduced Meg01 cells, shown for both undifferentiated and PMA-treated conditions

Supplementary Figure S13 Co-transfection of lenti_FLI1-WT and lenti_FLI1-Met100* plasmids



Legend Co-transfection of lenti_FLI1-WT and lenti_FLI1-Met100* plasmids into HEK293T cells, followed by immunoprecipitation using anti-FLI1-C-terminus (anti-FLI1-Cter) antibody. Blots stained with anti-FLI1-N-terminus (anti-FLI1-Nter) antibody

Who would dare to assert that we know all there is to be known?

Gallileo Gallilei (1564 - 1642 AD)

8

ETV6/TEL knockout leads to impaired megakaryoid gene regulation, which is phenocopied by the ETS-domain mutant ETV6-R369W via dominant negative actions

H. Zhang
E.J. Huisman
S. Rouschop
L. Kuijpers
C.C. Clark
A.F. Svendsen
C. van der Zwaan
N. Gillemans
W.F.J. van Ijcken
M.H. Cnossen
S. Philipsen
A.J. Hoogendijk
E. van den Akker

Under review in Journal of Thrombosis and Haemostasis

ABSTRACT

Megakaryopoiesis, the process by which hematopoietic stem cells give rise to megakaryocytes and platelets, is tightly regulated by the temporal expression of specific transcription factors. ETV6/TEL, a member of the ETS transcription factor family, has recently been implicated in autosomal dominant thrombocytopenia. ETV6 acts mainly as a transcriptional repressor, forming dimers through its pointed (PNT) domain, interacting with cofactors via its central domain, and binding DNA through its conserved ETS domain, where most pathogenic variants are found.

In this study, we describe two patients with inherited thrombocytopenia carrying the ETV6-R369W variant and aimed to investigate the roles of both wild-type ETV6 and this variant in megakaryopoiesis. RNA-sequencing of ETV6 knockout cells identified 424 genes upregulated and 203 downregulated compared with wild-type cells, consistent with ETV6's repressive function. Erythroid genes (*GYPA*, *CD36*, and *KLF1*) were reduced, while genes associated with hematopoietic lineage and platelet activation pathways (*CD44*, *KIT*, *GP6*, and *FLI1*) were increased, findings validated at both transcriptomic and protein levels. Overexpression of ETV6-R369W in wild-type Meg01 cells produced a similar pattern, suggesting a dominant-negative mechanism. Immunoprecipitation showed that ETV6-WT and ETV6-R369W dimerize and share cofactors including JUNB, ERG, and TRIM21. In $CD34^+$ cultures, ETV6-R369W accelerated megakaryocyte differentiation. CD44 was also elevated in patient platelets, suggesting its potential as a biomarker for ETV6-related thrombocytopenia.

Collectively, these findings demonstrate that ETV6 loss and the dominant-negative ETV6-R369W variant dysregulate hematopoietic progenitor and platelet activation markers, contributing to defective megakaryocyte differentiation and maturation.

Key words

ETV6/TEL; blood platelet disorder; thrombocytopenia; thrombopoiesis; megakaryopoiesis

INTRODUCTION

Megakaryopoiesis is orchestrated by a network of transcription factors, including *RUNX1*, *FLI1*, *GATA1*, *GFI1B*, *ETV6*, *EVI1*, *HOXA11*, and *IKZF5*. Genetic variants in these regulators are increasingly recognized as underlying causes of inherited platelet disorders, particularly with the advent of high-throughput sequencing. Among these, ETV6 variants have emerged as a significant contributor to thrombocytopenia. ETV6 is a member of the ETS transcription factor family, which also includes FLI1, ERG, and ETS1, and is critical for embryonic development, hematopoiesis, and maintenance of hematopoietic stem cells (HSCs). In mice, complete loss of *Etv6* causes embryonic lethality due to vascular abnormalities and bone marrow failure.(1) Targeted deletion within hematopoietic cells using the Gata1-Cre system specifically disrupts megakaryocyte maturation.(2)

ETV6-related thrombocytopenia is an autosomal dominant disorder characterized by moderate thrombocytopenia, mild bleeding (such as epistaxis, easy bruising, or menorrhagia), red cell macrocytosis, and a markedly elevated lifetime risk (~30%) of developing hematologic malignancies, most commonly B cell acute lymphoblastic leukemia.(3) Bone marrow analysis in affected individuals typically reveals erythroid dysplasia and an accumulation of immature, hypolobulated megakaryocytes, indicative of defective maturation.(4) While platelet counts generally remain above 75×10^9/L and platelet size is often normal, some patients present with macrothrombocytes and impaired platelet function, including defects in spreading, clot retraction, and aggregation.(5, 6)

The ETV6 protein contains a PNT domain that mediates dimerization, an ETS domain for DNA binding, and a flexible linker domain that likely contributes to its regulatory versatility.(7, 8) ETV6 has been shown to be a transcriptional repressor and studies in cell line models, primarily non-hematopoietic of origin, have shown potential interactions with corepressors such as L3MBTL1, KAP1, SIN3A, NCoR, SMRT, TIP60, and HDAC3 through its distinct domains.(9-14) Disease-associated mutations are predominantly missense variants affecting the central or ETS domains, although truncating mutations are also observed.(4-6, 15-20) These genetic lesions impair nuclear localization, DNA binding, and repressive activity, resulting in defective megakaryocyte maturation and reduced proplatelet formation.(2, 4-6, 15, 16, 19, 20) Platelet dysfunction is likely caused by disrupted expression of cytoskeletal regulators such as CDC42 and RHOA.(6)

Despite these insights, the molecular role of ETV6 in megakaryopoiesis remains poorly defined. In this study, we address this gap by generating ETV6 knockout Meg01 cell models. Additionally, we describe a patient harboring a heterozygous ETV6-R369W mutation and investigate its pathogenicity by expressing either wild-type ETV6 or ETV6-R369W in Meg01 cells. We further assessed their effects on megakaryocytic differentiation using $CD34^+$ progenitor cells. Our data indicate that ETV6-R369W acts in a dominant-negative

manner by sequestering ETV6-WT and shared cofactors, ultimately disrupting transcriptional regulation, suppressing erythroid gene expression, and impairing megakaryocyte development.

MATERIALS AND METHODS

Cell culture

Meg01 cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific), supplemented with 10% fetal calf serum (FCS, Bodinco) and 1% penicillin/streptomycin (P/S, Sigma), under standard conditions at 37 °C in a humidified atmosphere with 5% CO_2. HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific) with the same FCS and P/S supplementation. For lentiviral production, HEK293T cells were adapted to the Meg01 growth medium.

Transduction

Lentiviral particles were generated by transfecting HEK293T cells with either the empty control vector or the ETV6 expression construct, together with packaging plasmids psPAX2 (Addgene #12260) and VSV.G (Addgene #14888), using polyethylenimine (PEI, Sigma) as the transfection reagent. Viral supernatants were harvested at 48 and 72 hours post-transfection and subsequently used to transduce Meg01 cells.

For CD34 transduction, lentiviral supernatants were ultracentrifuged in 26 mL volumes at 20,000 RCF for 3 hours at 4 °C in polyallomer tubes (Beckman Coulter). Viral pellets were resuspended in 500 µL StemSpan II (Stemcell Technologies) and stored at -80 °C. HEK293T cells (75,000/well) were seeded in 96-well plates and transduced with serial dilutions of the concentrated virus (5-fold increments starting from 1:5). After 48 hours, cells were harvested using TrypLE, washed, and resuspended in FACS buffer (PBS + 5 mM EDTA + 5% BSA). GFP expression was measured using a SONY ID7000 spectral analyzer. Titer was calculated from the dilution yielding 5-20% GFP^+ cells. Theoretical titer (TU/µL) = (GFP^+ cells × dilution factor) / virus volume (µL), Volume for MOI = (target cell number × desired MOI) / titer (TU/µL)

$CD34^+$ to Megakaryocyte Differentiation

$CD34^+$ cells were isolated from mobilized peripheral blood (MPB) and enriched using magnetic-activated cell sorting (MACS) with CD34 magnetic beads (Miltenyi Biotec), following the manufacturer's protocol. Thawed cells were resuspended in StemSpan II, centrifuged (250 RCF, 5 min), and adjusted to 1-2 × 10^6 cells/mL in StemSpan II supplemented with 1:100 SCF (culture supernatant from 293T cells expressing ectopic SCF), 50 ng/mL romiplostim (Nplate, Amgen), 100 ng/mL FLT3L (Stemcell Technologies), and

20 ng/mL IL-6 (Stemcell Technologies). One mL per well was plated in a 12-well plate and incubated at 37 °C for 48 h.

24-well non-TC plates were coated with 0.5 µg/mL retronectin (2 h, RT, TaKaRa), blocked with 2% HSA (30 min, Sanquin), and incubated with lentivirus (MOI = 57). Plates were spinoculated (1500 RCF, 3 h, 37 °C), then seeded with 8×10^5 $CD34^+$ cells in cytokine-containing medium (same as above plus 100 U/mL P/S), spun (200 RCF, 5 min), and incubated overnight. Medium was fully replaced after 24 h and cells were cultured an additional 32 h.

On day 4, cells were transferred to new wells in StemSpan II with 50 ng/mL Nplate, 10 ng/mL IL-1β (Stemcell Technologies), 1 µM StemRegenin 1 (SR-1, Stemcell Technologies), and 100 U/mL P/S. Cultures were maintained at $1\text{-}1.5 \times 10^6$ cells/mL with medium refreshment every 2-3 days. On days 4, 8, and 11, 100 µL of culture was harvested, stained with flow cytometry antibodies (Table 1), and analyzed on a SONY ID7000 cytometer using FlowJo v10.10.

Table 1 Antibodies and dyes used for flowcytometry

Antigen	Fluorophore	Dilution	Manufacturer	Catalogue number
CD41a	R718	1:100	BD	#568058
CD61	PerCP-Cy5.5	1:100	BD	#564173
CD42b	BV711	1:100	BD	#740779
KIT	Pacific Blue	1:100	BD	#550412
CD44	PE	1:100	Miltenyi	#130-113-335
CD44	APC	1:2000	eBioscience	#17-0441-83
CD235a	APC	1:1000	Miltenyi	#130-100-262
CD235a	PE	1:2500	Origene	#DM066R
CD36	APC	1:100	BD	#550956

Flow cytometry

Meg01 cells were stained in FACS buffer (PBS supplemented with 0.1% BSA and 0.5 mM EDTA) at 4 °C for 30 minutes using the antibodies in Table 1. Following staining, cells were washed and analyzed using a BD FACSymphony™ A5 flow cytometer. Flow cytometry data were processed and analyzed with FlowJo software (BD Biosciences).

Western blot and immunoprecipitation

Wild-type and ETV6 knockout Meg01 clones were lysed in CARIN buffer,(21) supplemented with orthovanadate (BioLabs), PMSF (Sigma-Aldrich), protease inhibitors (Merck), okadaic acid (Calbiochem), and benzonase (Sigma-Aldrich). For western blot analysis, lysates were denatured at 95 °C for 5 minutes in sample buffer (Invitrogen), separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen), and transferred using the iBlot-NC system

(Thermo Fisher Scientific). Membranes were probed with the rabbit antibodies targeting the N- and C- terminal regions of ETV6 (kind gifts from Dr. Jacques Ghysdael, Curie Institute, Paris, France). Goat anti-rabbit (Dako, p0448) was used as the secondary antibody. RhoGDI (Thermo Fisher Scientific, MA5-17032) was used as a loading control.

To assess ETV6 dimerization, immunoprecipitation was performed using mouse anti-V5 (Absea) followed by capture with Dynabeads Protein G (Thermo Fisher Scientific). Bound proteins were eluted by heating in sample buffer at 95 °C and probed with rabbit anti-ETV6-N-terminal and goat anti-V5 (Abcam) antibodies. Fluorescent secondary antibodies (donkey anti-goat IRDye 800CW and donkey anti-rabbit IRDye 680RD; Li-Cor) were used for detection. For mass spectrometry-based immunoprecipitation, lysates were incubated with rabbit anti-ETV6-C-terminal antibody prior to bead enrichment. Beads were washed five times in PBS before processing for mass spectrometry. Blots were probed with rabbit anti-ETV6-N-terminal antibody, and TidyBlot-HRP (BIO-RAD) was used to minimize inference from denatured IgG.

Bulk RNA sequencing analysis

RNA libraries were prepared following previously published protocols,(22) and sequencing was performed on the Illumina HiSeq 2500 platform. Sequencing reads were aligned to the human reference genome GRCh38 (hg38) using the STAR aligner. Low-quality reads were excluded, and differential expression analysis was conducted using the glmLRT function in the edgeR package, applying a fold-change cutoff of ≥1.5 and a false discovery rate (FDR) threshold of 0.01.

Gene enrichment analysis

Differentially expressed genes between Meg01 cells transduced with EV, ETV6-WT, or ETV6-R369W were ranked according to their $\log_2$ fold change. This ranked gene list was used as input for gene set enrichment analysis (GSEA), performed with the clusterProfiler R package, utilizing the C8 cell type signature gene sets from the Molecular Signatures Database (MSigDB). To investigate shared molecular pathways, genes overlapping between RNA sequencing and mass spectrometry datasets were further analyzed using the ClusterGVis R package for K-means clustering and gene ontology (GO) enrichment analysis.

Statistics

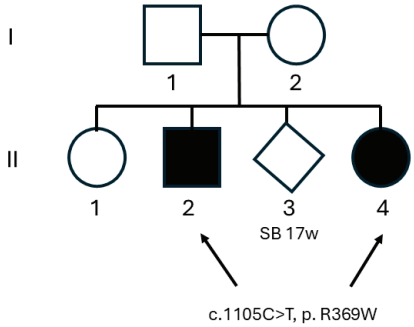
Statistical analyses were conducted using GraphPad Prism version 10. Comparisons between two groups with replicates were evaluated using unpaired t-tests, with multiple comparisons adjusted via the Holm-Šídák method. For experiments involving more than two groups, two-way ANOVA was applied, followed by Tukey's or Šídák's post hoc tests. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics

We describe a family in which two out of three children were diagnosed with inherited thrombocytopenia due to a pathogenic *ETV6* variant (NM_001987.4(ETV6): c.1105C>T, p.(Arg369Trp)), resulting in a frameshift mutation (Fig. 1, Pedigree). This variant was not detected in either parent; germline mosaicism was not investigated. The mother had a history of bleeding symptoms, including heavy menstrual and postpartum bleeding requiring iron supplementation, although coagulation disorders were ruled out. The father had no history of excessive bleeding.

Figure 1 Pedigree of individuals carrying the ETV6 c.1105C>T (p.R369W) mutation



Legend Family tree showing inheritance of the ETV6 p.R369W variant

The first child, a daughter, had normal platelet counts. The second child, a boy and the index patient, first presented at age 5 with recurrent severe epistaxis and petechiae. He also experienced frequent respiratory and ear infections in early childhood, managed with oral antibiotics and without hospitalization. He attended special education due to delayed language development. Blood tests revealed chronic thrombocytopenia ranging from 70-150 x 10^9/L until age 9, with a subsequent decline to 30-60 x 10^9/L and a gradual increase in MPV (mean platelet volume) from 9.6 fL (age 6) to 11.5 fL (age 13). Platelet function test was not feasible due to severity of the thrombocytopenia. He exhibited an elevated bleeding tendency, including petechiae, hematomas, epistaxis, oral bleeding, and postoperative bleeding, resulting in a bleeding score of 8 using the ISTH bleeding assessment tool (normal for children: 0–2). Genetic testing at age 13, as part of the DoReMi study, revealed the *ETV6* variant. No bone marrow examination was performed at diagnosis, but clinical surveillance was intensified. From age 14, epistaxis aggravated and the patient reported fatigue interfering with daily activities. There were no signs of abnormal enlarged lymph nodes or hepatosplenomegaly. Repeated peripheral blood tests revealed no abnormalities, especially no peripheral blasts. Immunophenotyping showed absent CD13 expression on monocytes, without association to any known disease entity. At age

15, due to persistent complaints, a bone marrow biopsy was performed and revealed >60% blasts, consistent with aleukemic pre-B cell acute lymphoblastic leukemia (ALL; $CD19^+/CD10^+/CD34^+/CD45^-/TdT^+$) with a complex karyotype and no ABL-class fusions. He was treated under the 'ALLtogether' protocol, showing early MRD negativity, complete remission, and no markedly elevated bleeding tendency despite chemotherapy-induced severe thrombocytopenia.

A third pregnancy ended in intrauterine fetal death at 18 weeks due to massive fetal hemorrhage (no further data available). The third living child, a girl, was found to have thrombocytopenia at 11 months during evaluation for recurrent virus-induced asthma and respiratory upper and lower tract infections, requiring hospitalizations at ages 2 and 3 for intravenous antibiotics. Platelet counts remained between 70-110 x 10^9/L, with a gradual increase in MPV (9.2 – 10.8). Platelet function test via light transmission aggregometry demonstrated responses to all agonists, albeit with reduced amplitude, due to low PRP (platelet-rich plasma, 109 x 10^9/L, normally required >150 x 10^9/L). She also exhibited an elevated bleeding tendency, with symptoms similar to her older brother, resulting in a bleeding score of 6. Ongoing surveillance according to our hospital's predisposition protocol has revealed no abnormalities to date.

ETV6 knockout in Meg01 cells suppresses erythroid programs and enhances megakaryocytic Identity

To investigate the role of ETV6 and its R369W variant in megakaryopoiesis, we generated ETV6 knockout (KO) Meg01 clones by nucleofecting ribonucleoprotein complexes (homemade Cas9 and dual gRNAs targeting exon 5; Supplemental Table 1). PCR, Sanger sequencing, and western blotting confirmed successful editing and complete loss of ETV6 protein (Fig. 2A; Supplemental Fig. 2A). MDS analysis after RNA-seq of three WT and three KO clones revealed distinct transcriptomic profiles (Supplemental Fig. 2B), with 424 genes upregulated and 203 downregulated in KO clones, consistent with ETV6's repressive function (Supplemental Fig. 2C&D; Supplemental Table 2A). Upregulated genes included known targets such as *CLIC5*, *BIRC7*, and *WBP1L* (Supplemental Fig. 2D&E). Cell-type enrichment (Descartes database) showed that upregulated genes were associated with megakaryocyte and myeloid signatures, while downregulated genes were enriched for erythroid and endothelial programs (Fig. 2B; Supplemental Table 2B), consistent with gene set enrichment analysis (Supplemental Fig. 2F; Supplemental Table 2C). Erythroid genes such as *KLF1*, *GYPA*, and *CD36* (also expressed in megakaryocytes) were downregulated, while megakaryocyte-associated genes including *FLI1*, *ITGB3*, *GP6* and *CXCR4* were upregulated (Fig. 2C; Supplemental Table 2A). KEGG analysis revealed enrichment in platelet activation and hematopoietic lineage pathways, involving genes such as *ITGB3*, *GP6*, *PLEK*, *RGS18*, *KIT*, *CD44*, and *ITGA6* (Supplemental Fig. 2G; Supplemental Table 2D). Gene ontology analysis highlighted upregulated processes including cell adhesion, migration, and proliferation (Supplemental Table 2E). Notably, several genes associated

with megakaryocyte and proplatelet formation, platelet function, and thrombocytopenia, including *PTK2B*, *S1PR1*, and *SLFN14*, were downregulated in KO cells (Supplemental Table 2A). Proteomic profiling confirmed loss of ETV6 (Fig. 2D; Supplemental Table 2F). Integration of transcriptomic (Fig. 2E) and proteomic (Fig. 2F) data revealed concordant expression changes, with decreased KLF1, CD36, and PTK2B, and increased FLI1, GP6, KIT, and CD44 (Fig. 2D; Supplemental Table 2F). Flow cytometry further supported these findings, showing reduced $CD235a^+$ and $CD36^+$ cells, with trends toward increased CD44 mean fluoresence intensity (MFI) and higher levels of KIT^+ cells in the KO clones (Fig. 2G; Supplemental Fig. 3). In summary, ETV6 knockout Meg01 clones exhibit reduced erythroid identity and increased expression of megakaryocytic and early hematopoietic progenitor markers, accompanied by downregulation of key maturation genes, indicative of conflict in cell identify and disruptive lineage commitment.

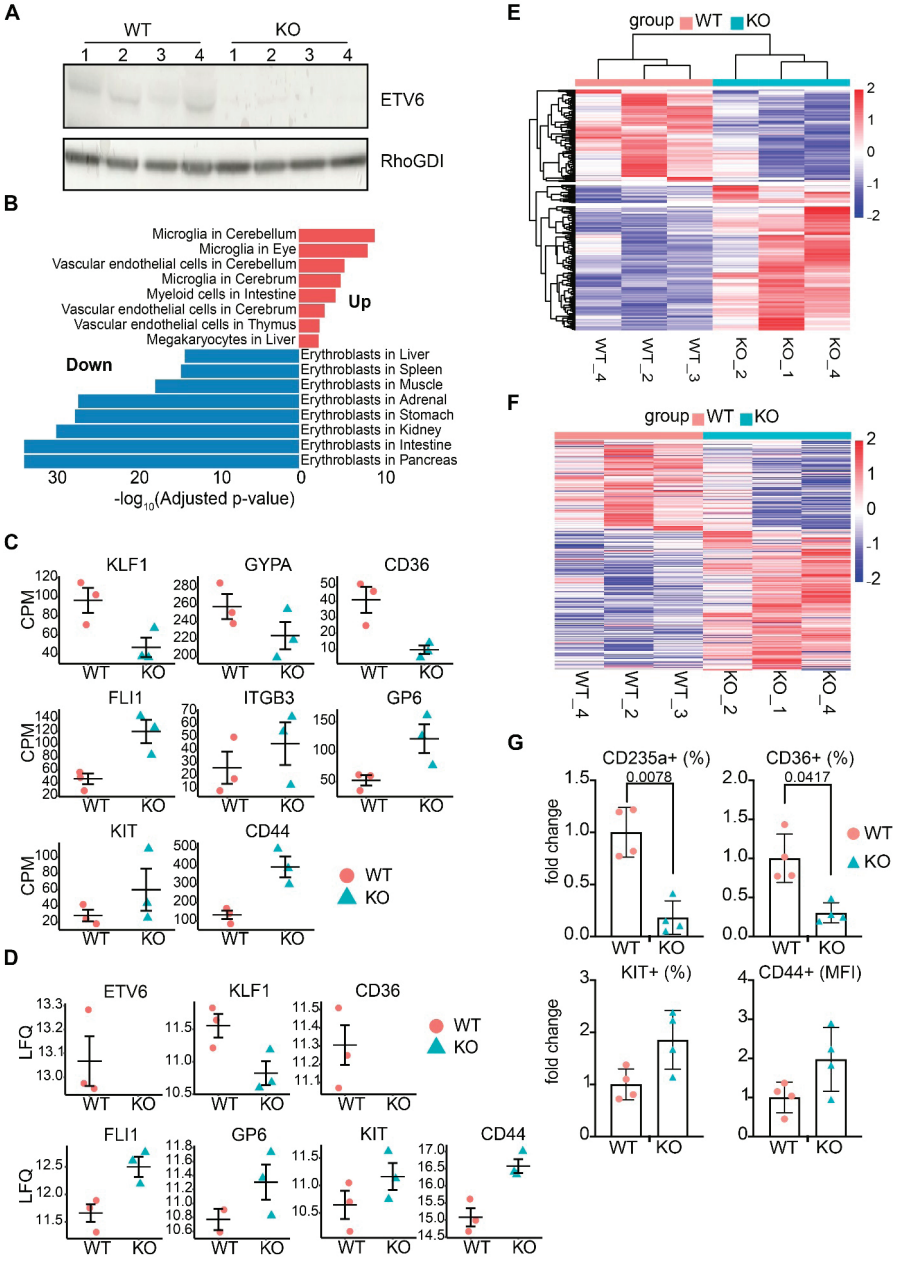
Table 2 Laboratory results of hemostatic tests

Parameters	Results P2.2	Results P2.3	Normal age references
Blood count and platelet indices			
Platelet count, mean (range; x 10^9/L)	18 (0-95)	81 (71-148)	150-370
MPV at age 5yr, (range; L/L)	9.6	8.4	N 8-12
MPV at age 10yr, (range; L/L)	10.3	9.4	1-5
MPV at age 13yr, (range; L/L)	11.5	10.8	12-16.5
IPF, mean (range; %)	6 (5-8)	2 (2-3)	3.5-10.0
Hemoglobin, mean (range; g/dL)	13.0 (3.6-9.4)	13.3 (11.7-13.9)	
Leucocyte count, mean (range; x 10^9/L)	4.0 (1.3-10.2)	5.4 (4.8-6.2)	
Other platelet related tests			
TPO, mean (range; U/mL)	32 (30-34)	10	4-31
PFA-EPI (sec)	170	134 (115-147)	<150
LTA, descriptive	N.A. due to severe thrombocytopenia	Normal response[1] to ADP and epinephrin Acceptable response to collagen, TRAP, ristocetin, and U46199. Weak response to Arachidonic acid (40%)	ADP and epinephrin >50% final amplitude; other agonists >70%

Abbreviations ADP = adenosine diphosphate, IPF = immature platelet fraction, LTA = light transmission aggregometry, MPV = mean platelet volume, N = normal, P = patient, PFA-EPI = Platelet function analyzer 200© - epinephrin, PRP = platelet rich plasma, TPO = thrombopoietin, U = unit

Legend [1] Due to mild thrombocytopenia, PRP of 109 x 10^9/L, not meeting preferred international standards and interpretation performed with caution

Figure 2 ETV6 knockout in Meg01 cells suppresses erythroid and enhances megakaryocytic features



Abbreviations CPM = counts per million, KO = knockout, LFQ = label-free quantification, MFI = mean fluorescence intensity, WT = wild-type

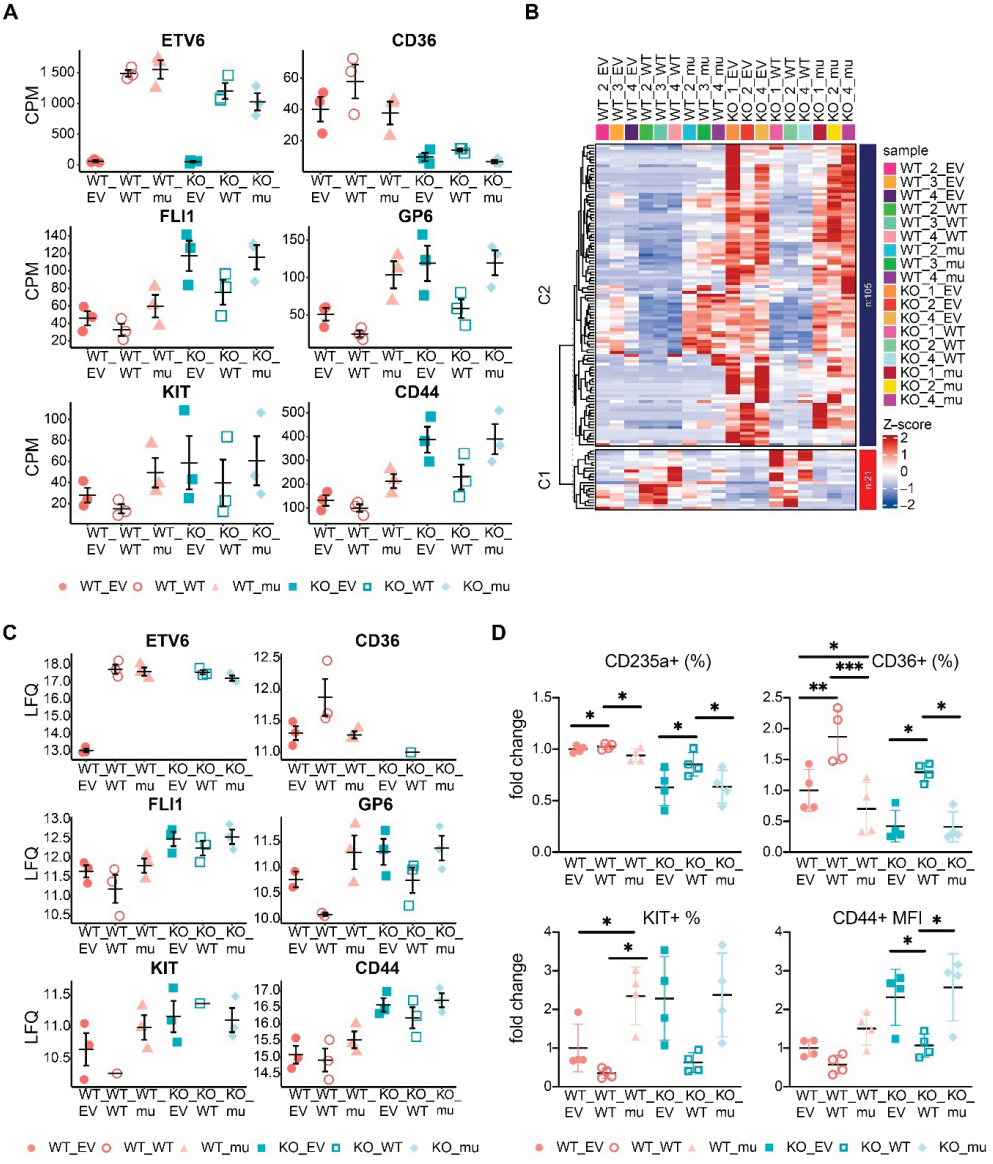
Legend (A) Western blot analysis of ETV6 expression in four wild-type and four KO clones. RhoGDI served as a loading control. (B) Cell type enrichment analysis showing cell types associated with significantly upregulated (shown in red) and downregulated (shown in blue) gene expression pro-

files in ETV6 KO clones. (C) Normalized counts per million for erythroid markers (*KLF1*, *GYPA*, *CD36*), megakaryocytic markers (*FLI1*, *ITGB3*, *GP6*), and progenitor markers (*KIT*, *CD44*) in WT and KO clones. (D) LFQ values for ETV6, erythroid (KLF1, CD36), megakaryocytic (FLI1, GP6), and progenitor (KIT, CD44) proteins in WT and KO clones. (E) Heatmap of differentially expressed genes (DEGs) with detectable protein expression between WT and KO clones. (F) Heatmap of LFQ values corresponding to the DEGs in panel (E); sample and gene order is preserved. (G) Fold changes in the percentage of $CD235a^+$, $CD36^+$, KIT^+ cells, as well as the MFI of CD44+ cells in KO clones relative to WT. Data are presented as mean ± standard deviation and were analyzed using multiple unpaired t-tests with Holm–Šídák correction for multiple comparisons. Adjusted p-values are indicated

ETV6-R369W promotes hematopoietic progenitor identity and platelet activation gene expression in Meg01 cells

To address the molecular mechanisms underlying the ETV6-R369W phenotype, we overexpressed an empty vector (EV), ETV6-WT, or ETV6-R369W in both wild-type and ETV6 KO clones. MDS analysis of the RNA-sequencing data showed that wild-type and KO clones segregated separately, regardless of whether ETV6-WT or ETV6-R369W was overexpressed (Supplemental Fig. 4A). Using the differentially expressed genes identified between WT and KO clones transduced with EV, we observed that all WT conditions clustered together and all KO conditions clustered together (Supplemental Fig. 4B; Supplemental Table 3A), consistent with the separation seen in the MDS analysis. As expected, *ETV6* was among the upregulated genes, confirming successful transduction (Fig. 3A). To gain better resolution, we clustered the differentially expressed genes within all WT groups and among KO groups separately, which revealed two major clusters. Cluster 1 contained genes repressed by ETV6-WT and upregulated by ETV6-R369W, while Cluster 2 contained genes upregulated by ETV6-WT (Fig. 3B; Supplemental Table 3B). KEGG pathway analysis of genes in Cluster 1 revealed enrichment for hematopoietic lineage and platelet activation pathways (Supplemental Table 3C), including genes such as *FLI1*, *GP6*, *CXCR4*, *KIT*, and *CD44* (Fig. 3B; Supplemental Table 3B). Additionally, we observed that the erythroid marker *CD36* was upregulated by ETV6-WT but downregulated by ETV6-R369W (Fig. 3B). Notably, these transcriptomic findings were also supported by proteomics (Fig. 3C; Supplemental Table 3D). We next examined the subset of genes in Clusters 1 and 2 for which corresponding proteins were detected in the proteomics dataset and confirmed that transcript and protein expression patterns were concordant (Supplemental Fig. 4C&D). To further validate these results, we performed flow cytometry on transduced cells, confirming that ETV6-WT promoted an erythroid phenotype (increased CD235a and CD36) and suppressed hematopoietic progenitor markers (KIT and CD44), whereas ETV6-R369W had the opposite effect (Fig. 3D; Supplemental Fig. 4E and 5). Interestingly, platelet proteomics also revealed upregulation of CD44 in both patients carrying the ETV6-R369W mutation (Supplemental Fig. 6). Together, our data demonstrate that ETV6-WT upregulates erythroid marker expression and downregulates platelet activation and hematopoietic progenitor markers, while ETV6-R369W induced the reciprocal expression pattern, suggesting a dominant-negative function of the mutant.

Figure 3 Expression of ETV6-R369W in Meg01 cells mimics the ETV6 knockout phenotype



Abbreviations EV = empty vector, KO = knockout, WT = wild-type

Legend (A) Normalized CPM values of ETV6, CD36, FLI1, GP6, KIT, and CD44 across all WT and KO sample groups transduced with either EV, ETV6-WT, or ETV6-R369W (WT_EV, WT_WT, WT_R369W; KO_EV, KO_WT, KO_R369W). (B) Heatmap of differentially expressed genes within the WT (WT_EV, WT_WT, WT_R369W) and KO (KO_EV, KO_WT, KO_R369W) groups following lentiviral transduction with EV, ETV6-WT, or ETV6-R369W. (C) LFQ values of ETV6, CD36, FLI1, GP6, KIT, and CD44 in all sample groups. (D) Percentages of $CD235a^{+}$, $CD36^{+}$, KIT^{+} cells and CD44 MFI in all WT and KO groups. Data represent mean ± standard deviation and were analyzed by two-way ANOVA followed by Tukey's post hoc test. Significance levels: *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001

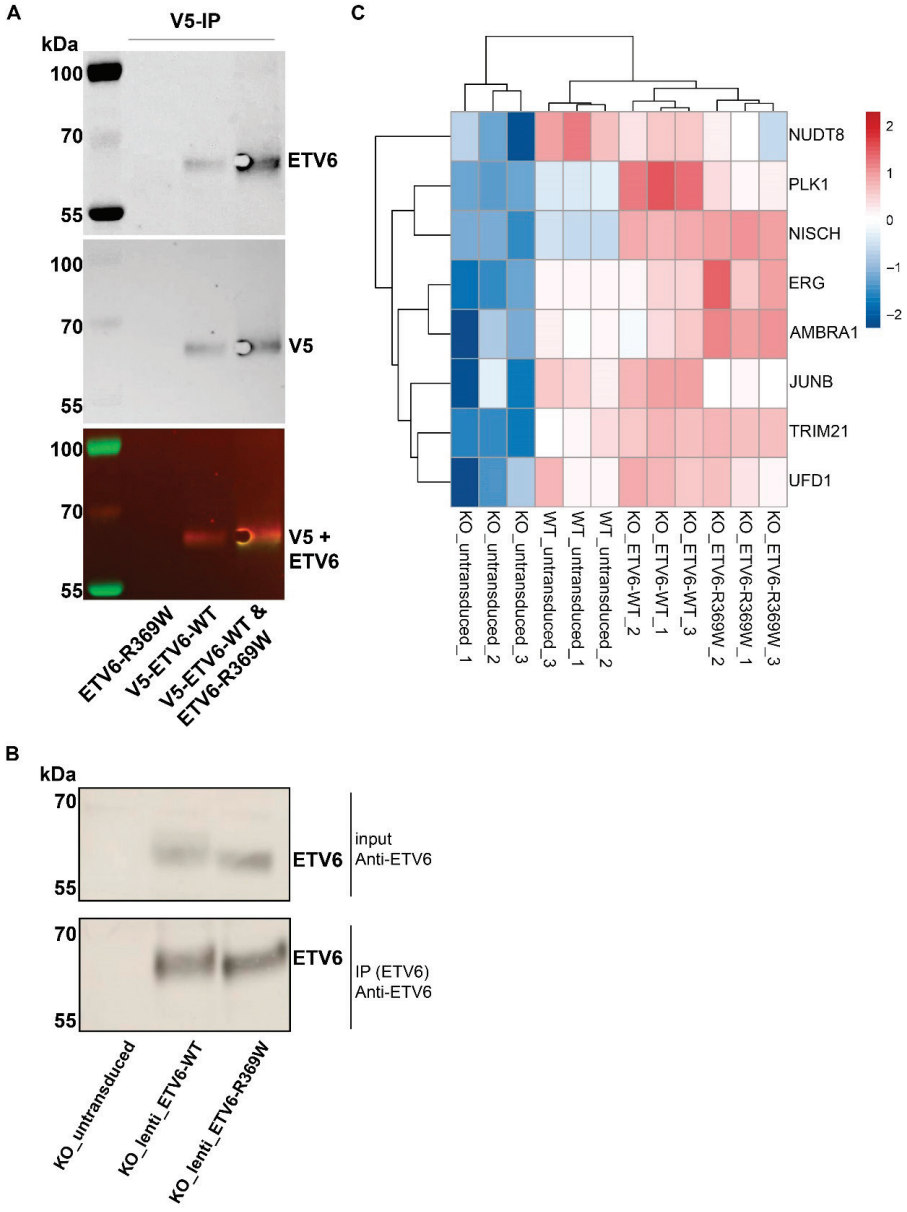
ETV6-R369W exerts dominant negative effects through dimerization with wild-type ETV6 and sequestration of cofactors

To investigate the molecular mechanism of ETV6-R369W, we first examined whether it can interact with wild-type ETV6. ETV6-knockout Meg01 cells were co-transduced with ETV6-WT or ETV6-R369W along with V5-tagged ETV6-WT or V5-tagged ETV6-R369W, generating four combinations to assess dimerization. Co-immunoprecipitation using anti-V5 antibodies followed by Western blot analysis showed that both wild-type and mutant ETV6 could interact with each other as well as with themselves, indicating their ability to form dimers (Fig. 4A; Supplemental Fig. 7A). We then performed proteomic profiling of immunoprecipitated overexpressed ETV6-WT and ETV6-R369W in ETV6-deficient cells. Mass spectrometry revealed several proteins that co-immunoprecipitated with both forms of ETV6, including the known interactor FLI1 and GAB2, and newly identified partners such as transcription factors ERG, JUNB, ERF, AHR, and NFKB2, as well as kinases PLK1, SRC, and TYK2 (Fig. 4B; Supplemental Fig. 7B; Supplemental Table 4). The interactome also comprised proteins involved in the ubiquitination pathway, including the deubiquitinase USP25; several E3 ubiquitin ligases such as TRIM21, TRIM32, STUB1, NEDD4, NEDD4L, CUL3, FBXO3, DCAF7, and DCAF8; as well as proteins linked to ubiquitin-mediated degradation, such as UFD1 and AMBRA1. To confirm these interactions in a more physiological context, we immunoprecipitated endogenous ETV6 from wild-type Meg01 cells. Several of the previously identified proteins including PLK1, TRIM21, ERG, AMBRA1, UFD1, and JUNB, were validated in this setting (Fig. 4B). Collectively, these findings indicate that ETV6-R369W retains the ability to dimerize with wild-type ETV6 and may act in a dominant-negative manner by sequestering shared cofactors, thereby impairing normal ETV6-mediated regulatory functions.

ETV6-R369W accelerates megakaryocyte differentiation from CD34$^+$ stem and progenitors

To examine the effect of the ETV6-R369W variant on megakaryopoiesis, we transduced mobilized peripheral blood CD34$^+$ cells with either ETV6-WT or ETV6-R369W using a lentiviral system, and then differentiated them toward the megakaryocyte lineage following our established protocol,((23) Fig. 5A). CD34$^+$ cells were cultured in expansion medium, and transduction was performed on day 2 post-recovery. By day 4, cells were switched to megakaryocyte differentiation medium. Transduction efficiency, assessed by the percentage of GFP$^+$ cells, peaked on day 4+4 across all groups, with the most pronounced increase observed in the EV group and the most rapid decline in the ETV6-R369W group (Fig. 5B; Supplemental Fig. 8A). We assessed early megakaryocyte populations by CD41/CD61 co-expression and late-stage populations by CD41/CD42 co-expression at days 4+4, 4+8, and 4+11. Notably, at day 4+4, expression of ETV6-R369W led to a significant increase in both early (GFP$^+$/CD41$^+$/CD61$^+$) and late (GFP$^+$/CD41$^+$/CD42$^+$) megakaryocyte populations compared to ETV6-WT (Fig. 5C; Supplemental Fig. 8B). We also examined CD44 MFI within GFP$^+$/CD41$^+$ cells. At day 4+4, CD44 MFI was higher in ETV6-WT-transduced cells,

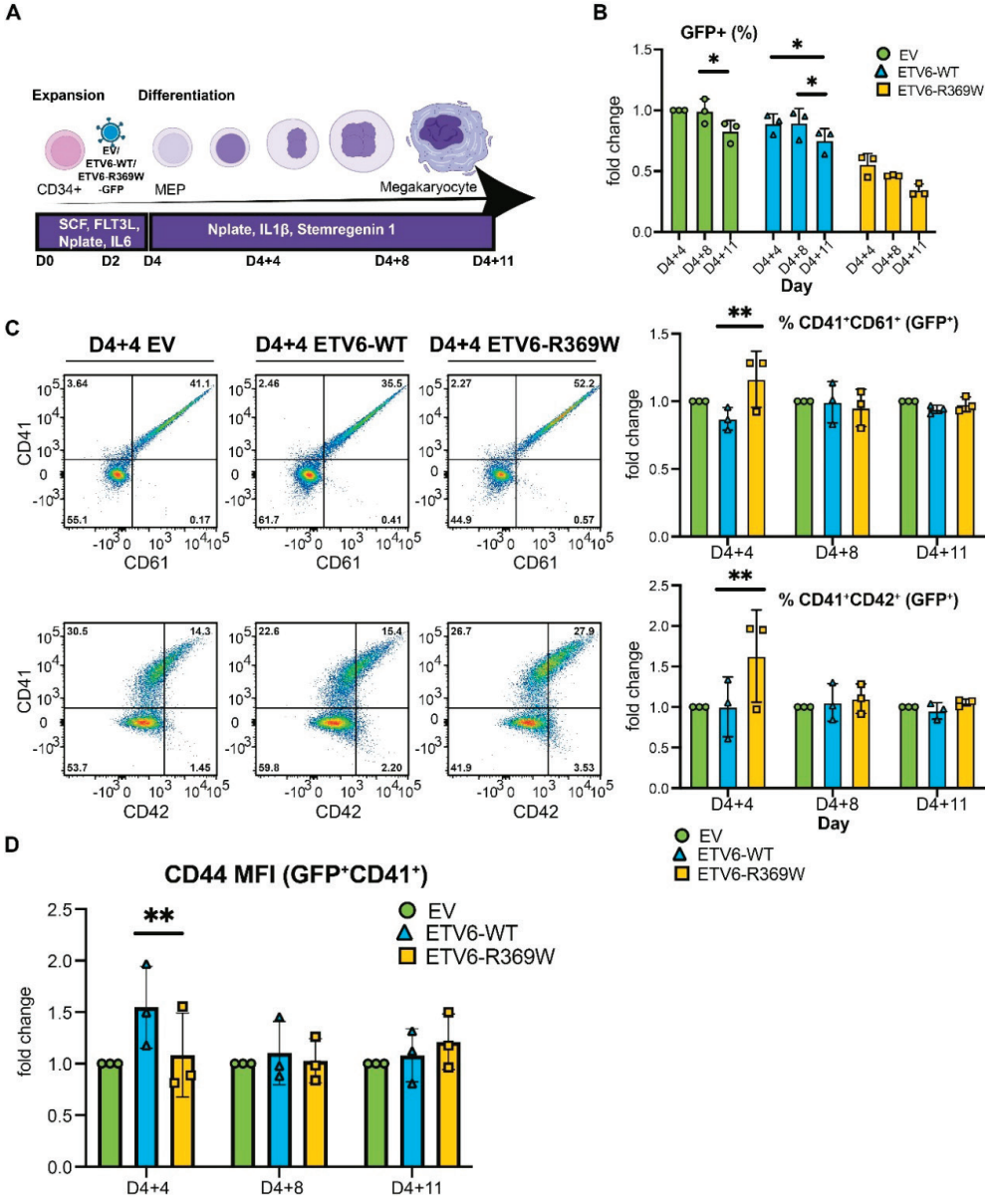
Figure 4 ETV6-R369W interacts with ETV6-WT and shares protein cofactors



Abbreviations IP = immunoprecipitation, KO = knockout, WT = wild-type

Legend (A) Co-immunoprecipitation (co-IP) of V5-tagged ETV6-WT with ETV6-R369W using an anti-V5 antibody, followed by western blot analysis with anti-V5 and anti-ETV6 N-terminal antibodies. (B) IP of endogenous ETV6 using an anti-ETV6 C-terminal antibody, detected with an anti-ETV6 N-terminal antibody by western blot. (C) IP-mass spectrometry (IP-MS) analysis of proteins co-precipitating with ETV6 in KO cells transduced with either ETV6-WT or ETV6-R369W. Proteins that were also detected in untransduced WT cells are shown.

Figure 5 ETV6-R369W accelerates megakaryocytic differentiation in CD34+ cells



Abbreviations EV = empty vector, MFI = mean fluorescence intensity, WT = wild-type

Legend (A) Schematic of CD34+ cell differentiation into megakaryocytes with lentiviral transduction of EV, ETV6-WT, or ETV6-R369W. Timeline includes key growth factors and cytokines. (B) Fold change in GFP+ cell percentages on day 4+4, 4+8, and 4+11 (D4+4, D4+8, D4+11), normalized to EV-transduced donor at D4+4. (C) Fold change in the percentages of $CD41^+CD61^+$ (early) and $CD41^+CD42^+$ (late) megakaryocytes within the GFP^+ population at D4+4, D4+8, and D4+11, normalized to the corresponding donor's EV control at each time point. Representative flow cytometry plots at D4+4 are shown. (D) MFI of CD44 in GFP^+CD41^+ cells across all time points, normalized to donor EV control. Data in (B–D) are presented as mean ± standard deviation. Statistical analysis was performed using two-way ANOVA with Tukey's (B) or Šídák's (C–D) multiple comparisons test. Significance levels: *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001

consistent with a less mature megakaryocyte phenotype (Fig. 5D; Supplemental Fig. 8C). By day 4+11, when differentiation stages were comparable across all conditions, CD44 MFI was instead elevated in the ETV6-R369W group. This aligns with increased CD44 expression observed in ETV6-R369W-transduced Meg01 cells and patient-derived platelets (Fig. 3D and 5D; Supplemental Fig. 6 and 8). Collectively, these results demonstrate that the ETV6-R369W variant accelerates megakaryocyte differentiation and enhances CD44 expression in $CD34^{+}$-derived megakaryocytes.

DISCUSSION

ETV6 is an ETS family transcription factor that functions primarily as a transcriptional repressor. In ETV6 KO Meg01 cells, we observed upregulation of established ETV6 targets (*CLIC5*, *BIRC7*, *WBP1L*) and enrichment of genes involved in proliferation, adhesion, and migration, consistent with its reported roles in tumor suppression and immune regulation. Gene ontology analysis further revealed upregulation of inflammatory response pathways, including interferon-stimulated genes, aligning with ETV6's proposed role in repressing inflammatory signaling in hematopoietic cells.(19, 24, 25)

Megakaryocytes and HSCs share key transcriptional regulators, including the ETS family members FLI1, ETV6, and ERG, reflecting the capacity of HSCs to differentiate directly into megakaryocytes under stress conditions.(26-29) In mice, *Etv6* knockout causes yolk sac angiogenesis defects and loss of bone marrow hematopoiesis. Our previous work demonstrated high *ETV6* expression in iPSC-derived hemogenic endothelium,(30) and another study showed that it specifies HSCs in the dorsal aorta via VEGF signaling and supports their maintenance.(31) In line with this developmental role, ETV6 KO Meg01 cells displayed reduced endothelial features, including downregulation of *KDR* (*VEGFR2*), which was also decreased in the ETV6-R369W variant. Erythroid features were diminished in both KO and ETV6-R369W cells, likely due to FLI1 upregulation following loss of its negative regulation by ETV6, which was recently shown to bind to the FLI1 promoter. (25) This aligns with previous studies in cell lines, mice, and zebrafish, where ETV6 was shown to promote erythroid differentiation.(32-34) While patients in this study did not exhibit overt erythroid defects, other ETV6 mutations have been associated with abnormal erythropoiesis, suggesting a role for ETV6 in red cell differentiation.(4, 6)

Overexpression of ETV6-WT in KO Meg01 cells produced minimal changes in gene and protein expression, possibly due to the need for epigenetic reprogramming. Among the genes upregulated by ETV6, we identified EGR1, a transcription factor that recruits the DNA demethylase TET1 to activate downstream targets.(35) ETV6 binds the EGR1 promoter, and EGR1 expression differed between WT and homozygous (p.P214L) cells.(24, 25) Given that transcriptomic and proteomic analyses were performed only four days af-

ter transduction, additional time may have been required to observe epigenetic effects. In contrast, the immediate changes seen with ETV6-R369W revealed its dominant-negative function. Both ETV6 KO and ETV6-R369W cells showed upregulation of hematopoietic progenitor markers (KIT, CD44) and platelet activation markers (GP6, CXCR4), creating a conflicted lineage identity that disrupts megakaryopoiesis. Consistent with this, CD44 upregulation was observed in $CD34^+$ cultures and patient platelets, suggesting its potential as a biomarker for ETV6-related thrombocytopenia. Despite elevated platelet activation signatures in ETV6 KO cells, several genes essential for megakaryocyte maturation, proplatelet formation, and platelet function (*PTK2B*, *S1PR1*, *SLFN14*) were downregulated. These genes are critical for thrombopoiesis, as evidenced by severe thrombocytopenia in *S1pr1*-deleted mice and in SLFN14 mutation carriers, and by impaired platelet activation in *Ptk2b* deficient mouse.(36-38) Finally, whereas ETV6 KO Meg01 cells displayed increased proliferation, ETV6-R369W expression caused a rapid loss of GFP^+cells, indicating a growth disadvantage, which was also observed in ETV6 KO $CD34^+$ cells (data not shown).

ETV6-R369W has previously been shown to impair DNA binding and cause cytoplasmic mislocalization. Here, we demonstrate that ETV6 R369W dimerizes with ETV6-WT and shares common cofactors, reinforcing its dominant-negative effect. These include known interactors such as FLI1, whose transcriptional activity is inhibited by ETV6.(38, 39) and GAB2, a component of the Grb2-Gab2 complex that associates with the Tel-Abl fusion protein via Tyr314 of Tel.(40) We also identified additional transcription factors ERG, JUNB, and ERF, as well as AHR and NFKB2. Both ERG and JUNB are essential for endothelial and HSC development.(41-43) Bloom et al. demonstrated that genes adjacent to ETV6 binding sites are enriched for TNF-NFKB signaling pathways, and our identification of NFKB2 as an ETV6 interactor supports a direct role for ETV6 in repressing inflammatory signaling.(25) AHR-null mice display reduced platelet count and megakaryocyte ploidy.(44) Additional interactors included kinases (PLK1, SRC, TYK2) and ubiquitination-related proteins (e.g., USP25, TRIM21, NEDD4, CUL3, DCAF7, AMBRA1), several of which are directly linked to platelet biology. For example, PLK1 loss impairs platelet production,(45) and *Dcaf7* deletion in mice causes thrombocytopenia.(46)

In summary, ETV6-R369W exerts a dominant-negative effect through dimerization with ETV6-WT and sequestration of cofactors, leading to retention of progenitor characteristics and premature platelet activation. In $CD34^+$ cultures, the variant accelerates megakaryocyte differentiation.

ACKNOWLEDGMENT

This study was supported by the Dutch Scientific Research organization (NWO) in the framework of the NWA-ORC Call grant agreement NWA.1160.18.038 (SYMPHONY), and by the ZonMw TRACER consortium as part of the PSIDER program (grant number 10250022110001).

DATA AVAILABILITY

Sequencing data are available at GEO under accession number **GSE301595**. Proteomic data are available via ProteomeXchange with identifier PXD063984. Original data will be made available upon request.

REFERENCES

1. Wang LC, Kuo F, Fujiwara Y, Gilliland DG, Golub TR, Orkin SH. Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. Embo J. 1997;16(14):4374-83.
2. Hock H, Meade E, Medeiros S, Schindler JW, Valk PJM, Fujiwara Y, Orkin SH. Tel/Etv6 is an essential and selective regulator of adult hematopoietic stem cell survival. Gene Dev. 2004;18(19):2336-41.
3. Hock H, Shimamura A. in hematopoiesis and leukemia predisposition. Seminars in Hematology. 2017;54(2):98-104.
4. Noetzli L, Lo RW, Lee-Sherick AB, Callaghan M, Noris P, Savoia A, et al. Germline mutations in are associated with thrombocytopenia, red cell macrocytosis and predisposition to lymphoblastic leukemia. Nat Genet. 2015;47(5):535-U143.
5. Melazzini F, Palombo F, Balduini A, De Rocco D, Marconi C, Noris P, et al. Clinical and pathogenic features of -related thrombocytopenia with predisposition to acute lymphoblastic leukemia. Haematologica. 2016;101(11):1333-1342.
6. Poggi M, Canault M, Favier M, Turro E, Saultier P, Ghalloussi D, et al. Germline variants in underlie reduced platelet formation, platelet dysfunction and increased levels of circulating CD34 progenitors. Haematologica. 2017;102(2):282-94.
7. Lopez R, Carron C, Oury C, Gardellin P, Bernard O, Ghysdael J. TEL is a sequence-specific transcriptional repressor. J Biol Chem. 1999;274(42):30132-8.
8. Green SM, Coyne HJ, McIntosh LP, Graves BJ. DNA Binding by the ETS Protein TEL (ETV6) Is Regulated by Autoinhibition and Self-association. J Biol Chem. 2010;285(24):18496-504.
9. Wood LD, Irvin BJ, Nucifora G, Luce KS, Hiebert SW. Small ubiquitin-like modifier conjugation regulates nuclear export of TEL, a putative tumor suppressor. P Natl Acad Sci USA. 2003;100(6):3257-62.
10. Boccuni P, MacGrogan D, Scandura JM, Nimer SD. The human L(3)MBT polycomb group protein is a transcriptional repressor and interacts physically and functionally with TEL (ETV6). J Biol Chem. 2003;278(17):15412-15420.
11. Nakamura Y, Yamagata T, Maki K, Sasaki K, Kitabayashi I, Mitani K. TEL/ETV6 binds to corepressor KAP1 via the HLH domain. Int J Hematol. 2006;84(4):377-80.
12. Wang LL, Hiebert SW. TEL contacts multiple co-repressors and specifically associates with histone deacetylase-3. Oncogene. 2001;20(28):3716-25.
13. Chakrabarti SR, Nucifora G. The leukemia-associated gene TEL encodes a transcription repressor which associates with SMRT and mSin3A. Biochem Bioph Res Co. 1999;264(3):871-7.
14. Nordentoft I, Jorgensen P. The acetyltransferase 60 kDa -acting regulatory protein of HIV type 1-interacting protein (Tip60) interacts with the translocation E26 transforming-specific leukaemia gene (TEL) and functions as a transcriptional co-repressor. Biochem J. 2003;374:165-73.
15. Topka S, Vijai J, Walsh MF, Jacobs L, Maria A, Villano D, et al. Germline ETV6 Mutations Confer Susceptibility to Acute Lymphoblastic Leukemia and Thrombocytopenia. PLoS Genet. 2015;11(6):e1005262.
16. Zhang MY, Churpek JE, Keel SB, Walsh T, Lee MK, Loeb KR, et al. Germline ETV6 mutations in familial thrombocytopenia and hematologic malignancy. Nat Genet. 2015;47(2):180-5.
17. Bigot T, Gabinaud E, Hannouche L, Sbarra V, Andersen E, Bastelica D, et al. Single-cell analysis of megakaryopoiesis in peripheral CD34 cells: insights into ETV6-related thrombocytopenia. Journal of Thrombosis and Haemostasis. 2023;21(9):2528-44.

18. Moriyama T, Metzger ML, Wu G, Nishii R, Qian MX, Devidas M, et al. Germline genetic variation in and risk of childhood acute lymphoblastic leukaemia: a systematic genetic study. Lancet Oncol. 2015;16(16):1659-66.
19. Fisher MH, Kirkpatrick GD, Stevens B, Jones C, Callaghan M, Rajpurkar M, et al. germline mutations cause HDAC3/NCOR2 mislocalization and upregulation of interferon response genes. Jci Insight. 2020;5(18).
20. Faleschini M, Ammeti D, Papa N, Alfano C, Bottega R, Fontana G, et al. ETV6-related thrombocytopenia: dominant negative effect of mutations as common pathogenic mechanism. Haematologica. 2022;107(9):2249-54.
21. Iacono G, Abay A, Murillo JSG, Aglialoro F, Yagci N, Varga E, et al. Differentiating erythroblasts adapt to mechanical stimulation by upregulation of cholesterol biosynthesis via S1P/SREBP-induced HMGCR expression. Sci Rep-Uk. 2024;14(1).
22. Heshusius S, Grech L, Gillemans N, Brouwer RWW, den Dekker XT, van IWFJ, et al. Epigenomic analysis of KLF1 haploinsufficiency in primary human erythroblasts. Sci Rep. 2022;12(1):336.
23. Varga E, Hansen M, Akker E van den, et al. Erythropoiesis and Megakaryopoiesis in a Dish. Cell Culture. Published online November 5, 2018.
24. Zhou CJ, Uluisik R, Rowley JW, David C, Jones CL, Scharer CD, et al. Germline mutation promotes inflammation and disrupts lymphoid development of early hematopoietic progenitors. Exp Hematol. 2022;112:24-34.
25. Bloom M, Oak N, Baskin-Doer R, Feng RP, Iacobucci I, Baviskar P, et al. ETV6 represses inflammatory response genes and regulates HSPC function during stress hematopoiesis in mice. Blood Advances. 2023;7(18):5608-23.
26. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science. 2016;351(6269):139-+.
27. Sanjuan-Pla A, Macaulay IC, Jensen CT, Woll PS, Luis TC, Mead A, et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature. 2013;502(7470):232-+.
28. Carrelha J, Meng YR, Kettyle LM, Luis TC, Norfo R, Alcolea V, et al. Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. Nature. 2018;554(7690):106-+.
29. Manso BA, Medina P, Smith-Berdan S, Baena ARY, Bachinsky E, Mok L, et al. A rare HSC-derived megakaryocyte progenitor accumulates via enhanced survival and contributes to exacerbated thrombopoiesis upon aging. bioRxiv. 2024.
30. Zhang H, Hansen M, di Summa F, von Lindern M, Gillemans N, Ijcken WFJV, et al. LSD1/KDM1A and GFI1B repress endothelial fate and induce hematopoietic fate in induced pluripotent stem cell-derived hemogenic endothelium. Haematologica. 2024;109(12):3975-88.
31. Ciau-Uitz A, Pinheiro P, Gupta R, Enver T, Patient R. Tel1/ETV6 Specifies Blood Stem Cells through the Agency of VEGF Signaling. Dev Cell. 2010;18(4):569-78.
32. Eguchi-Ishimae M, Eguchi M, Maki K, Porcher C, Shimizu R, Yamamoto M, Mitani K. Leukemia-related transcription factor TEL/ETV6 expands erythroid precursors and stimulates hemoglobin synthesis. Cancer Sci. 2009;100(4):689-97.
33. Takahashi W, Sasaki K, Kvomatsu N, Mitani K. TEL/ETV6 accelerates erythroid differentiation and inhibits megakaryocytic maturation in a human leukemia cell line UT-7/GM. Cancer Sci. 2005;96(6):340-8.
34. Rasighaemi P, Onnebo SMN, Liongue C, Ward AC. ETV6 (TEL1) regulates embryonic hematopoiesis in zebrafish. Haematologica. 2015;100(1):23-31.
35. Sun ZX, Xu XG, He JL, Murray A, Sun MA, Wei XR, et al. EGR1 recruits TET1 to shape the brain methylome during development and upon neuronal activity. Nat Commun. 2019;10.

36. Canobbio I, Cipolla L, Consonni A, Momi S, Guidetti G, Oliviero B, et al. Impaired thrombin-induced platelet activation and thrombus formation in mice lacking the Ca-dependent tyrosine kinase Pyk2. Blood. 2013;121(4):648-57.
37. Zhang L, Orban M, Lorenz M, Barocke V, Braun D, Urtz N, et al. A novel role of sphingosine 1-phosphate receptor S1pr1 in mouse thrombopoiesis. J Exp Med. 2012;209(12):2165-81.
38. Kwiatkowski BA, Bastian LS, Bauer TR, Tsai S, Zielinska-Kwiatkowska AG, Hickstein DD. The family member Tel binds to the Fli-1 oncoprotein and inhibits its transcriptional activity. J Biol Chem. 1998;273(28):17525-30.
39. Kwiatkowski BA, Zielinska-Kwiatkowska AG, Bauer TR, Hickstein DD. The ETS family member Tel antagonizes the Fli-1 phenotype in hematopoietic cells. Blood Cell Mol Dis. 2000;26(1):84-90.
40. Million RP, Harakawa N, Roumiantsev S, Varticovski L, Van Etten RA. A direct binding site for Grb2 contributes to transformation and leukemogenesis by the Tel-Abl (ETV6-Abl) tyrosine kinase. Molecular and Cellular Biology. 2004;24(11):4685-95.
41. Loughran SJ, Kruse EA, Hacking DF, de Graaf CA, Hyland CD, Willson TA, et al. The transcription factor Erg is essential for definitive hematopoiesis and the function of adult hematopoietic stem cells. Nat Immunol. 2008;9(7):810-9.
42. Passegué E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell. 2004;119(3):431-43.
43. Licht AH, Pein OT, Florin L, Hartenstein B, Reuter H, Arnold B, et al. JunB is required for endothelial cell morphogenesis by regulating core-binding factor β. J Cell Biol. 2006;175(6):981-91.
44. Lindsey S, Papoutsakis ET. The aryl hydrocarbon receptor (AHR) transcription factor regulates megakaryocytic polyploidization. Brit J Haematol. 2011;152(4):469-84.
45. Trakala M, Partida D, Salazar-Roa M, Maroto M, Wachowicz P, de Cárcer G, Malumbres M. Activation of the endomitotic spindle assembly checkpoint and thrombocytopenia in Plk1-deficient mice. Blood. 2015;126(14):1707-14.
46. Melo-Cardenas J, Bezavada L, Cotton A, Crispino JD. DDB1 and CUL4 Associated Factor 7 (DCAF7) Is Essential for Hematopoiesis. Blood. 2022;140:8586-7.

SUPPLEMENTARY METHODS

Patient data and study design

This study was approved by the Medical Ethics Committee of Erasmus University Medical Center (MEC-2016-218) and conducted in compliance with the Medical Research Involving Human Subjects Act. Written informed consent was obtained from the patient in accordance with applicable national legislation. Clinical information, including medical history, bleeding phenotype, complete blood counts, and hemostatic evaluations, was extracted from electronic medical records.

Bleeding assessment

Bleeding symptoms were scored on the day of inclusion using the Bleeding Assessment Tool of the International Society on Thrombosis and Haemostasis (ISTH-BAT). This bleeding score has been validated for PDs.(1) It scores 13 specified bleeding items on absence (score 0) or presence and severity of bleeding (scores 1-4). The final bleeding score is the sum of all bleeding items. The cut-off values for an abnormal bleeding score is a total score of ≥3 in children <18 years.(2)

Laboratory tests for hemostatic function

At the time of inclusion, 26.2 mL of peripheral blood was collected by venipuncture and allocated as follows: 3.0 mL in EDTA tubes for complete blood counts, cell indices, and molecular analyses; 0.5 mL in serum tubes for thrombopoietin (TPO) measurement; and 22.7 mL in sodium-citrate tubes for coagulation and platelet function testing. Blood counts were performed using the Sysmex XN-9100 analyzer (Sysmex, Netherlands) and confirmed with a peripheral blood smear. Serum TPO was quantified using an in-house ELISA (Sanquin, Netherlands). Citrated plasma was used to assess activated partial thromboplastin time (aPTT; Actin FS), prothrombin time (PT; Thromborel S), and fibrinogen levels (Thrombin Reagent), all conducted on the Sysmex CS5100 platform (Siemens Healthcare, Germany). Platelet function analyzer (PFA-200) testing with the collagen-epinephrine cartridge (PFA-EPI; Siemens Healthcare) was considered normal if closure time was <150 seconds. Light transmission aggregometry (LTA) was performed using a Chrono-Log 490 aggregometer (Stago Benelux, Netherlands) with a panel of agonists: collagen (2 and 5 µg/mL), U46619 (4 µM), TRAP (40 µM), ADP (5 and 10 µM), epinephrine (5 µM), ristocetin (0.31 and 1.25 mg/mL), and arachidonic acid (1 µM). Aggregation responses were considered normal if the final amplitude reached ≥50% with weak agonists (ADP, epinephrine) and ≥70% with strong agonists (collagen, TRAP, high-dose ristocetin, arachidonic acid, U46619), in the absence of a second wave or reversibility, and <10% response to low-dose ristocetin.(1)

Molecular diagnosis

Genomic DNA was extracted using either the Gentra Puregene kit or the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. Targeted next-generation sequencing (tNGS) was performed using a custom-designed Ampliseq panel covering the coding regions of 73 genes implicated in platelet formation and function (Thermo Fisher Scientific, Waltham, MA).(3) Library preparation and sequencing were conducted on the Ion GeneStudio™ S5 system according to standard protocols. Variant calling and annotation were carried out using Ion Reporter software, and all detected variants were interpreted following the ACMG guidelines for classification.(4)

IVTsgRNA production and nucleofection

In Vitro Transcribed single guide RNAs (IVTsgRNAs) were synthesized in vitro following established protocols.(5) Briefly, DNA oligonucleotides containing a T7 promoter, crRNA sequence (Table 1), and a complementary overhang for tracrRNA were amplified by two-step PCR (98°C for 10 s, 68°C for 10 s) for 35 cycles. Transcription was performed using 5 µL of PCR product and HiScribe T7 kit (NEB) at 37°C for 20 hours. After DNase I treatment (37°C, 15 min; 80°C inactivation), RNA was purified with the NEB RNA Clean-Up Kit (T2040) and quantified by Nanodrop. IVTsgRNAs were diluted to 0.5 µg/µL and stored at –80°C. For genome editing, ~2×10^5 Meg01 cells were nucleofected with RNP complexes (3 µg Cas9 + 1 µg IVTsgRNA) and IDT enhancer (1:50) in a custom nucleofection buffer. (4) Electroporation was conducted using the LONZA 4D-Nucleofector (program EN-102). Cells were then cultured in RPMI 1640 with 10% FCS and 1% P/S in 48-well plates.

Polymerase Chain Reaction (PCR) and Sanger sequencing

PCR amplification was carried out using 5–50 ng of genomic DNA, gene-specific primers (Table 1), and Taq DNA Polymerase (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's protocol. Thermocycling was performed on a Veriti 96-Well Thermal Cycler with initial denaturation at 94°C for 3 minutes, followed by 35–40 cycles of 94°C for 45 seconds, annealing at 60–65°C for 30 seconds, and extension at 72°C for 60–90 seconds, with a final elongation step at 72°C for 10 minutes. PCR products were purified either enzymatically using Exonuclease I and Shrimp Alkaline Phosphatase (NEB) or via gel extraction using the NucleoSpin Gel and PCR Clean-up Kit (MACHEREY-NAGEL). Sanger sequencing was conducted with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) on an Applied Biosystems 3730 DNA Analyzer.

Generation of *ETV6* lentiviral construct

A DNA fragment encoding either ETV6-WT or ETV6^{R369W}, flanked by a 5′ Kozak sequence and an XbaI site and a 3′ HpaI site, was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Following bacterial transformation, plasmid DNA was extracted, digested with XbaI and HpaI, and the resulting FLI1 inserts were gel-

purified. These inserts were ligated into a LentiV2GFP empty vector (previously digested with the same enzymes) for lentiviral expression.(6) To generate N-terminal V5-tagged ETV6 constructs, PCR was performed using the lentiviral plasmids as templates. The forward primer introduced a V5 epitope tag upstream of the FLI1 start codon, along with a Kozak sequence and an XbaI site, while the reverse primer included the terminal coding sequence and an HpaI site. Amplified fragments were purified (NucleoSpin Gel and PCR Clean-up Kit, MACHEREY-NAGEL), digested with XbaI and HpaI, and cloned into the LentiV2GFP backbone.

Proteomics sample preparation and analysis

Immunoprecipitated (IP) Samples

IP beads were resuspended in 50 µL of 1 M urea and 100 mM Tris-HCl (pH 8; Invitrogen). Protein reduction was carried out using 10 mM dithiothreitol (DTT; Thermo Fisher Scientific) for 20 minutes at 25°C, followed by alkylation with 50 mM iodoacetamide in the dark for 10 minutes at the same temperature. On-bead digestion was initiated by adding 375 ng of MS-grade Trypsin-LysC (Thermo Fisher Scientific) and incubating for 2 hours at 25°C. The resulting supernatant was collected, and the beads were subsequently incubated again in 1 M urea with 10 mM DTT in 100 mM Tris-HCl (pH 8) for 5 minutes at 25°C. The supernatants from both steps were pooled and subjected to overnight digestion (18 h at 25°C) with an additional 150 ng of Trypsin-LysC. Digestion was stopped by adding 12 µL of 10% (v/v) trifluoroacetic acid (TFA; Thermo Fisher Scientific). A total volume of 75 µL of the peptide solution was loaded onto Evotip Pure columns according to the manufacturer's protocol.

Whole Cell Lysates

Cell pellets were lysed in a buffer containing 1% sodium deoxycholate (SDC; BioWORLD), 10 mM Tris(2-carboxyethyl)phosphine (TCEP; Thermo Fisher Scientific), 40 mM chloroacetamide (Sigma-Aldrich), and 100 mM Tris-HCl (pH 8; Life Technologies). Lysates were heated to 95°C for 5 minutes, cooled, and then sonicated for 10 minutes. Protein concentration was determined using a BCA assay (Thermo Fisher Scientific). For each sample, 10 µg of protein was digested overnight (18 h at 25°C) with Trypsin Gold (Promega) at a 1:50 enzyme-to-protein ratio. Samples were then acidified with TFA (final concentration 1%; Thermo Fisher Scientific) and centrifuged at 10,000 × g for 5 minutes to pellet the precipitated SDC. The resulting peptide-containing supernatants were transferred to clean tubes, and 500 ng of peptide material was loaded onto Evotip columns.

Peptides were separated using an Evosep One system operated in the preset 60 samples-per-day mode, with an 8 cm Performance Column (EV-1109; 150 µm inner diameter, 1.5 µm particle size). Mass spectrometry data were acquired on a timsTOF-HT instrument (Bruker Daltonics) using the dia-PASEF mode. Ion mobility accumulation and ramp times

were set at 100 ms. Each acquisition cycle consisted of 16 data-independent acquisition (DIA) windows spanning 0.7–1.5 $1/k_0$ and 421–1594 m/z. The size of each window was adjusted based on precursor ion density. Collision energy was applied as a linear function of ion mobility (20 eV at 0.6 $1/k_0$ and 59 eV at 1.60 $1/k_0$). Raw mass spectrometry data were analyzed using DIA-NN version 1.8.1. Protein and peptide identification was performed by querying the filtered human SwissProt database (release 2021.22.04) using a library-based spectral search with default settings. The maximum number of allowed variable modifications was set to two. Protein inference was based on "Protein names (from FASTA)," and quantification followed the "Robust LC (high precision)" strategy. Further data analysis was carried out in R (version 4.4.3) using RStudio (version 2024.12.1). Proteins were filtered to retain only proteotypic entries with at least two unique peptides and detected in at least 75% of samples within one experimental condition.

Label-free quantification (LFQ) intensities were $\log_2$-transformed. Missing values were imputed from a normal distribution (width = 0.3, shift = 1.8), assuming these values represented low-abundance proteins near the detection threshold. Differential expression analysis was conducted using the LIMMA package, applying Benjamini–Hochberg correction for multiple testing (adjusted p-value < 0.05) and a significance cutoff of $|\log_2$ fold change$| > 1$. Data visualization and further analysis were performed using R (v4.4.3) and RStudio (2024.12.1).

REFERENCES

1. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. Journal of Thrombosis and Haemostasis. 2013;11(6):1183-9.
2. Elbatarny M, Mollah S, Grabell J, Bae S, Deforest M, Tuttle A, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. Haemophilia. 2014;20(6):831-5.
3. Kreft IC, Huisman EJ, Cnossen MH, van Alphen FPJ, van der Zwaan C, van Leeuwen K, et al. Proteomic landscapes of inherited platelet disorders with different etiologies. J Thromb Haemost. 2023;21(2):359-72 e3.
4. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine. 2015;17(5):405-24.
5. Verhagen HJMP, Kuijk C, Rutgers L, Kokke AM, van der Meulen SA, van Mierlo G, et al. Optimized Guide RNA Selection Improves Cas9 Gene Editing of Human Hematopoietic Stem and Progenitor Cells. Crispr J. 2022;5(5):702-16.
6. Zhang H, Hansen M, di Summa F, von Lindern M, Gillemans N, Ijcken WFJV, et al. LSD1/KDM1A and GFI1B repress endothelial fate and induce hematopoietic fate in induced pluripotent stem cell-derived hemogenic endothelium. Haematologica. 2024;109(12):3975-88.

Supplementary Table S1 gRNAs and primers used in this study

Gene	gRNAs	Description	Sequence
ETV6	g-exon5#1	gRNA - antisense	ACAGAGGGTAGGACTCCTGG|TGG
	g-exon5#2	gRNA - antisense	GGAGCGGTGCAACAGTTCAA|TGG
	Primers		
	ex5-F	PCR primer - forward intron 4	TCTGTGATTGTCTTTCCCTCTG
	ex5-R	PCR primer - reverse exon 5	TCCCGATGAGAGAGGTTGAT

Supplementary Table S2

Only online

Legend (A) Differentially expressed genes (DEGs) between ETV6 wild-type (WT) and knockout (KO) clones. (B) Cell type enrichment for upregulated and downregulated DEGs in KO clones. (C) Gene set enrichment analysis (GSEA) of C8 cell type gene sets for DEGs between WT and KO clones. (D) KEGG pathway enrichment for upregulated genes in KO clones. (E) Gene ontology (GO) biological process enrichment for upregulated and downregulated genes in KO clones. (F) Label-free quantification (LFQ) protein values for WT and KO clones.

Supplementary Table S3

Only online

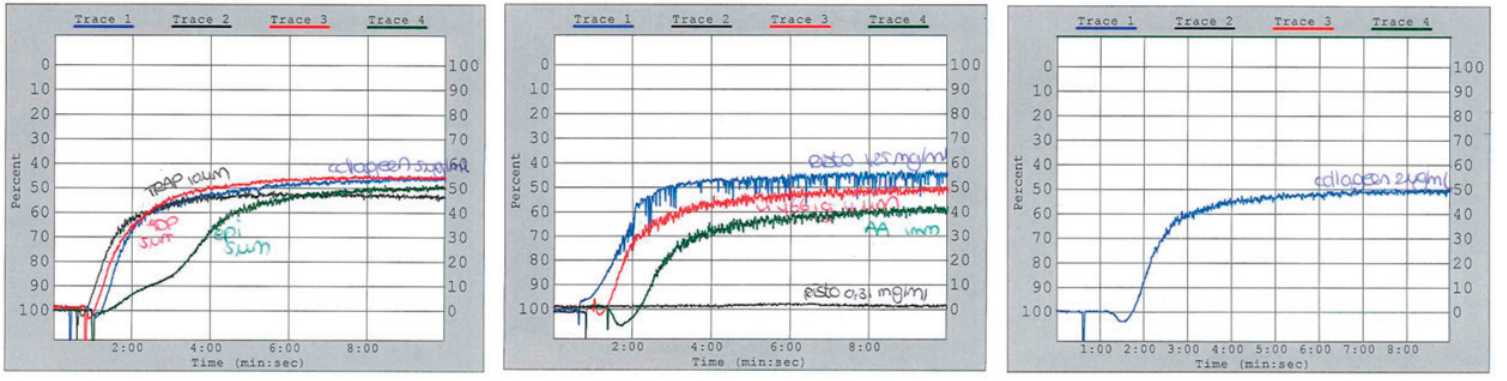
Legend (A) Normalized CPM values for WT and KO groups transduced with empty vector (EV), WT ETV6, or ETV6 p.R369W (mu) (WT_EV, WT_WT, WT_mu; KO_EV, KO_WT, KO_mu). (B) Clustering of DEGs within WT and KO groups after transduction with EV, WT ETV6, or ETV6 p.R369W. (C) KEGG pathway enrichment analysis for genes in Cluster 2 from panel B. (D) LFQ protein values for all WT and KO groups after transduction.

Supplementary Table S4

Only online

Legend LFQ protein values of ETV6-interacting proteins in KO cells transduced with WT ETV6 or ETV6 p.R369W, and from endogenous ETV6 immunoprecipitated in WT cells.

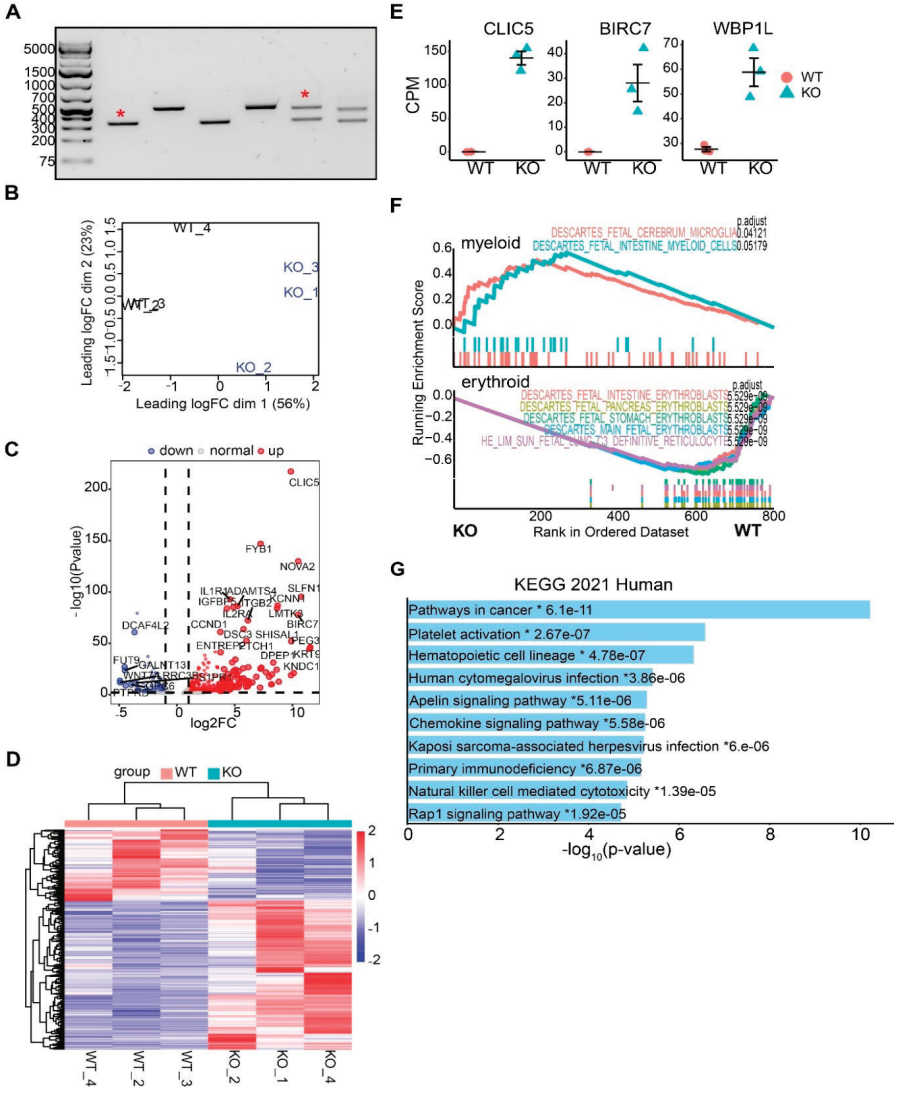
Supplementary Figure S1 Light transmission aggregometry results



Abbreviations AA = arachidonic acid, Epi = epinephrin, Risto = ristocetin

Legend Light transmission aggregometry (LTA) results for patient P1.2 showing adequate platelet function. Due to mild thrombocytopenia, PRP reached only 109 × 10^9/L (preferred ≥150 × 10^9/L), requiring cautious interpretation. Responses to ADP and epinephrine were normal (>50%), collagen, TRAP, ristocetin, and U46619 were in the acceptable range (50–60%; normally >70%), and arachidonic acid showed a weak response (40%; normally >70%)

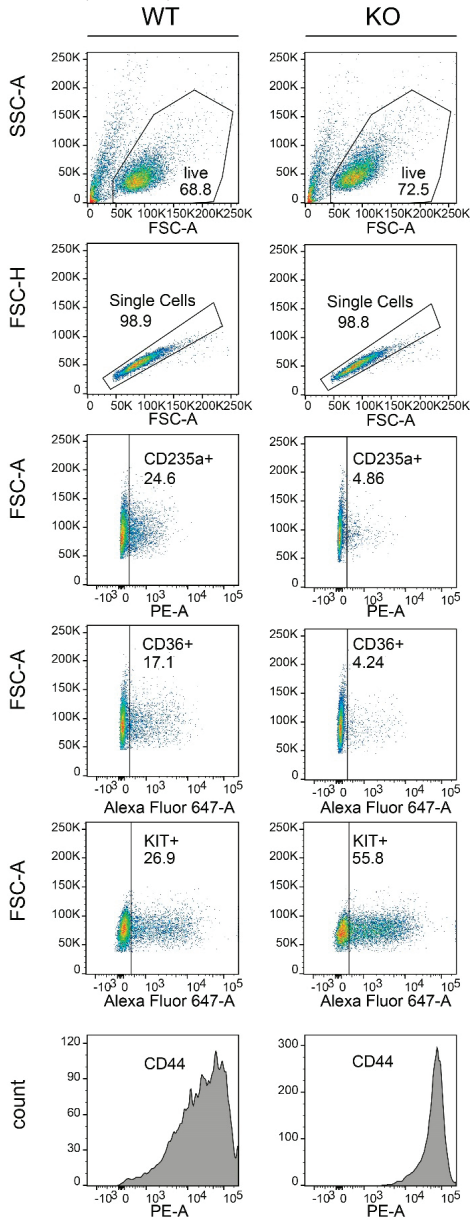
Supplementary Figure S2 Differential gene expression between ETV6 WT and KO clones



Abbreviations KO = knockout, WT = wild-type

Legend (A) PCR fragments spanning ETV6 exon 5 in CRISPR-edited clones, with KO1 and KO2 indicated. The upper WT-size band in KO2 contained a 2 bp insertion (confirmed by Sanger sequencing). (B) Western blot of ETV6 expression in four WT and four KO clones; RhoGDI served as a loading control. (C) Multidimensional scaling (MDS) of RNA-seq data for ETV6 WT and KO clones. (D) Volcano plot of DEGs (fold change >1.5, $p < 0.01$), with upregulated genes in red and downregulated genes in blue. (E) Heatmap of DEGs between WT and KO clones. (F) Normalized CPM values for *CLIC5*, *BIRC7*, and *WBP1L*. WT samples are shown as red circles; KO samples as blue triangles. (G) GSEA showing upregulation of myeloid and downregulation of erythroid signatures in KO cells. KEGG pathway enrichment for upregulated genes in KO cells

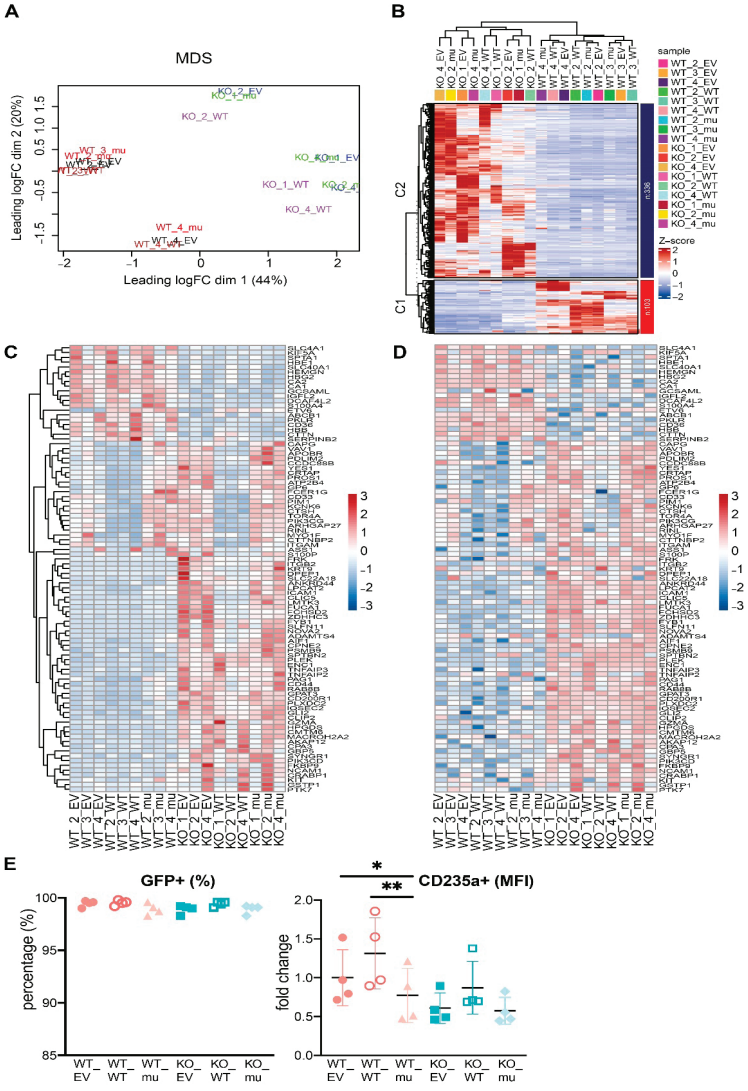
Supplementary Figure S3 Flow cytometry plots



Abbreviations KO = knockout, WT = wild-type

Legend Flow cytometry plots of CD235a, CD36, KIT, and CD44 expression in WT and KO ETV6 clones

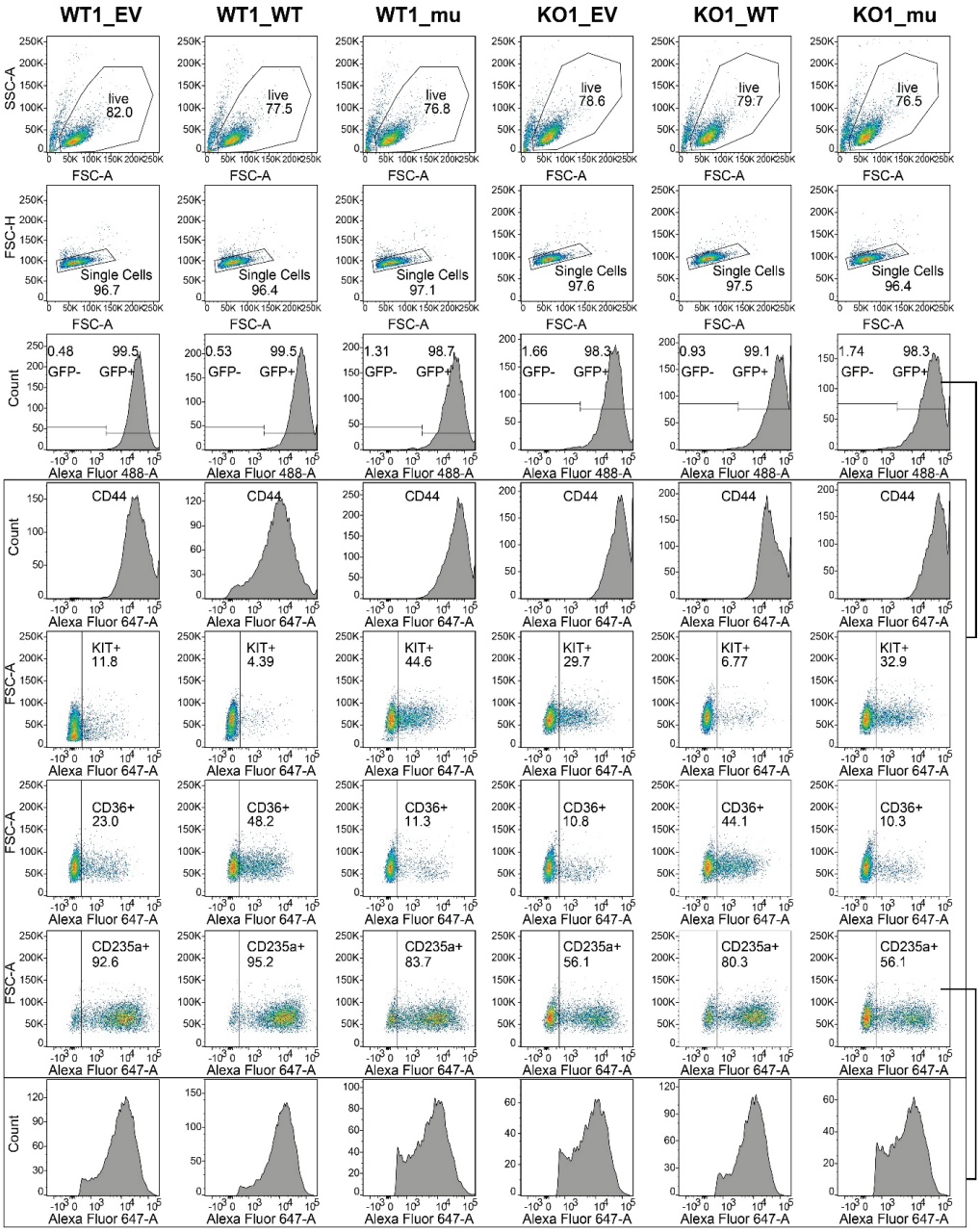
Supplementary Figure S4 ETV6 p.R369W expression in Meg01 cells recapitulates the KO phenotype



Abbreviations EV = empty vector, GFP = green fluorescent protein, KO = knockout, MDS = multidimensional scaling, MFI = mean intensity fluorescence, WT = wild-type

Legend (A) MDS analysis for WT and KO groups transduced with EV, WT ETV6, or ETV6 p.R369W (mu). (B) Heatmap of DEGs identified from WT_EV vs KO_EV, showing expression patterns across all transduced groups. (C) Heatmap of DEGs from Fig. 2B with detectable protein expression. (D) Heatmap of LFQ values corresponding to panel C, preserving sample and gene order. (E) Percentage of GFP$^+$ cells and CD235a (GFP$^+$) mean fluorescence intensity in all groups. Data represent mean ± standard deviation and were analyzed by two-way ANOVA followed by Tukey's post hoc test. Significance levels: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Symbols: WT_EV (red filled circles), WT_WT (red open circles), WT_mu (red filled triangles), KO_EV (blue filled squares), KO_WT (blue open squares), KO_mu (blue filled diamonds)

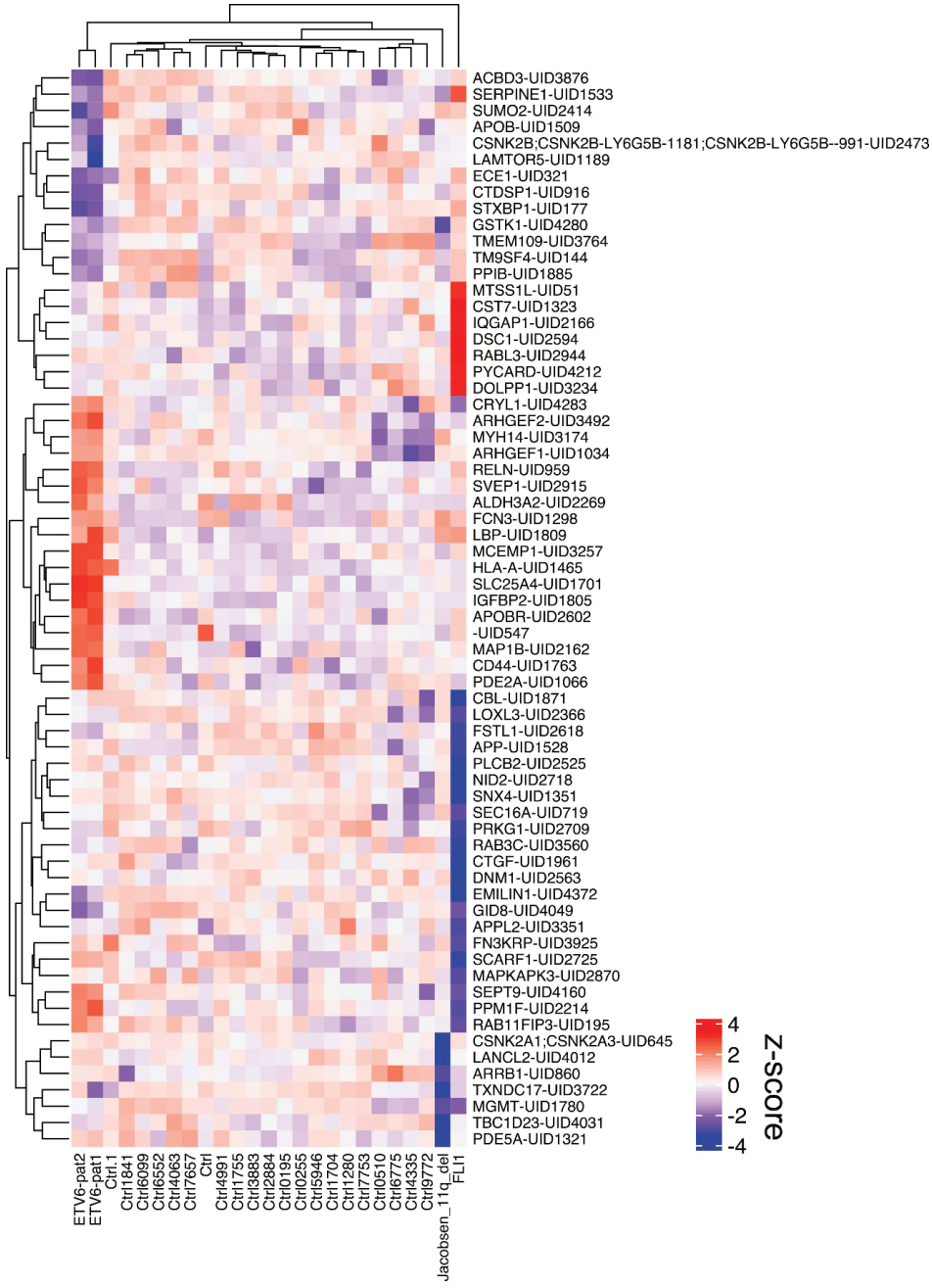
Supplementary Figure S5 Flow cytometry plots of CD235a, CD36, KIT, and CD44 expression in WT and KO ETV6 clones adding empty vector



Abbreviations CD = cluster of differentiation

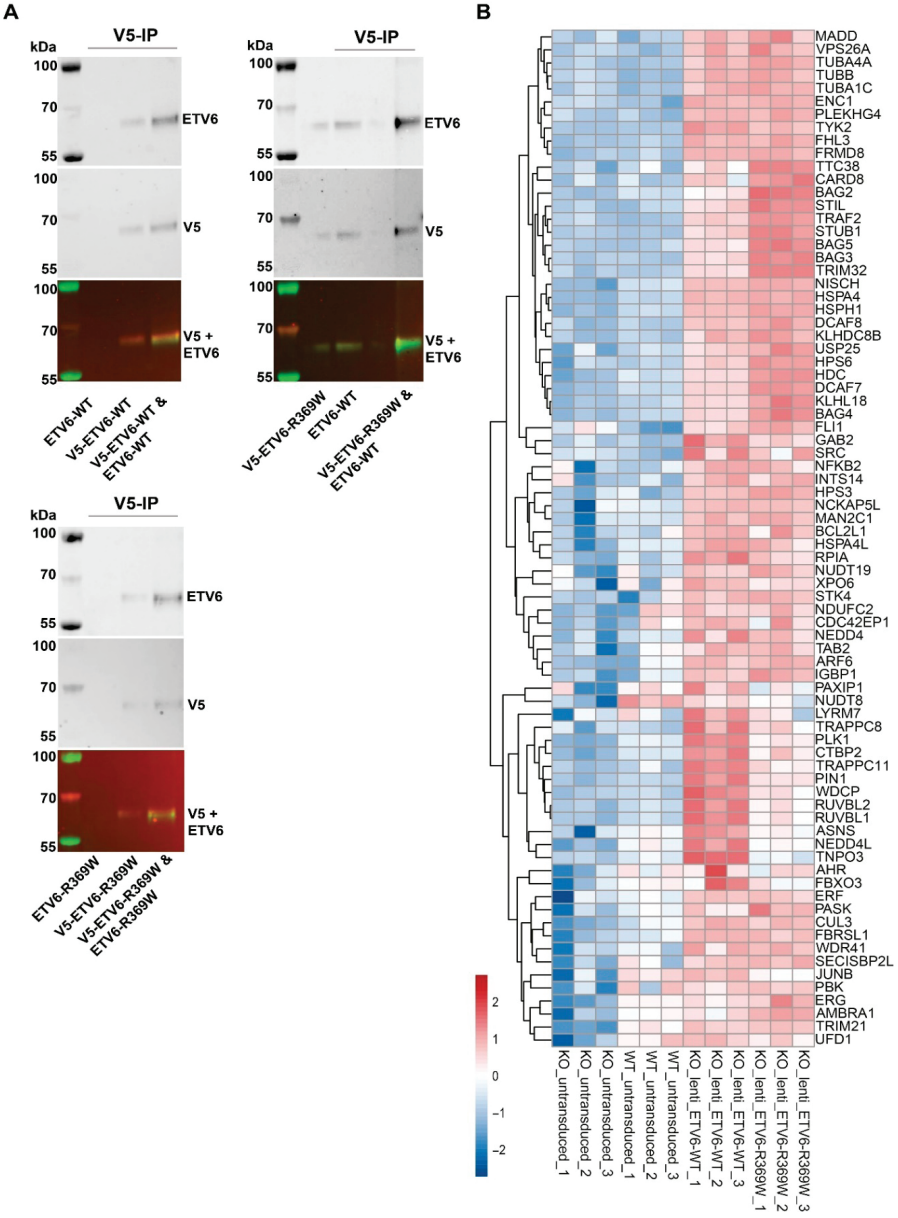
Legend Flow cytometry plots of CD235a, CD36, KIT, and CD44 expression in WT and KO ETV6 clones transduced with empty vector (EV), WT ETV6, or ETV6 p.R369W (mu) (WT_EV, WT_WT, WT_mu; KO_EV, KO_WT, KO_mu)

Supplementary Figure S6 ETV6 p.R369W interacts with WT ETV6 and shares protein cofactors



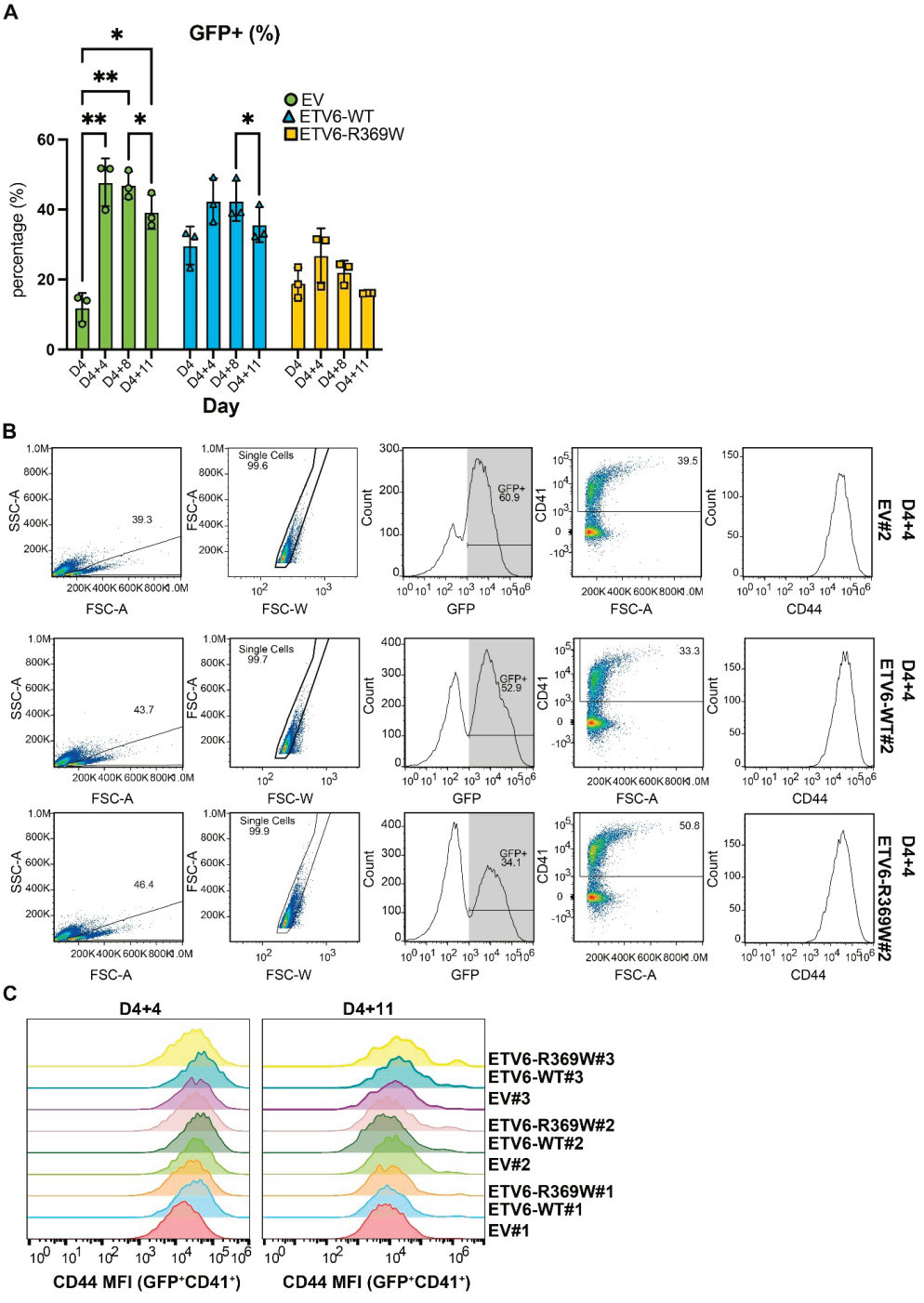
Legend (A) V5-tagged WT ETV6 (V5-ETV6-WT) or ETV6 p.R369W (V5-ETV6-R369W) were co-immunoprecipitated with ETV6-WT or ETV6-R369W using an anti-V5 antibody and detected by western blotting with anti-V5 (IRDye 680RD, red) and anti-ETV6 N-terminal (IRDye 800CW, green) antibodies. (B) IP-MS of proteins co-precipitating with ETV6 in KO cells transduced with ETV6-WT or ETV6-R369W

Supplementary Figure S7 ETV6 p.R369W expression in CD34^{+} cells accelerates megakaryocyte differentiation



Legend (A) Percentage of GFP^{+} cells at D4, D4+4, D4+8, and D4+11. Data shown as mean ± SD, two-way ANOVA with Tukey's multiple comparisons test. Significance: *P < 0.0332, **P < 0.0021, ***P < 0.0002, ****P < 0.0001. Symbols: EV (green filled circles), ETV6-WT (blue filled triangles), ETV6-R369W (yellow filled squares) (B) Representative D4+4 flow cytometry plots (donor 2) for EV, ETV6-WT, and ETV6-R369W showing gating strategy. (C) CD44 mean fluorescence intensity in GFP^{+}CD41^{+} cells on D4+4 and D4+11 for all donors.

Supplementary Figure S8



Research means "to search again". Why not?
Sometimes, a new interpretation emerges that is of vast importance

Isaac Asimov (1920-1992 AD)

PART II

THERAPEUTIC DILEMMAS

Prevention is better than cure

Desiderius Erasmus (1466 – 1536 AD)

9

Implementing HLA and HPA-matched platelet transfusions for patients with Glanzmann and Bernard-Soulier syndrome as standardized care? A Dutch survey and recommendations

E.J. Huisman
N. Holle
M. Schipperus
M.H. Cnossen
M. de Haas
L. Porcelijn
J.J. Zwaginga

Transfusion 2024;64(5):824-838

ABSTRACT

Background

Glanzmann thrombasthenia (GT) and Bernard-Soulier syndrome (BSS) patients require frequent platelet transfusions and hence have an increased risk for alloimmunization against donor Human Leukocyte Antigens (HLA) when no HLA-matching is performed. Knowing that Human Platelet Antigens (HPA) are located on the platelet glycoproteins that can be absent in these patients, preventive HPA-matching may also be considered. Uniform recommendations on this topic lack in transfusion guidelines making standard practice unclear, therefore, we aimed to provide a framework for matched platelet transfusions.

Study design and methods

We conducted a targeted literature search and a national survey of Dutch (pediatric) hematologists from July to September 2021.

Results

We found 20 articles describing platelet transfusion policies in 483 Glanzmann thrombasthenia patients and 29 BSS-patients, both adults and children. Twenty surveys were returned for full analysis. All responders treated patients with platelet disorders, including Glanzmann thrombasthenia (n=36 reported) and BSS (n=29 reported). Of respondents, 75% estimated the risk of antibody formation as 'likely' for HLA and 65% for HPA. Formation of HLA antibodies was reported in 5 Glanzmann thrombasthenia and in 5 BSS patients, including one child. Fifteen respondents gave preventive HLA matched platelets in elective setting (75%). Three respondents additionally matched for HPA in Glanzmann thrombasthenia patients (15%). Main argument for matched platelet transfusions was preventing alloimmunization to safeguard the effectivity of 'random' donor-platelets in acute settings.

Conclusion

Elective HLA matching for Glanzmann thrombasthenia and BSS-patients is already conducted by most Dutch (pediatric) hematologists. HPA matching is mainly applied when HPA antibodies are formed. Based on the current literature and the survey, recommendations are proposed.

Keywords

Antibody formation [MESH]; antigens, human platelet [MESH]; Bernard-Soulier syndrome [MESH]; blood platelet disorders [MESH]; Glanzmann thrombasthenia [MESH]; Human antigens, platelet [MESH]; HLA antigens [MESH]; platelet transfusion [MESH];

INTRODUCTION

Patients with inherited platelet disorders (IPD) require platelet transfusions for the treatment of acute bleeding or for the prevention of major bleeding during surgery and child delivery.(1, 2) Repetitive platelet transfusions may lead to alloimmunization against human leucocyte antigens (HLA) or human platelet antigens (HPA). In this regard, IPD-patients with the specific IPD-diagnoses Glanzmann Thrombasthenia (GT) and Bernard-Soulier syndrome (BSS) are considered a high-risk group for alloimmunization. (3, 4) Glanzmann thrombasthenia is characterized by either a dysfunctional or (partial) absence of the glycoprotein (GP)-complex IIb/IIIa, also named αIIbβ3 integrin or CD41/CD61.(5, 6) This complex is essential in the platelet-fibrinogen binding.(6) Bernard-Soulier syndrome is caused by either a dysfunctional or (partial) absent GP-complex Ib/IX/V, also named CD42a/CD42b.(7) This complex is essential for the platelet-von Willebrand factor (VWF) binding. Although hemostatic treatments as tranexamic acid, desmopressin and for Glanzmann thrombasthenia patients also recombinant factor VII exist, platelet transfusions are often inevitable during life.(8) Both Glanzmann thrombasthenia and Bernard-Soulier syndrome are categorized by flowcytometric GP-expression in 3 subtypes: in type 1 glycoproteins are absent or have less than 5% of the normal expression, in type 2 expression is ≥5-25% of normal range and in type 3 expression is ≥25-100%; but although sometimes normally expressed, glycoproteins may be dysfunctional in type 2 and 3.

Risk and incidence of HLA antibodies in Glanzmann thrombasthenia and Bernard-Soulier syndrome

Because platelets carry HLA-class I and HPA, there is a general risk of alloimmunization against non-self (donor) HLA-class I and HPA. The incidence of HLA antibody formation is further modulated by the number of platelet transfusions, remaining leucocytes in the blood products, individual immune characteristics of the receiver and potential antigen mismatch between donor and receiver.(9) When HLA antibodies are formed, platelet refractoriness and moderate to severe transfusion reactions may occur.(3) No large datasets are currently available on the incidence of HLA alloimmunization in Glanzmann thrombasthenia or Bernard-Soulier syndrome patients. Results from a number of small cohort studies with Glanzmann thrombasthenia and Bernard-Soulier syndrome patients, demonstrate a broad incidence of HLA antibodies formation of 9 to 24%.(1, 10-13)

Risk and incidence of HPA antibodies in Glanzmann thrombasthenia and Bernard-Soulier syndrome

Although 41 HPA antigens are known,(14) five HPA's are regarded as most clinically relevant due to their allele frequencies in Caucasian population, expression levels and immunogenicity: HPA-1a, 1b, 2b, 3a and 5b.(15) Immunization against these antigens and subsequent production of antibodies may lead to platelet transfusion refractoriness,

post transfusion purpura and feto-neonatal alloimmune thrombocytopenia.(16) For Glanzmann thrombasthenia and Bernard-Soulier syndrome patients these may be even more relevant, as these HPA antigens are located on the glycoprotein-complexes that are largely or completely absent on Glanzmann thrombasthenia and Bernard-Soulier syndrome platelets.(6, 7, 14, 17, 18) (See Table 1) Theoretically, Glanzmann thrombasthenia and Bernard-Soulier syndrome patients may therefore have a higher risk of HPA antibody formation compared with the general population. In daily practice risk assessment depends on additional factors such as remaining expression of HPA antigens on other tissues than platelets, as is the case if Glanzmann thrombasthenia is caused by mutations leading to absent expression of GPIIb. In genotypically HPA-1a positive patients, HPA-1a will still be expressed as vitronectin receptor (α_v/β_3) on endothelium and other blood cells.(2, 14, 19, 20) Reported incidences of HPA antibodies range from 2.5 to 9% in small case-series.(12, 21)

Table 1 HPA-antigens on platelet glycoproteins involved in Glanzmann thrombasthenia and Bernard-Soulier syndrome

HPA-scystem	HPA-antigen	Original terminology	Relevant	Glycoprotein	CD	Platelet disease	Antigen frequency*
HPA-1	HPA-1a	Zwa, PIA1 Zwb	+	GPIIIa	CD61	Glanzmann	98%
	HPA-1b	PIA2	+				28%
HPA-2	HPA-2a	Kob Koa	-	GPIb	CD42b	BSS	99%
	HPA-2b	Siba	-				16%
HPA-3	HPA-3a	Baka,	+	GPIIb	CD41	Glanzmann	84%
	HPA-3b	Leka Bakb	+				63%
HPA-4	HPA-4a	Yuka, Pena	-	GPIIIa	CD61	Glanzmann	100%
	HPA-4b	Yukb, Penb	+				<1%
	HPA-6b	Caa, Tua	-	GPIIIa	CD61	Glanzmann	<1%
	HPA-7b	Moa	-	GPIIIa	CD61	Glanzmann	<1%
	HPA-8b	Sra	-	GPIIIa	CD61	Glanzmann	<1%
	HPA-9b	Maxa	-	GPIIb	CD41	Glanzmann	<1%
	HPA-11b	Groa	-	GPIIIa	CD61	Glanzmann	<1%
HPA-15	HPA-16b	Duva,	-	GPIIIa	CD61	Glanzmann	<1%
	17b, 19b	Vaa, Sta					
	21b, 23b	Nos, Hug					
	26b, 29b	Seca, Khab					
	32b, 33b	Domb, Bla					
	34b, 35b	Bzha, Efsa					
	HPA-20b	Kno	-	GPIIb	CD41	Glanzmann	<1%
	22b, 24b	Sey, Cab2a+					
	27b, 28b 30b	Cab3a+, War Laba					
	HPA-31b	Cab4b+	-	GPIX	CD42a	BSS	<1%

Abbreviations BSS = Bernard-Soulier syndrome, CD = Cluster of Differentiation, Glanzmann = Glanzmann thrombasthenia, GP = Glycoprotein, HPA = Human Platelet Antigen

Legend *Prevalence measured in cohort with Caucasian ethnicity

Risk of developing iso-antibodies in Glanzmann thrombasthenia and Bernard-Soulier syndrome

Glanzmann thrombasthenia and Bernard-Soulier syndrome patients may develop iso-antibodies directed against common epitopes on the GP as well.(22) Such iso-antibodies appear as (pan-)positive results in platelet antibody tests such as the Monoclonal Antibody-specific Immobilization of Platelet Antigen (MAIPA) with no apparent specificity. All donor platelets will be recognized by these iso-antibodies and no matching strategy can be performed to prevent refractoriness. It is not known if prevention of HLA or HPA antibody formation has a role in prevention of iso-antibodies. But transfusions with HLA and HPA matched platelets may be useful to prevent HLA and HPA antibody formation. To date, no recommendations are given in the national guidelines in the Netherlands for platelet transfusion management in these patient groups. Therefore, we performed a targeted literature search to create an overview of current knowledge. Secondly, we investigated how Dutch hematologists apply HLA and/or HPA matched platelets in pediatric and adult Glanzmann thrombasthenia and Bernard-Soulier syndrome patients. We subsequently combined the survey results with current evidence from literature recommending a practical approach.

MATERIALS AND METHODS

Literature search

We conducted a targeted literature search, using the following search terms "Glanzmann, Glanzmann Thrombasthenia, Bernard-Soulier syndrome, inherited platelet (function) disorders, congenital platelet (function) disorders, human leucocyte antigen, Human platelet antigen, (platelet) refractoriness" in the PubMed database. Only articles that were available in English as full text and involved Glanzmann thrombasthenia and Bernard-Soulier syndrome patients were analyzed.

Data collection

The survey was conducted among (pediatric) hematologists in the Netherlands specialized in the management of IPDs and blood transfusion management. The survey was designed by one investigator (EH) and peer-reviewed in two rounds by two other investigators (JZ and MS). Our survey was forwarded digitally via an online questionnaire (www.survio.com) program to all seven Dutch Hemophilia Treatment Centers as well as several working parties (WP): WP of the pediatric hematologists of the Dutch Society for Pediatrics (NVK), WP of benign hematologists of the Dutch Society for Transfusion (NVB) and the association of Dutch Hemophilia doctors (NVHB). The survey was completed either by individual practitioners or collectively by cooperating physicians working in the same medical center.

Data analysis

We have used descriptive statistics for the survey results, using total number and percentage if indicated.

RESULTS

Literature

We found 20 articles describing a total of 483 Glanzmann thrombasthenia patients and 29 Bernard-Soulier syndrome patients, both adults and children.(2, 11-13, 21-36), See Table 2) Two publications focused on pregnant Glanzmann thrombasthenia and Bernard-Soulier syndrome patients and neonatal outcome.(12, 13) Presence of HLA antibodies in eight case-series and one review of ≥5 patients were reported in 49/265 (18.5%) pediatric and adult Glanzmann thrombasthenia patients (range 6.5-86%),(2, 11, 12, 21-25, 31) and in one case-series of 3/18 Bernard-Soulier syndrome patients (17%).(13) It was often not clear if the use of non-leukocyte-reduced platelet transfusions were additional risk factors for the development of HLA antibodies, so this was left out of the overview in Table 2. The impact of the HLA antibodies causing platelet refractoriness and leading to additional treatment was not always reported and ranged from 0 till 33%.(11, 23-26, 29)

Presence of HPA antibodies in Glanzmann thrombasthenia patients were reported in one case-series and one case-report.(2) In the case-series, five out of seven French Gipsy Glanzmann thrombasthenia patients had a genetic variant in the ITGA2B gene (coding for integrin α2b or CD41).(2) This variant is strongly linked to a homozygous HPA-1bb genotyping. In the one case-report from 1989 a combination of HPA-1a and HPA-1b antibodies were detected in a pregnant Glanzmann thrombasthenia patient. However, it is likely that this antibody pattern would currently be classified as iso-antibodies, for discrimination between iso-antibodies and HPA antibodies was not performed or technically not possible then.(12) The other case-series do not mention HPA antibody testing.

Table 2 Overview of literature on occurrence of HLA and HPA-antibodies in patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome according to sample size

Journal	**Patient and disease characteristics, n(%)**			**Risk factors for antibody formation**			**Reported incidences in exposed group, n(%)**			**Clinical outcome, n(%)**	
First Author (year)	IPD	Number; (% females)	IPD subtype*	Pregnancies	Platelet transfusion product / LR	Platelet transfusion frequency	HLA Abs	HPA Abs	Iso Abs**	Platelet refractoriness	Neonatal outcome
		Case-series Glanzmann Thrombasthenia									
Zotz (2019)[22] GT registry	GT	131 children (46%)	Type I: 35 Type II: 13 Type III: 3 Variant: 3 Unknown: 80	na (child)	Platelets nos, LR nm	nm	3/46 (6,5%)[‡]	Nm	20/46 (44%)	1/3 (33%)	na
Poon (2015)[23] GT registry	GT	96 adults and children (54%)	Type I: 48 Type II: 6 Type III: 1 Unknown: 41	nm	Platelets nos, LR nm	nm	16/96 (17%)	nm	35/96 (37%)	23/96 (24%)	nm
Poon (2004)[24] Survey	GT	59 adults (59%)	Type I: 39 Type II: 7 Type III: 3 Unknown: 10	nm	Platelets nos, LR nm	nm	13/54 (24%)[‡‡]	nm	21/54 (39%)	17/59 (29%)	nm
Siddiq (2011)[12] Review	GT	35; adult (100%)	Unknown	40	SDP n=6; HLAm n=7; platelets nos n=27; WB in n=1; LR nm	Table 1: 1-≥120 in n=38; n=1 history n.m., n=1 not given	8/35 (23%)	1/40 (2.5%) HPA1a and 1b)[†]	9/35 (26%)	nm	IVIG in 3 pregnancies Plasma-exchange in 2 pregnancies 6 healthy neonates. 4 IU deaths due to ICH 1 IU death after amniocentesis 1 conjunctival bleeding 3 FNAIT, 1 marked bruises

Table 2 Overview of literature on occurrence of HLA and HPA-antibodies in patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome according to sample size (*continued*)

Journal	**Patient and disease characteristics, n(%)**			**Risk factors for antibody formation**			**Reported incidences in exposed group, n(%)**			**Clinical outcome, n(%)**	
First Author (year)	IPD	Number; (% females)	IPD subtype*	Pregnancies	Platelet transfusion product / LR	Platelet transfusion frequency	HLA Abs	HPA Abs	Iso Abs**	Platelet refractoriness	Neonatal outcome
Fiore (2012)[21]	GT	24; adults and children (58%)	Type I: 16 (Manouche) Type I: 5 Type II: 2 Variant: 1	3	Platelets nos, LR nm	nm	nm	nm	Manouche: 13/16 (81%) Other: 2/8 (25%)	nm	nm
Santoro (2010)[11]	GT	17; adults (53%)	Type I: 14 Type III: 3	7	Platelet concentrate multiple donors; 1 transfusion = defined as 1 unit/10kg; LR nm	Table 1: 4-335 units (n=14, n=2 not given)	3/14 (219%)	nm	2/14 (14%) ‡‡	0/4 (0%)	5 live births, 1 voluntary abortion, 1 early abortion at 13 wks. No bleeding complications
Fiore (2021)[2]	GT	7; adults and children (100%)	type I: 6 (Manouche) type I: 1 (other)	0 (n=6)	Platelets nos, LR nm	nm (n=6, 1 unknown)	1/7 (14,3%)	5/7 anti-HPA-1a (71%)	7/7 (100%)	nm	Na
Friend (2020)[25]	GT	2; children (50%)	Unknown	na (child)	Platelets nos, LR nm	nm (n=2)	0/1 (0%)	nm	0/1 (0%)	0/1 (0%)§§	na
		Case-reports Glanzmann Thrombasthenia									
Soni (2019)[26]	GT	1; adult woman	Type I: 1	1	1 unit SDP; others nm; LR nm	≥8 units	n.m.	nm††	1 (100%)	0/1 (0%)	1 IU death due to ICH
Cid (2017)[27]	GT	1; adult woman	Unknown	0	Platelets nos, LR nm	nm	1/1 (100%)	Nm	1/1 (100%)	nm	na due to hysterectomy due to heavy menstrual bleeding < age of 20 yrs

Table 2 Overview of literature on occurrence of HLA and HPA-antibodies in patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome according to sample size (*continued*)

Journal	**Patient and disease characteristics, n(%)**			**Risk factors for antibody formation**			**Reported incidences in exposed group, n(%)**			**Clinical outcome, n(%)**	
First Author (year)	IPD	Number; (% females)	IPD subtype*	Pregnancies	Platelet transfusion product / LR	Platelet transfusion frequency	HLA Abs	HPA Abs	Iso Abs**	Platelet refractoriness	Neonatal outcome
Léticée (2005)[28]	GT	1; adult woman	Type 1: 1	2	Platelets nos, LR nm	nm	1/1 (100%)	nm	1/1 (100%)	0/1 (0%)§	1st pregnancy early abortion 2nd pregnancy IU death due to ICH
Ito (1991)[29]	GT	1; adult woman	Unknown	1	WB and HLAm, LR nm	≥15 units	1/1 (100%)	nm	1/1 (100%)	0/1 (100%)	1 healthy neonate (after antibody removal therapy)
		Case-series combined Glanzmann Thrombasthenia and Bernard-Soulier syndrome									
Gabe (2022)[20]	GT	11; adults (82%)	Type 1: 6 Variant: 3 Unknown: 2	3	Platelet concentrate (MD), LR applied	3-521 units	1/11 (9%)	n1/11 (9%)	1/11 (9%)¶	Not reported, 3 transfusion reactions (TRALI, allergy, NHTR)	Nm
	BSS	4; adults (100%)	Unknown: 4	0		0-28 units	1/4 (25%)	nm			
Almeida (2003)[30]	GT	5; children (?%)	Type I: 4 Type III: 1	na (child)	Unknown, LR nm	1-4 (n=3); 0 units	4/5 (80%)¶¶	0/5	0/3 (0%)	n.m. 3 with transfusion reactions (NHTR)	na
	BSS	1; child (?%)	Unknown: 1	na (child)			1/1 (100%)	0/1			na

Table 2 Overview of literature on occurrence of HLA and HPA-antibodies in patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome according to sample size (*continued*)

Journal	**Patient and disease characteristics, n(%)**			**Risk factors for antibody formation**			**Reported incidences in exposed group, n(%)**			**Clinical outcome, n(%)**	
First Author (year)	IPD	Number; (% females)	IPD subtype*	Pregnancies	Platelet transfusion product / LR	Platelet transfusion frequency	HLA Abs	HPA Abs	Iso Abs**	Platelet refractoriness	Neonatal outcome
		Case-series Bernard-Soulier syndrome									
Peitsidis (2010)*13* Systematic review	BSS	18; adult (100%)	Unknown	30	HLAm + platelets nos, n=1; SPD n=1; platelets nos n=10; blood nos n=1; LR nm	nm	3/10 (30%)	nm	7/10 (70%)	nm	IVIG in 5 pregnancies. 1 IU death due to GI-bleeding; 1 neonatal death <6h due to ICH; 4 neonates: FNAIT 18 neonates: normal platelet counts
Uotila (2008)*31*	BSS	2; adult (100%)	Unknown	6	Unknown, LR nm	nm	1/2 (50%)	n.m. in 1, excluded in the other	1/2 (50%)	nm	IVIG 1x/week in both pregnancies. 1st pregnant woman: normal neonatal platelets in 3; 2nd pregnant woman: FNAIT and bleeding nos in 3
		Case-reports Bernard-Soulier syndrome									
Pascual (2011)[35]	BSS	1; adult woman	Unknown	2	HLAm, LR assumed	nm	1/1 (100%)	n.m.	0/1 (0%)	1 (100%)	2 neonates with normal platelet counts
Sandrock (2010)[32]	BSS	1; adult man	Type 1: 1	na (male)	Unknown, LR nm	nm	1/1 (100%)	nm	nm	nm	na
Fujimori (1999)[33]	BSS	1; adult woman	Unknown	nm	Unknown, LR nm	nm	nm	nm	1/1	nm	1 neonatal death 6h after delivery due to ICH and FNAIT

Table 2 Overview of literature on occurrence of HLA and HPA-antibodies in patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome according to sample size (*continued*)

Journal	Patient and disease characteristics, n(%)			Risk factors for antibody formation			Reported incidences in exposed group, n(%)			Clinical outcome, n(%)	
First Author (year)	IPD	Number; (% females)	IPD subtype*	Pregnancies	Platelet transfusion product / LR	Platelet transfusion frequency	HLA Abs	HPA Abs	Iso Abs**	Platelet refractoriness	Neonatal outcome
Peng (1991)[34]	BSS	1; adult woman	Unknown	4	Unknown, LR nm	nm	nm	nm	1/1	nm	1st neonate healthy, 2nd neonate IU death due to GI-bleeding and FNAIT, 3rd FNAIT, 4th, normal platelet count after weekly IVIG in 4th pregnancy

Abbreviations BSS = Bernard-Soulier Syndrome; FNAIT = feto-neonatal allo-immune thrombocytopenia; GI = gastro-intestinal; GT = Glanzmann thrombasthenia; ICH = intracranial hemorrhage; h = hours; HLA = Human leucocyte antigen; HLAm = Human leucocyte antigen-matches platelets IU = intra-uterine; IPD = Inherited Platelet Disorder; IVIG = intravenous immunoglobulines; MD = multiple donor; n = number; na = not applicable; NHTR = Non-hemolytic transfusion reaction; nm = not mentioned; nos = not otherwise specified; PC = (random) platelet concentrate; SDP = single donor platelet transfusion; TRALI = transfusion-related acute lung injury; WB = whole blood transfusion.

Legends *Type 1 is defined as <5% glycoprotein expression compared to wild type, type 2 as 5-25% versus wild-type, type III as 25-100% versus wild-type, also called variant; **anti-αIIbβ3 for GT patients; anti-GPIb/IX for BSS patients; § platelet transfusions were avoided because of iso-antibody presence, no refractoriness reported in early years; §§ not mentioned in the second case; ¶ mentioned as HPA-antibody in the article, but later described as anti-αIIbβ3); 7 children were included, but one patient had a storage pool defect and is excluded in this table. In the article 6/7 including the patient with SPD had developed HLA antibodies after transfusion.; ‡ International registry in Asia, Africa, Europe and North America. In 25 children antibodies were reported but only in 3 patients this was determined as HLA-antibodies with refractoriness in 1/3. Therefore, we have only included this number in the table. ‡‡HLA antibody tests were not known in all included patients; † anti-PLA is the former name of anti-HLA-1; †† incompatibility of HPA-15 ab between parents was shown, but presence of anti-HPA-15b was not examined; *** some women received both HLA-matched en platelets nos or SPD and platelets nos Also, no information on platelet product selection was given on historically transfused women.

Neonatal complications resembling FNAIT were also reported in Glanzmann thrombasthenia patients, ranging from postnatal low platelet numbers (n=3) and mild bleedings as mucosal, conjunctival or skin bruises (n=5) till severe gastro-intestinal or intracranial bleedings (n=5) and fetal or neonatal death.(11, 12, 27, 29) In Bernard-Soulier syndrome patients reported complications ranged from low postnatal platelet counts (n=7) till severe gastro-intestinal bleedings and intracranial bleeds (n=5) and neonatal death due to bleeding complications (n=4).(13, 32, 34, 35) To note, none of the reports mentioned preventive atraumatic approach during labor nor if Glanzmann thrombasthenia or Bernard-Soulier syndrome was ruled out in the neonate. Maternal iso-antibodies were considered causal for fetal or neonatal bleeding complications in six of the Glanzmann thrombasthenia patients and in ten of the Bernard-Soulier syndrome -patients of whom the pregnancy was complicated with FNAIT.(11-13, 22, 27, 29, 30, 32, 34, 35) Two pregnant Bernard-Soulier syndrome patients received weekly IVIG and in one Glanzmann thrombasthenia patient plasma-exchanges were performed. (12, 32, 37) Coexistence of maternal HPA antibodies was often unknown. Healthy neonates and normal neonatal platelets were seen as well (n=3 in Glanzmann thrombasthenia, n=18 in Bernard-Soulier syndrome).(11-13, 32, 35)

Survey results

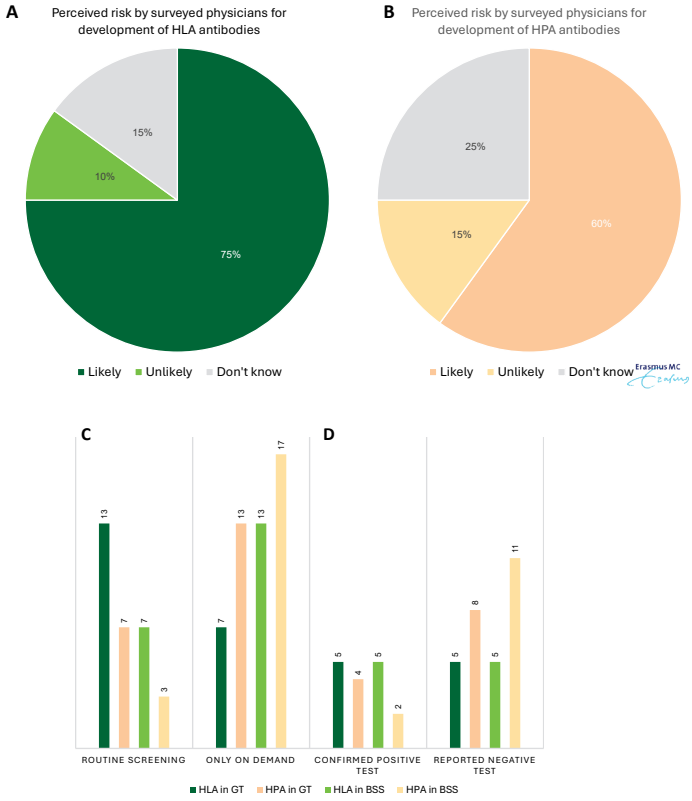
General demographics of survey respondents

Twenty surveys were fully completed and used for analysis. All respondents treated patients with IPDs: 50% had 1-10 patients with any type of IPD in follow-up (n=10), 35% had 10-25 patients (n=7) and 15% had >25 patients with IPD (n=3). Of the IPD-patients, Glanzmann thrombasthenia and Bernard-Soulier syndrome patients were treated in some but not all centers (range 0-4 Glanzmann thrombasthenia patients per center and 0-2 Bernard-Soulier syndrome patients per center). Overall, 36 Glanzmann thrombasthenia and 29 Bernard-Soulier syndrome patients were reported. Determination of incidence is not possible due to possible overlap in reported patients and the absence of specific patient characteristics.

HLA and HPA antibody screening and formation

Most respondents estimated the risk on HLA or HPA antibody formation as likely (estimated risk for development of HLA antibodies in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients was 75%; and 60% for HPA, see Figure 1, 1A and 1B). This high-risk estimation was reflected in current practice: to prevent alloimmunization in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients, 15 respondents routinely gave HLA-matched platelets in elective setting (15/20, 75%). Three of these respondents also preventively matched for HPA (3/20, 15%). The other five (5/20) did not give HLA-matched platelet transfusions when no HLA antibodies were formed or signs of clinical refractoriness present.

Figure 1 Risk assessment by surveyed physicians for HLA or HPA antibody development in Glanzmann Thrombasthenia and Bernard-Soulier syndrome



Abbreviations BSS = Bernard-Soulier syndrome; GT = Glanzmann Thrombasthenia; HLA = Human Leucocyte Antigen; HPA = Human Platelet Antigen

Legend Total number of responders n=20

(A) Perceived risk by surveyed physicians for development of HLA antibodies,

(B) Perceived risk by surveyed physicians for development of HPA antibodies,

(C) HLA and HPA antibody tests and routine care

D) Number of positive or negative antibody tests reported.

Preventive matching did not lead to a regular screening for the formation of HLA or HPA antibodies during follow-up in all centers. If surveillance was part of routine medical care, HLA antibody screening was most frequently performed in patients with Glanzmann thrombasthenia (n=13/20, 65% at least once) and less frequently in Bernard-Soulier syndrome patients (n=7; 35%). For HPA antibody screening, this was 35% in Glanzmann thrombasthenia (n=7) and rarely done in Bernard-Soulier syndrome (n=3, 15%). See Figure 1C. Positive test results on HLA antibody-formation were reported equally in 5 Glanzmann thrombasthenia and in 5 Bernard-Soulier syndrome patients. For Bernard-Soulier syndrome, one pediatric hematologist added as a note that HLA antibodies were confirmed in one 12 year old child who had received 3 units of non-leucocyte depleted platelet transfusions in Iraq. HPA antibodies were found less frequent: four times in Glanzmann thrombasthenia patients, and two times in Bernard-Soulier syndrome patients. One pediatric hematologist added as a note that HPA antibodies were found in a 14 year old child who had received 14 units of 5-donor concentrated platelets before the age of 13, indicating that HPA antibodies can occur at younger age. Negative test results were found as well: 5 times respondents mentioned that they had tested their Glanzmann thrombasthenia patients and found no HLA antibodies, and 5 times as well no HLA antibodies were found in Bernard-Soulier syndrome patients. See Figure 1D. Determination of incidence is not possible due to low numbers and possible overlap in reported patients. The presence of iso-antibodies has not been surveyed, because no transfusion strategy can be performed to prevent or overcome iso-antibodies.

Motivations for HLA and HPA antibody testing and preventive matched platelet transfusions

If performed, reasons for testing HLA and HPA antibodies varied from routine screening during follow-up (n=11), routine screening for scheduled platelet transfusion in (n=7) or clinical platelet refractoriness (n=7). Transfusion reactions or pregnancy were not reported as reason.

Motivations to transfuse or not to transfuse HLA and/or HPA-matched platelets in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients were very heterozygous (Table 3). In general, the reasons for preventive HLA matching resulted from the expectation that Glanzmann thrombasthenia and Bernard-Soulier syndrome were more likely to develop HLA more than HPA antibodies (n=16). This was supported by the wish to preserve the use of ready-available random platelet transfusions, e.g. in case an acute bleeding occurs (n=10). This motivation was explained in more detail by the argument that there are not many alternative treatments, other than recombinant FVII for Glanzmann thrombasthenia patients (n=1).

Table 3 Pros and cons with regard to HLA matched platelets in Glanzmann and Bernard-Soulier syndrome as formulated by Dutch (pediatric) hematologists

In favor of preventive HLA matching	Against preventive HLA matching
Prevention of HLA antibody formation because I consider this group a high-risk group for developing HLA antibodies due to frequent platelet transfusions (n=16)	There is no or only sparse data on the incidence of HLA antibodies, proving that alloimmunization for HLA is higher in Glanzmann or BSS patients (n=1)
Prevention of HLA antibody formation in elective settings leaves the possibility to use random concentrated platelet transfusion units in acute settings (n=10)	HLA matching is not suitable in acute settings (n=1)
Prevention of HLA antibody formation in order to preserve HLA matched platelet transfusions for treatment, because there are no/ few other treatment options for IPD-patients (n=1)	My personal experience is that the risk for HLA alloimmunization is low, as I have still to observe a positive HLA antibody test in Glanzmann or BSS patient (n=1)
	I consider the risk to be low after universal leukodepletion (n=2)
	There is insufficient data on the incidence of HLA alloimmunization in children (n=1)
	Potentially children are less at risk for formation of HLA antibodies, therefore this is less relevant for young children (n=1)
	Matching for HLA or HPA does not solve the formation of complete GPIIB/IIIA-antibodies in Glanzmann patients (n=1)
	It is not recommended in the national transfusion guidelines (n=2)
	Creates a precedential space for other IPDs (n=1)

Table 3 Pros and cons with regard to HLA matched platelets in Glanzmann and Bernard-Soulier syndrome as formulated by Dutch (pediatric) hematologists (*continued*)

In favor of preventive HPA matching	Against preventive HPA matching
Same arguments as HLA (n=2)	I consider the chance of HPA antibody formation considerably lower than HLA antibody formation (n=6)
I have one adolescent patient with Glanzmann who developed a HPA antibody at young age, but I do not know if this is an exception or the patient is high-risk due to her disease (n=1)	I consider the risk for HPA antibody formation low, as I have never encountered a positive HPA antibody test in this group of patients (n=1)
	Incidence numbers of HPA antibody formation are not available in the literature (n=2)
	Personally, I have a lack of knowledge on the topic (n=5)
	It is not recommended in the national transfusion guidelines (n=5)
	It is not possible to match on HLA and HPA at the same time; if indicated I prefer HLA matching (n=1)

Abbreviations BSS = Bernard-Soulier syndrome, Glanzmann = Glanzmann Thrombasthenia, GP = Glycoprotein, HLA = Human Leucocyte Antigen, HPA = Human Platelet Antigen, IPD = Inherited Platelet Defects, n = number

Availability of HLA and/or HPA-matched platelets takes approximately 48 hours between donation and transfusion in The Netherlands. In case of emergency, this time is not available.

The motivations to refrain from transfusing HLA or HPA-matched platelets were variable and less supportive for the preventive use of HPA-matching than for HLA-matching. The main argument against HLA and HPA-matching was the absence of clear incidence numbers in adults and children (n=5). Furthermore, to match for HLA was considered less urgent in the era of universal leukocyte filtration (n=2). Two respondents remarked that matching for HPA did not prevent the development of iso-antibodies which they considered a more serious risk than HLA antibody formation. Finally, not all physicians were aware of the possibility to transfuse combined HLA and HPA-matched platelets, but in The Netherlands, donors can be selected based on both HLA and HPA antigens.

Use of corrected count increment in Glanzmann thrombasthenia and Bernard-Soulier syndrome

When HLA or HPA matched platelets are transfused, the Dutch transfusion guidelines advise to measure the corrected count increment (cci) as a measure for effect or refractoriness in thrombocytopenic patients. Bernard-Soulier syndrome patients show mildly

decreased till severely decreased platelet counts, but Glanzmann patients often have a normal platelet count. We therefore investigated whether the Glanzmann thrombasthenia and Bernard-Soulier syndrome patients were tested for platelet refractoriness using cci: this was routinely done by n=16 as "a standard procedure to evaluate effect (n=13)", or as "a quality requirement after HLA or HPA matched platelets (n=3)". The arguments not to use the cci routinely were: "only useful in patients with severe thrombocytopenia (n=1)", and "only used when there are clinical signs of refractoriness (n=3)".

HLA matching in other IPDs than Glanzmann thrombasthenia or Bernard-Soulier syndrome

In addition to the specific treatment strategies in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients, we surveyed the use of preventive HLA and HPA-matching in other IPDs than Glanzmann thrombasthenia or Bernard-Soulier syndrome. The other IPD forms were not specified by the respondents. Most physicians did not match for HLA in other IPDs (13/20, 65%), but seven respondents mentioned to give all IPD patients HLA and/or HPA matched platelets. Several reasons were mentioned: transfusion-depending IPDs (n=3), other IPDs not classified (n=3), routine practice, because all IPDs follow the same care pathway as Glanzmann thrombasthenia patients (n=1).

DISCUSSION

Conclusions

In current guidelines, recommendations for HLA-matching are based on only three small cohort studies in Glanzmann thrombasthenia patients reporting the occurrence of HLA antibody formation.(11, 22, 38) Unfortunately, these studies do not provide sufficient insight into the effectiveness of prophylactic transfusion strategies with HLA-matched products.(39) In absence of high-quality evidence on prophylaxis while facing the practical and therapeutic dilemmas of HLA antibodies when formed, some treatment guidelines (8, 20, 39-43) or national transfusion guidelines have implemented prophylactic matching.(44, 45) But some national transfusion guidelines do not, as is the case in the Dutch International Federation of Medical Specialists (IFMS) guideline,(46) the Joint UK (47) or Canadian guidelines on transfusions.(48) Recently published cohort-studies confirm that 14-30% of Glanzmann thrombasthenia and Bernard-Soulier syndrome patients will develop HLA antibodies, some already in childhood (reported incidence of 7%).(2, 11-13, 21, 23-25, 31) This life-long incidence is higher for male Glanzmann thrombasthenia and Bernard-Soulier syndrome patients than the population-risk, but approaches incidences in post-pregnant healthy women, as HLA antibody formation occurs in 7% of healthy non-transfused male donors (95% CI 6.3-7.8),(49, 50) and in 24-33% of healthy female donors after pregnancy.(49, 51) The impact of the developed HLA antibodies, however, in patients with a platelet disorder is clearly much higher than for

healthy persons in the general population, as the chance to receive a platelet transfusion as prophylaxis or during severe bleeding is much higher for Glanzmann thrombasthenia and Bernard-Soulier syndrome patients. Not to notice the lack of alternative treatment options to prevent or treat serious bleeding. This makes preventive HLA-matching a desirable strategy in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients. This impact is reflected in our survey results, showing that the formation of platelet antibodies is estimated as a likely risk by a large proportion of respondents leading to preventive matching for HLA antigens in elective setting. This consensus is not unanimous for all Dutch hematologists, because of the absence of true incidence numbers in thorough, prospective trial results leaving the risk assessment incomplete. Although true incidence numbers can also not be deducted from our survey, we can conclude that despite universal leukocyte reduction in our country HLA antibodies still may occur in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients, leaving this issue relevant.

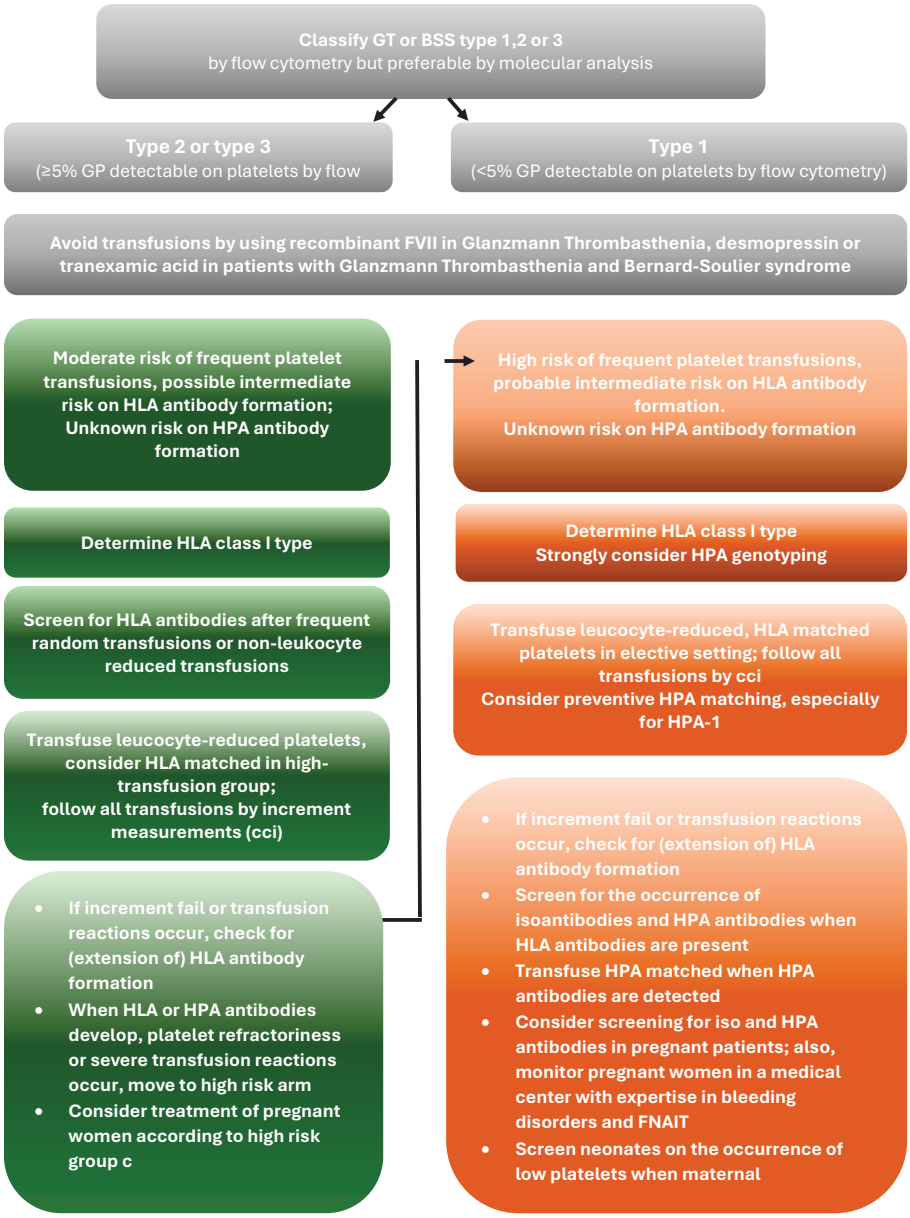
Preventive HPA matching is not advised in current guidelines and, as the survey shows, less frequently applied by Dutch (pediatric) hematologists. True incidence numbers cannot be presented from current case-series, nor deducted from our survey. It was mentioned only once in the survey to occur in an adolescent with Glanzmann thrombasthenia. In general, it remains unclear if this is the result of a lower true incidence, difficulty in screening when collateral iso-antibodies are present or less intense surveillance. All we can conclude is that HPA antibody formation may occur and in risk groups such as pregnant Glanzmann thrombasthenia and Bernard-Soulier syndrome patients can lead to extra treatment due to the risk of platelet refractoriness and neonatal complications as major bleedings resembling FNAIT.(12 13) Because serological tests in Glanzmann thrombasthenia patients can be incongruent to HPA genotype, genotyping HPA-antigens should preferentially be performed.(2, 17, 52)

The occurrence of iso-antibodies and their pathogenicity has not been studied in our survey, but our literature review has demonstrated that it will be of importance to investigate the existence of iso-antibodies, because they may lead to platelet transfusion refractoriness and in pregnant Glanzmann thrombasthenia or Bernard-Soulier syndrome patients are associated with fetal and neonatal complications. Although not advised in a recent guideline on managing pregnancy in Glanzmann thrombasthenia patients,(53) we consider it of importance to screen at least all Glanzmann thrombasthenia and Bernard-Soulier syndrome patients with frequent platelet transfusions and female Glanzmann thrombasthenia and Bernard-Soulier syndrome patients. Besides maternal and perinatal care in pregnant women with Glanzmann thrombasthenia and Bernard-Soulier syndrome, we conclude that neonatal care needs special attention, as is demonstrated by the case-series and cases described in Table 2.

Recommendations

Combining the results of the current body of evidence with the survey, we propose the following recommendations (See Figure 2):

Figure 2 Flowchart of HLA or HPA platelet matching in patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome



Abbreviations BSS = Bernard-Soulier syndrome, cci = corrected count increment, CD = cluster of Differentiation, FNAIT = feto-neonatal alloimmune thrombocytopenia, GP = glycoprotein, GT = Glanzmann Thrombasthenia, HLA = human leucocyte antigen, HPA = human platelet antigen

Recommendations on screening in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients

- Perform HLA class I antigen typing in all patients, children and adults. Preferably at diagnosis, but certainly when platelet refractoriness occurs or HLA class I antibodies have been detected;
- Screen at least once for the occurrence of HLA class I antibodies in patients who in the past have been transfused with random or non-leukocyte reduced platelet transfusions. Also, screen patients with platelet refractoriness that do not yet receive HLA-matched platelets or have a HLA-donor match based on test-results that do not fully match the own HLA-haplotype. Consider extra screening in pregnancies. It is not clear from what age children will benefit from this surveillance;
- Perform HPA genotyping at diagnosis for at least HPA-1, and if possible also HPA-2, 3 and 5.
- Screen for the occurrence of iso-antibodies and HPA antibodies when HLA antibodies have been formed. Also, screen in case of platelet refractoriness despite HLA-matching. Consider this extended screening in pregnancy to assess peripartum and neonatal risk strategy;
- Screen neonates on the occurrence of postnatal low platelets when maternal iso-antibodies or HPA antibodies are present.

Recommendations on treatment in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients

- Avoid platelet transfusions in Glanzmann thrombasthenia patients e.g. by using recombinant FVII when possible;
- Give preventive transfusion of HLA-matched platelets in elective setting in Glanzmann thrombasthenia and Bernard-Soulier syndrome patients;
- Transfuse HLA-matched platelets when HLA antibodies have been formed unless medical care cannot be delayed while waiting for matched platelets in an acute medical setting;
- Transfuse HPA-matched platelets when HPA antibodies have been formed or platelet refractoriness remains under HLA-matched platelets;
- Consider additionally HPA-matching of platelets in patients with a HPA profile predisposing to the formation of clinically relevant HPA antibodies such as anti-HPA-1a; Discuss the need for preventive matching in a team with expertise on HPA-immunogenicity
- Perinatal management should be applied to all pregnant women in a medical center with expertise in bleeding disorders and FNAIT. A postnatal transfusion strategy for the neonate should be made in advance when HPA antibodies are detected during pregnancy.

Our study has several limitations. It does not yield the possibility to deduct incidence numbers for antibody formation in the Dutch Glanzmann thrombasthenia and Bernard-Soulier syndrome patients. Overlap of reported patients remains possible because responding physicians may have been involved in treatment of the same patients in different institutes. Therefore, we do not give an incidence number in this article. Although we have based our recommendations on the current body of evidence, trial results from literature remain meager and this topic will need more research. With the absence of thorough incidence numbers of HLA and HPA alloimmunization as well as iso-antibody formation at one side and the lack of identification for risk factors such as age of the patient, HPA genotype profile, non-platelet expression of HPA and the underlying molecular Glanzmann thrombasthenia and Bernard-Soulier syndrome genotypes, it remains impossible to detect the patients that will benefit the most from preventive matching. Obtaining more detailed information on this patient characteristics and disease characteristics in relation to platelet refractoriness and FNAIT should be the main targets of future research to define the risk groups that will benefit the most from preventive matching programs. In line with this, high-quality trials providing data on preventive matching should follow. The lack of these data makes a meta-analysis impossible to implement.

While awaiting these research results, we have chosen a conservative strategy in order to prevent as much alloimmunization against HLA and HPA as possible, for the possibility to match on HLA and if necessary, also for HPA-antigens is available in The Netherlands. The recommendations are based on the current Dutch blood bank facilities and may not be implementable in other settings.

REFERENCES

1. Di Minno G, Zotz RB, d'Oiron R, Bindslev N, Di Minno MN, Poon MC, Glanzmann Thrombasthenia Registry I. The international, prospective Glanzmann Thrombasthenia Registry: treatment modalities and outcomes of non-surgical bleeding episodes in patients with Glanzmann thrombasthenia. Haematologica 2015;100: 1031-7.
2. Fiore M, Bayat B, Phuangtham R, Blouin L, Huguenin Y, Bein G, Santoso S. Immunization against alphaIIb beta3 and alphav beta3 in Glanzmann thrombasthenia patients carrying the French Gypsy mutation. J Thromb Haemost 2021;19: 255-61.
3. Novotny VMJ. Prevention and management of platelet transfusion refractoriness. Vox Sanguinis 1999;76: 1-13.
4. Novotny VM, van Doorn R, Witvliet MD, Claas FH, Brand A. Occurrence of allogeneic HLA and non-HLA antibodies after transfusion of prestorage filtered platelets and red blood cells: a prospective study. Blood 1995;85: 1736-41.
5. Nurden P, Stritt S, Favier R, Nurden AT. Inherited platelet diseases with normal platelet count: phenotypes, genotypes and diagnostic strategy. Haematologica 2021;106: 337-50.
6. Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. Blood 2011;118: 5996-6005.
7. Savoia A, Pastore A, De Rocco D, Civaschi E, Di Stazio M, Bottega R, Melazzini F, Bozzi V, Pecci A, Magrin S, Balduini CL, Noris P. Clinical and genetic aspects of Bernard-Soulier syndrome: searching for genotype/phenotype correlations. Haematologica 2011;96: 417-23.
8. Bolton-Maggs PH, Chalmers EA, Collins PW, Harrison P, Kitchen S, Liesner RJ, Minford A, Mumford AD, Parapia LA, Perry DJ, Watson SP, Wilde JT, Williams MD, Ukhcdo. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. Br J Haematol 2006;135: 603-33.
9. Novotny VM. Prevention and management of platelet transfusion refractoriness. Vox Sang 1999;76: 1-13.
10. Poon MC, Di Minno G, d'Oiron R, Zotz R. New Insights Into the Treatment of Glanzmann Thrombasthenia. Transfusion Medicine Reviews 2016;30: 92-9.
11. Santoro C, Rago A, Biondo F, Conti L, Pulcinelli F, Laurenti L, Perrone MP, Baldacci E, Leporace A, Mazzucconi MG. Prevalence of allo-immunization anti-HLA and anti-integrin alphaIIbbeta3 in Glanzmann Thromboasthenia patients. Haemophilia 2010;16: 805-12.
12. Siddiq S, Clark A, Mumford A. A systematic review of the management and outcomes of pregnancy in Glanzmann thrombasthenia. Haemophilia 2011;17: E858-E69.
13. Peitsidis P, Datta T, Pafilis I, Otomewo O, Tuddenham EGD, Kadir RA. Bernard Soulier syndrome in pregnancy: a systematic review. Haemophilia 2010;16: 584-91.
14. Curtis BR, McFarland JG. Human platelet antigens - 2013. Vox Sang 2014;106: 93-102.
15. Porcelijn L, Huiskes E, de Haas M. Progress and development of platelet antibody detection. Transfus Apher Sci 2020;59: 102705.
16. Kjeldsen-Kragh J, Titze TL, Lie BA, Vaage JT, Kjaer M. HLA-DRB3*01:01 exhibits a dose-dependent impact on HPA-1a antibody levels in HPA-1a-immunized women. Blood Adv 2019;3: 945-51.
17. Jallu V, Bianchi F, Kaplan C. Fetal-neonatal alloimmune thrombocytopenia and unexpected Glanzmann thrombasthenia carrier: report of two cases. Transfusion 2005;45: 550-3.
18. Simsek S, Faber NM, Bleeker PM, Vlekke AB, Huiskes E, Goldschmeding R, von dem Borne AE. Determination of human platelet antigen frequencies in the Dutch population by immunophenotyping and DNA (allele-specific restriction enzyme) analysis. Blood 1993;81: 835-40.

19. Curtis BR. Recent progress in understanding the pathogenesis of fetal and neonatal alloimmune thrombocytopenia. Br J Haematol 2015;171: 671-82.
20. Bellucci S, Caen J. Molecular basis of Glanzmann's Thrombasthenia and current strategies in treatment. Blood Rev 2002;16: 193-202.
21. Gabe C, Ziza KC, Durazzo N, Pagani FM, Oliveira VB, Conrado MAV, Dezan MR, Mendrone A, Jr., Villaca PR, Dinardo CL, Rocha V. Detection of alloimmunization in Glanzmann Thrombasthenia and Bernard-Soulier Syndrome: Data from a Brazilian Center. Hematol Transfus Cell Ther 2022.
22. Fiore M, Firah N, Pillois X, Nurden P, Heilig R, Nurden AT. Natural history of platelet antibody formation against alphaIIbbeta3 in a French cohort of Glanzmann thrombasthenia patients. Haemophilia 2012;18: e201-9.
23. Zotz RB, Poon MC, Di Minno G, D'Oiron R, Glanzmann Thrombasthenia Registry I. The International Prospective Glanzmann Thrombasthenia Registry: Pediatric Treatment and Outcomes. TH Open 2019;3: e286-e94.
24. Poon MC, d'Oiron R, Zotz RB, Bindslev N, Di Minno MN, Di Minno G, Glanzmann Thrombasthenia Registry I. The international, prospective Glanzmann Thrombasthenia Registry: treatment and outcomes in surgical intervention. Haematologica 2015;100: 1038-44.
25. Poon MC, D'Oiron R, Von Depka M, Khair K, Negrier C, Karafoulidou A, Huth-Kuehne A, Morfini M, International Data Collection on Recombinant Factor V, Congenital Platelet Disorders Study G. Prophylactic and therapeutic recombinant factor VIIa administration to patients with Glanzmann's thrombasthenia: results of an international survey. J Thromb Haemost 2004;2: 1096-103.
26. Friend BD, Roach GD, Kempert PH, Moore TB. Successful Use of Hematopoietic Stem Cell Transplantation for 2 Pediatric Cases of Glanzmann Thrombasthenia and Review of the Literature. J Pediatr Hematol Oncol 2020;42: e521-e6.
27. Soni P, Mantri S, Prabhudesai A, Patil R, Shanmukhaiah C, Shetty S. Triple jeopardy: A case of Glanzmann's thrombasthenia with anti-GPIIb-IIIa antibodies and HPA incompatibility resulting in stillbirth. Thrombosis Research 2019;181: 141-4.
28. Cid AR, Montesinos P, Sanchez-Guiu I, Haya S, Lorenzo JI, Sanz J, Moscardo F, Puig N, Planelles D, Bonanad S, Sanz GF, Vicente V, Gonzalez-Manchon C, Lozano ML, Rivera J, Sanz MA. Allogeneic hematopoietic cell transplantation in an adult patient with Glanzmann thrombasthenia. Clin Case Rep 2017;5: 1887-90.
29. Leticee N, Kaplan C, Lemery D. Pregnancy in mother with Glanzmann's thrombasthenia and isoantibody against GPIIb-IIIa: Is there a foetal risk? European Journal of Obstetrics & Gynecology and Reproductive Biology 2005;121: 139-42.
30. Ito K, Yoshida H, Hatoyama H, Matsumoto H, Ban C, Mori T, Sugiyama T, Ishibashi T, Okuma M, Uchino H, et al. Antibody removal therapy used successfully at delivery of a pregnant patient with Glanzmann's thrombasthenia and multiple anti-platelet antibodies. Vox Sang 1991;61: 40-6.
31. Almeida AM, Khair K, Hann I, Liesner R. The use of recombinant factor VIIa in children with inherited platelet function disorders. British Journal of Haematology 2003;121: 477-81.
32. Uotila J, Tammela O, Makipernaa A. Fetomaternal platelet immunization associated with maternal Bernard-Soulier syndrome. Am J Perinatol 2008;25: 219-23.
33. Sandrock K, Knofler R, Greinacher A, Furll B, Gerisch S, Schuler U, Gehrisch S, Busse A, Zieger B. Novel Mutation in Bernard-Soulier Syndrome. Transfus Med Hemother 2010;37: 278-84.
34. Fujimori K, Ohto H, Honda S, Sato A. Antepartum diagnosis of fetal intracranial hemorrhage due to maternal Bernard-Soulier syndrome. Obstet Gynecol 1999;94: 817-9.

35. Peng TC, Kickler TS, Bell WR, Haller E. Obstetric complications in a patient with Bernard-Soulier syndrome. Am J Obstet Gynecol 1991;165: 425-6.
36. Pascual C, Bento L, Carretero F, Rodriguez Huerta A, Pérez Rus G et al: Successful Outcome of Two Pregnancies in a Woman with Bernard Soulier Syndrome. Blood 2011;118(21):4676.
37. Ito K, Yoshida H, Hatoyama H, Matsumoto H, Ban C, Mori T et al: Antibody Removal Therapy Used Successfully at Delivery of a Pregnant Patient with Glanzmann's Thrombasthenia and Multiple Anti-Platelet Antibodies. Vox Sanguinis 1991;61(1):40-46
38. Poon MC, d'Oiron R. Alloimmunization in Congenital Deficiencies of Platelet Surface Glycoproteins: Focus on Glanzmann's Thrombasthenia and Bernard-Soulier's Syndrome. Seminars in Thrombosis and Hemostasis 2018;44: 604-14.
39. Stanworth SJ, Navarrete C, Estcourt L, Marsh J. Platelet refractoriness--practical approaches and ongoing dilemmas in patient management. Br J Haematol 2015;171: 297-305.
40. Alamelu J, Liesner R. Modern management of severe platelet function disorders. Br J Haematol 2010;149: 813-23.
41. Chitlur M, Rajpurkar M, Recht M, Tarantino MD, Yee DL, Cooper DL, Gunawardena S. Recognition and management of platelet-refractory bleeding in patients with Glanzmann's thrombasthenia and other severe platelet function disorders. Int J Gen Med 2017;10: 95-9.
42. Poon MC, Di Minno G, d'Oiron R, Zotz R. New Insights Into the Treatment of Glanzmann Thrombasthenia. Transfus Med Rev 2016;30: 92-9.
43. Botero JP, Lee K, Branchford BR, Bray PF, Freson K, Lambert MP, Luo M, Mohan S, Ross JE, Bergmeier W, Di Paola J, ClinGen Platelet Disorder Variant Curation Expert P. Glanzmann thrombasthenia: genetic basis and clinical correlates. Haematologica 2020;105: 888-94.
44. https://transfusion.com.au/system/files/resource_library/platelet_refractoriness_checklist_2272.pdf
45. Estcourt LJ, Birchall J, Allard S, Bassey SJ, Hersey P, Kerr JP, Mumford AD, Stanworth SJ, Tinegate H, British Committee for Standards in H. Guidelines for the use of platelet transfusions. Br J Haematol 2017;176: 365-94.
46. https://richtlijnendatabase.nl/richtlijn/bloedtransfusiebeleid/startpagina_-_bloedtransfusiebeleid.html
47. https://www.transfusionguidelines.org/document-library/documents/cb-use-of-hla-hpa-selected-platelets-pdf
48. https://professionaleducation.blood.ca/en/transfusion/clinical-guide/platelet-transfusion-alloimmunization-and-management-platelet
49. Middelburg RA, Porcelijn L, Lardy N, Briet E, Vrielink H. Prevalence of leucocyte antibodies in the Dutch donor population. Vox Sang 2011;100: 327-35.
50. Porretti L, Cattaneo A, Coluccio E, Mantione E, Colombo F, Mariani M, Bottelli G, Mazzucchelli S, Pappalettera M, Speranza T, Migliari M, Cambie G, Prati D, Rebulla P. Implementation and outcomes of a transfusion-related acute lung injury surveillance programme and study of HLA/HNA alloimmunisation in blood donors. Blood Transfusion 2012;10: 351-9.
51. Triulzi DJ, Kleinman S, Kakaiya RM, Busch MP, Norris PJ, Steele WR, Glynn SA, Hillyer CD, Carey P, Gottschall JL, Murphy EL, Rios JA, Ness PM, Wright DJ, Carrick D, Schreiber GB. The effect of previous pregnancy and transfusion on HLA alloimmunization in blood donors: implications for a transfusion-related acute lung injury risk reduction strategy. Transfusion 2009;49: 1825-35.
52. Pober M, Kyrle PA, Panzer S. Genotyping Provides a Reliable Tool for the Determination of the Platelet Antigen System Hpa-1 in Glanzmann Thrombasthenia. British Journal of Haematology 1993;85: 112-5.
53. Fiore M, Sentilhes L, d'Oiron R. How I manage pregnancy in women with Glanzmann thrombasthenia. Blood 2022;139: 2632-41.

Life can only be understood backwards; but must be lived forwards

Søren Kierkegaard (1813 – 1855 AD)

10 Are thrombocytopenia and platelet transfusions associated with major bleeding in preterm neonates? A systematic review

S.F. Fustolo-Gunnink
E.J. Huisman
J.G. van der Bom
F.M.A. van Hout
A. Makileni
E. Lopriore
K. Fijnvandraat

Blood Reviews 2019;36:1-9

ABSTRACT

Over 75% of severely thrombocytopenic preterm neonates receive platelet transfusions to prevent bleeding, but transfusion guidelines are based mainly on expert opinion. The aim of this review was to investigate whether platelet counts, platelet transfusions or platelet indices are associated with major bleeding in preterm neonates.

We performed a systematic search of the EMBASE and MEDLINE databases until December 2017. We included randomized trials, cohort and case control studies. (Prospero: CRD42015013399).

We screened 8734 abstracts and 1225 full texts, identifying 36 eligible studies. In 30, timing of the platelet counts or transfusions in relation to the bleeding was unclear. Of the remaining six studies, two showed that thrombocytopenia was associated with increased risk of bleeding, two showed no such association, and three showed lack of an association between platelet transfusions and bleeding risk. No studies assessing platelet indices were found.

The study results suggest that prophylactic platelet transfusions may not reduce bleeding risk in preterm neonates.

INTRODUCTION

Major bleeding, such as severe intraventricular hemorrhage (IVH) or pulmonary hemorrhage, occurs in 6-10% of neonates admitted to neonatal intensive care units (NICUs).(1-6) IVH is thought to be a multifactorial process, with fragility of the germinal matrix vasculature and fluctuations in cerebral blood flow due to respiratory and hemodynamic instability as main risk factors.(7) IVH is often asymptomatic, and detected during routine ultrasound screening. Pulmonary hemorrhage is characterized by sudden increase in respiratory requirements with fresh blood in the endotracheal tube. It is also considered a multifactorial process, potential risk factors are the presence of patent ductus arteriosus, exposure to indomethacin and surfactant, among others.(8) Other, less common types of major bleeding include renal bleeding, gastro-intestinal bleeding (often related to necrotizing enterocolitis) and other types of intracranial bleeding. Major bleeds occur predominantly in preterm neonates, and can have devastating consequences, such as cognitive and motor disabilities, or death.(9)

As stated, there is general consensus that bleeding is a multifactorial process. However, the extent to which different clinical factors contribute to bleeding risk, is unclear, and has proven difficult to assess. Because platelets are involved in primary hemostasis, thrombocytopenia, which occurs in 20-35% of neonates in the NICU, is often considered a risk factor for major bleeding.(1-3, 10-13) Common causes of neonatal thrombocytopenia are intra uterine growth retardation (IUGR) – in which chronic uterine hypoxia is thought to suppress thrombopoiesis in favor of erythropoiesis – sepsis and necrotizing enterocolitis (NEC). IUGR-related thrombocytopenia is often present at birth, with platelet counts dropping further over the first days of life, with spontaneous recovery to normal platelet counts afterwards. NEC or sepsis induced thrombocytopenia occurs later in life, and platelet counts often drop quickly.(14)

Over 75% of neonates with severe thrombocytopenia (defined as a platelet count <50 x 10^9/L) are treated with platelet transfusions.(2, 15) However, there is no conclusive evidence to support the notion that neonatal thrombocytopenia is causally associated with bleeding, or that platelet transfusions can prevent neonatal bleeding. Neonatal platelet transfusion guidelines are therefore based mainly on expert opinion. This is reflected in the fact that platelet transfusion guidelines differ significantly between hospitals and between countries. In Europe, platelet transfusions are usually administered when platelet counts drop below 20-25 x 10^9/L, and higher thresholds are used when neonates are considered 'sick', or 'unstable.' In general, American guidelines state higher thresholds of 50 x 10^9/L up to 100 x 10^9/L. The AABB transfusion guideline does not address neonates.

Various studies in adults have suggested that bleeding risk increases substantially only when platelet counts drop below 10 x 10^9/L in hospitalized patients.(16-18) But whether

or not these results apply to neonates remains to be determined. Adult studies cannot be directly extrapolated to neonates, as the etiology of neonatal thrombocytopenia differs from that of adult thrombocytopenia.(14) Furthermore, the neonatal hemostatic system differs significantly from that of adults.(19) Preterm neonates are particularly at risk of bleeding because of the fragile vasculature in their germinal matrix.(7) In addition, neonatal platelet transfusions are given at relatively high volumes (10-15 mL/kg) compared to adult platelet transfusions, and common neonatal comorbidities differ from common adult comorbidities. And lastly, several reports have pointed out that neonates may experience more transfusion related adverse events than children or adults.(20-21) Thus, there is an urgent need for additional research.

A structured review published in 2014 assessed the association between platelet transfusion and bleeding in various patient groups, including neonates in the NICU.(22) However, the researchers did not distinguish between major and minor bleeds, and did not assess the relationship between platelet count and bleeding. Also, both term and preterm neonates were included and temporal ambiguity was not assessed. Temporal ambiguity occurs when it is unclear whether platelet transfusions were administered prior to or after the bleed. Finally, the search was performed up to 2012. Therefore, we have performed a systematic review with the following aims:

1) to investigate whether (and how) platelet count is associated with major bleeding in preterm infants.
2) to investigate whether prophylactic platelet transfusions decrease the risk of major bleeding in preterm infants.
3) to investigate whether (and how) other platelet indices such as platelet mass or mean platelet volume are associated with major bleeding in preterm neonates.

METHODS

Study design

We conducted and reported this systematic review according to the PRISMA guidelines. (23)

Eligibility criteria

We selected published reports of randomized controlled trials, cohort studies and case control studies. We defined a case control study as a study in which cases were neonates with major bleeding, and controls were neonates without major bleeding. We excluded case series, case reports, reviews, editorials and commentaries without original data, and abstract-only publications.

We included studies involving any population of hospitalized preterm neonates and defined preterm as being born at a gestational age of less than 37 weeks. In case of a mix of term and preterm neonates, we included studies in which at least 80% of the population was preterm, or studies in which the results of the preterm neonates could be extracted from the results section. We excluded studies in which >25% of neonates had congenital disorders, or in which >25% of neonates were being treated with extracorporeal membrane oxygenation, as these are very specific sub-populations and results of these studies cannot be extrapolated to the neonatal population as a whole. In addition, we excluded studies that assessed bleeding related to surgery only, unless significantly prolonged or more excessive than expected. We excluded studies investigating bleeding associated with retinopathy of prematurity, because these are not potentially life-threatening. Lastly, we excluded studies in which the authors did not present major and minor bleeding separately in the results section, and studies in which major bleeding was part of a composite outcome, and results for individual outcomes were not presented. We included all potential prognostic factors related to platelets. This included platelet count, platelet mass, mean platelet volume and platelet transfusions.

Where possible, we have graded the bleeds reported in the studies according to a modified WHO bleeding score described elsewhere (Table 1).(24) If the authors provided insufficient information to classify the bleeds according to this bleeding score, we used the criteria listed in the right column of Table 1. Major bleeding was defined as intracranial hemorrhage (leading to neurosurgical intervention or radiologic imaging showing midline shift), IVH filling 50% or more of the cerebral ventricle, pulmonary hemorrhage (fresh bleeding through an endotracheal tube with increased ventilatory requirements), severe rectal bleeding, and severe bleeding (fatal bleeding, life-threatening bleeding associated with shock, or bleeding requiring fluid boluses or red cell transfusion).

Table 1 Definition of major or severe hemorrhage according to modified WHO bleeding score or alternative criteria

Major/severe bleeding grading according to modified WHO bleeding score	Major/severe bleeding grading for studies that did not use the modified WHO bleeding score
Pulmonary hemorrhage	
Acute fresh bleed through the endoctracheal tube (or mouth, when not intubated) associated with increased ventilatory requirements	Any bleeding labeled as pulmonary hemorrhage will be included, as it is expected that only obvious, fulminant pulmonary hemorrhage will be identified as such. Where necessary and possible, we will make a distinction between mild and severe bleeding based on descriptions of the clinical condition and treatment requirements

Table 1 Definition of major or severe hemorrhage according to modified WHO bleeding score or alternative criteria (*continued*)

Major/severe bleeding grading according to modified WHO bleeding score	Major/severe bleeding grading for studies that did not use the modified WHO bleeding score
Rectal hemorrhage	
Frank rectal macroscopic bleeds	Frank rectal bleeds can occur as a consequence of NEC, and may not necessarily imply a major bleed. Where possible, we will make a distinction between severe versus mild bleeding, and include only the rectal bleeds described as severe or major
Intracranial hemorrhage	
When a) neurosurgical intervention is required, or b) radiological imaging show a midline shift, or c) in the presence of clinical signs and symptoms of neurological impairment with significant derangement of laboratory investigations	As in modified WHO score. Studies describing this type of bleeding only as 'large/major intracranial hemorrhage', without further specification, will also be included. We will attempt to classify these bleedings based on information available in the papers
Intraventricular hemorrhage	
H2 or H3 with ventricular dilatation (V1) OR H1, H2, H3 with parenchymal involvement (P3) OR any evolution of intracranial haemorrhage to H2V1, H3V1, or (H1, H2, H3) with parenchymal involvement (P3)	In most studies, IVH will be graded as grade I, II, III or IV. When only this information is present, we will include studies which assess IVH grade III and IV. If information about ventricular dilatation or parenchymal involvement is present, we will include according to the modified WHO score
Other types of bleeding	
Any bleeding classified as life threatening, associated with hypotension, hypovolaemia or any other haemodynamic instability and/or bleeding requiring volume boluses or red cell transfusion in the same 24 hours	As in modified WHO score. Studies describing other types of bleeding as 'large/major bleeding', without futher specification, will also be included. An attempt will be made to further classify these bleedings based on information available in the paper

Adapted from: Venkatesh et al. Arch Dis Child Fetal Neonatal Ed 2013,98, F260–F263.

Abbreviations IVH = intraventricular hemorrhage, WHO = World health Organization

We did not use language restrictions in our search. Our team of researchers was able to read studies written in English, Dutch, German, French, Spanish and Italian. Studies in other languages were excluded.

Search strategy

With the help of an experienced librarian and Cochrane information specialist, we conducted a broad electronic search in the MEDLINE and EMBASE databases using index terms and free text words, with no date or language restriction, in June 2014. Our search strategy included terms related to preterm neonates, bleeding, and study methods (Table 2). The search was downloaded into Endnote for bibliographic management. Full text screening was performed for those studies eligible following title and abstract screening. Using the digital search function, full text papers in which platelet related risk factors were mentioned were identified. In retrospect, 95% of abstracts would have

been identified using a focused search, adding terms for platelet-related entities such as thrombocytopenia and platelet transfusion. Therefore, in December 2017, we executed a search update through a focused search in the MEDLINE and EMBASE databases, using index terms and free text words, starting from June 2014, with no language restriction. The search update strategy included the same terms as the broad search, with addition of platelet-related factors. The search was executed and downloaded into Covidence (www.covidence.org) for online bibliographic management. We recorded sources for all identified citations and removed duplicates.

Table 2 Search strategy

Original, broad search (up to june 2014)
Database: EMBASE
1 exp bleeding/
2 (hemorrhage or haemorrhage or bleeding or bleedings).tw.
3 1 or 2
4 Prematurity/
5 Exp low birth weight/
6 (prematur* or preterm* or pre-matur* or pre-term* or vlbw or elbw or birth weight).tw.
7 Or/4-6
8 3 and 7
9 Case control study/ or clinical trial/ or "clinical trial (topic)"/ or longitudinal study/ or prospective study/ or retrospective study/
10 (cohort or follow up or case control or longitudinal or prospective or retrospective or clinical trial*).tw.
11 9 or 10
12 8 and 11
13 8 and 9
Database: MEDLINE
((("Case-Control Studies"[Mesh]) OR "Cohort Studies"[Mesh] OR clinical trials[mh] OR Cohort[tiab] OR follow up[tiab] OR case control[tiab] OR longitudinal[tiab] OR prospective[tiab] OR retrospective[tiab] OR clinical trial*[tiab])) AND ((hemorrhage[mh] OR hemorrhage[tiab] OR haemorrhage[tiab] OR bleeding[tiab] OR bleedings[tiab]) AND ("Infant, Premature"[MESH] OR infant, premature, diseases[mh] OR "Infant, Low Birth Weight"[Mesh] OR prematur*[tiab] OR preterm*[tiab] OR vlbw[tiab] OR elbw[tiab] OR birth weight[tiab]))
Focused search update (from june 2014 to december 2017)
Database: EMBASE
1 exp bleeding/
2 (h?emorrhag* or bleeding or bleedings).ti,ab,kw.
3 1 or 2
4 exp neonatal intensive care unit/
5 exp prematurity/
6 (NICU or "neonatal intensive care unit*" or premature or preterm).ti,ab,kw.
7 5 or 6
8 exp thrombocyte/
9 exp blood transfusion/
10 (platelet* or thrombocyt* or transfusion*).ti,ab,kw.
11 8 or 9 or 10
12 3 and 7 and 11
13 limit 12 to (conference abstract or conference paper or "conference review")
14 12 not 13

Table 2 Search strategy (*continued*)

Original, broad search (up to june 2014)
15 (canadian or elsevier or embase).cr.
16 14 and 15
17 remove duplicates from 16
Database: MEDLINE
1 exp Hemorrhage/
2 h?emorrhag*.ti,ab,kf.
3 (bleeding or bleedings).ti,ab,kf.
4 1 or 2 or 3
5 exp Intensive Care Units, Neonatal/
6 exp Infant, Premature/
7 (NICU or "neonatal intensive care unit*" or premature or preterm).ti,ab,kf.
8 5 or 6 or 7
9 exp Blood Platelets/
10 (platelet* or thrombo* or transfusion*).ti,ab,kf.
11 9 or 10
12 4 and 8 and 11
13 remove duplicates from 12
14 exp "blood platelets"/ or exp "blood transfusion"/ or (platelet* or thrombocyt* or transfusion*).ti,ab,kf.
15 4 and 8 and 14
16 remove duplicates from 15

Selection process

Using Endnote and Covidence to organize and track the selection process, multiple reviewers (EH, SFG and SM) screened titles and abstracts based on a predefined screening questionnaire (Table 3). Every abstract that was excluded by one reviewer, was double checked by one of the other reviewers, with the exception of a selection of 616 abstracts (7% of all abstracts), which were screened by one reviewer. Disagreements were resolved by consensus. We screened in full all articles deemed to be relevant, or whose relevance could not be determined from the abstract. To assess eligibility, we screened the results section, tables and figures according to the predetermined inclusion criteria. We resolved any disagreement among the authors by consensus, when necessary, including a discussion with a third author.

Table 3 Abstract selection screening tool

If one or more of the following questions are answered with 'no', the study will be excluded. In order to avoid excluding papers in which bleeding was assessed but not reported in the abstract, we will include papers if one or more of the answers are 'unclear'. In those cases, the results section of the paper will be searched to obtain the necessary information.
1) **Does the study address preterm neonates?** Yes/no/unclear If the study includes <80% preterm neonates, it will be excluded, unless preterm neonate specific data can be extracted from the overall results. The official definition of 'neonate' is: an newborn infant in the first 28 days of life. However, for this study we will use the following definition: neonates that were preterm at birth (gestational age <37 weeks) will be called neonates until the moment of discharge from the neonatal ward, or time of death. This definition of preterm neonate applies to our full protocol.

2) **Does the study address bleeding as an outcome?**
Yes/no/unclear
If bleeding is not mentioned in the abstracts, the following rules will be applied. Abstracts will be rated as 'unclear' if they mention assessment of perinatal outcome, neonatal outcome, neonatal complications or morbidity. Abstracts will be excluded if they only mention fetal outcome, obstetrical outcome, pregnancy outcome, maternal outcome, or similar terms. These are expected to refer mainly to obstetrical and not to neonatal outcome. If postnatal neonatal outcomes that occur on the NICU, such as neonatal infection or NEC, are reported, but the terms stated above (perinatal outcome, etc.) are not mentioned, abstracts will be rated as 'unclear' as well.

3) **Does the study address one or more platelet-related risk factors or predictors for bleeding?**
Yes/no/unclear
Exclusion criteria are described above

Data extraction

We extracted the data using a predeveloped and piloted online data extraction form. Six authors (SG, FH, EH, KF, EL and JB) performed data extraction, each paper was extracted twice. We resolved any disagreement by consensus or by consultation with one of the other authors. Some papers presented results from several combined studies. In this case, where necessary, data were extracted from the original reports.

Temporal ambiguity

Temporal ambiguity occurs when it is unclear whether exposure (e.g. thrombocytopenia or platelet transfusions) occurred prior to or after the outcome (major bleeding). If it occurs after the bleeding, the exposure might be a result - and not a cause - of the bleeding, rendering causal interpretation of the study results impossible. This is a frequently encountered problem in observational studies assessing the relationship between platelet count or transfusion and major bleeding in preterm neonates. In this review, we have attempted to address this issue for all included studies. We realize that assessing the relationship between platelet count or transfusion and major bleeding may not have been the primary aim of some of the included studies. We also realize that this may be a problem of reporting, rather than conduct, as analyses may have been performed correctly, but were not fully described in the manuscript. However, we have excluded papers with temporal ambiguity from this review, in order to retain only those studies in which causal relationships can be detected more accurately.

2.7 Data synthesis and presentation

We presented the data in tabular or graphic format according to: type of bleeding, type of platelet-related prognostic factor, study design, year of publication, whether or not multivariate analyses were performed, whether or not temporal ambiguity was present, number of participants, number of events, and other relevant grouping criteria. We aimed to pool and meta-analyze study results, when possible. We described potential gaps in evidence and provided suggestions for future research.

RESULTS

Literature search results

A total of 8734 abstracts was reviewed, 1225 full-texts were assessed and 36 studies were included. More details of the screening process are provided in Figure 1 and more details of all 36 eligible studies are provided in Table Supplementary Table S1. Of these 36, 30 were subject to temporal ambiguity, and therefore not assessed in this review. The rationale behind these exclusions is provided in Table Supplementary Table S2 in the supplementary materials.

Overview of included studies

Six studies, published between 1993 and 2017 were included (Table 4, Fig 2). One RCT,(25) one cohort study,(26) and four case control studies.(2, 27-29) In total, these studies included 1580 neonates, of whom a total of 174 (11%) suffered major bleeding. Studies were performed in Canada, the USA (two studies), the UK, Germany and one international trial in Europe. Median or mean gestational age (where reported) ranged from 26 to 28 weeks. One paper presented a secondary analysis of 2 RCTs.(27) The association between platelet mass or mean platelet volume and bleeding could not be assessed, as the respective papers were all excluded based on temporal ambiguity (Supplementary Table S2).

Figure 1 PRISM Flowchart of selection and inclusion of articles

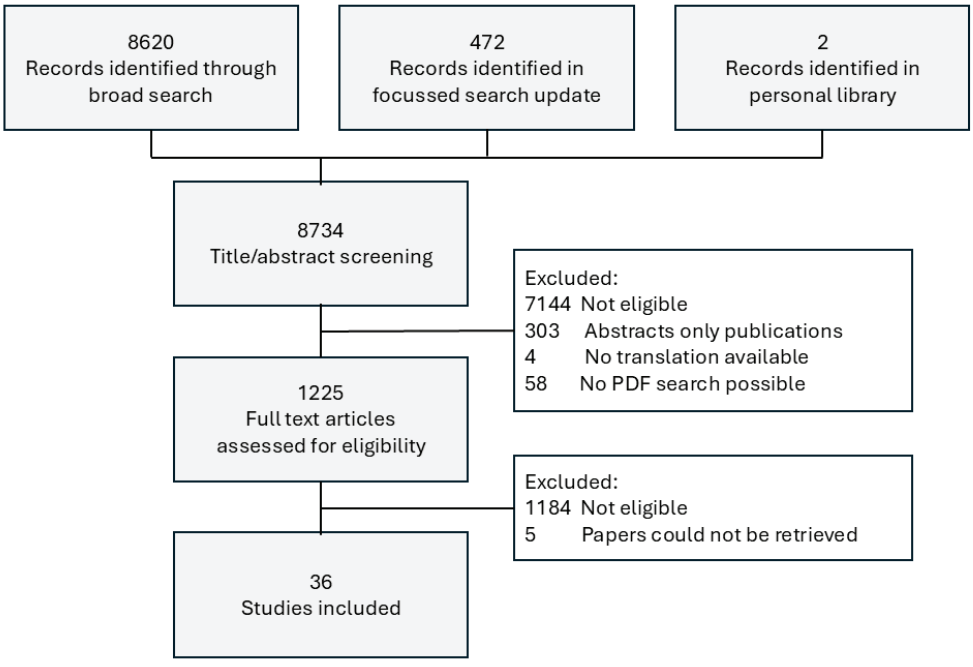
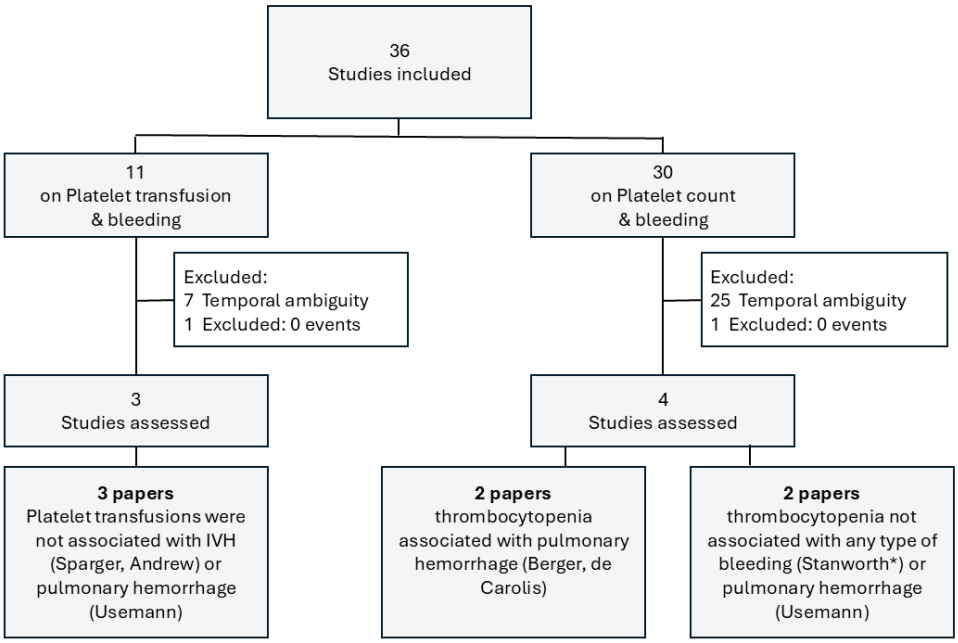


Figure 2 Flowchart of assessment of 36 included articles



Platelet count and major bleeding

Four case control studies assessed the association between platelet count and major bleeding, of which three focused on pulmonary hemorrhage only, and one on all types of major bleed combined.

The first case control study,(29) showed that a platelet count <100 x 10^9/L was associated with pulmonary hemorrhage (odds ratio 4.0, 95% CI 1.4 – 13.3) in a multivariate logistic regression model. In this study, 50 consecutive neonates with pulmonary hemorrhage were matched with controls for birth year and birth weight. Other variables in the logistic regression model were gestational age 24-26 weeks, antenatal glucocorticoids and resuscitation with positive pressure ventilation. Statistical analyses were not corrected for matching and no information about treatment with platelet transfusions was provided. It is unclear whether the platelet counts recorded were the last counts prior to the pulmonary hemorrhage.

The second case control study,(27) showed that pulmonary hemorrhage was associated with thrombocytopenia (<150 x 10^9/L) in the first 12 hours of life (OR 20.6, 95% CI 2.5 – 172.9). In this study, data from two randomized controlled trials assessing the effect of surfactant on neonatal respiratory distress syndrome in 99 neonates were combined. A total of 18 neonates without pulmonary hemorrhage in the study population were removed from the final analysis, without further clarification. In addition, no information

about treatment with platelet transfusion was provided, and results were not adjusted for confounding.

The third case control study,(28) showed no association between platelet count at birth and pulmonary hemorrhage. In this study, 20 consecutive neonates with birthweight <1000 grams and pulmonary hemorrhage were matched for gestational age and birth weight with 40 controls without pulmonary hemorrhage. Platelet count at birth was compared between both groups (170 x 10^9/L versus 183 x 10^9/L, p-value 0.51). Platelet count at onset of pulmonary hemorrhage was also reported, but not included in this review, as it is unclear whether this count is a cause or result of the bleeding (temporal ambiguity). More than 20 potential prognostic factors for pulmonary hemorrhage were compared between cases and controls, and results were not adjusted for confounders.

Finally, the fourth case control analysis,(2) in a cohort of neonates with severe thrombocytopenia (<60 x 10^9/L) showed no association between lowest platelet count and major bleeding. In this study, the lowest platelet count prior to major bleeding in neonates with major bleeding was compared to lowest platelet count during the thrombocytopenic episode in neonates without major bleeding. Results were reported in a graph, no statistical analyses were performed. The median platelet counts in neonates with major, minor or no bleeding were comparable, with overlapping interquartile ranges, suggesting no association between platelet count and bleeding. The number of platelet transfusions in each study group was provided but included both therapeutic and prophylactic platelet transfusions.

In short, four papers assessed the association between platelet count and bleeding, of which two suggest that pulmonary hemorrhage may be associated with thrombocytopenia, and two suggest that platelet count is not associated with pulmonary hemorrhage, or all types of major bleeding, respectively. No meta-analysis was performed due to the various limitations and heterogeneous study populations.

Table 4 Overview of six included studies in which the association between platelet count and major bleeding is assessed

Author	Year	Population	No total	No groups	Study groups	Outcome	Type of bleed	Result of analysis	Risk, Odds, Hazard ratio	Adjusted for confounders
Studies in which the association between PLATELET COUNT and major bleeding is assessed										
Berger 2000	Case control	GA <34 weeks.[2]	148	50 98	Cases: PH. Controls: no PH	Not reported	PH	PH associated with platelet count <100	OR 4.0 (1.4-13.3)	Yes
De Carolis 1998	Case control	GA < 30 weeks, included in one of 2 surfactant RCTs	79	10 69	Cases: PH. Controls: no PH.	113 (82-120)[3] 183 (138-220)	PH	PH associated with lower median platelet count[4]	OR_c[5] 20.6 (2.5-172.9)[6]	No
Stan worth 2009	Case control	Platelet count < 60	169	15 123 31	Group A: major hemorrhage Group B: minor hemorrhage Group C: no hemorrhage	<50 in 13 neonates	ALL	No analysis, counts reported in table and figure in paper	No analysis, but overlapping inter-quartile ranges	NA
Use mann 2017	Case control	BW <1000 g	60	20 40	A: pulmonary hemorrhage B: no pulmonary hemorrhage	170 (61-446) 183 (45-443)[7]	PH	No association	NA	No

Table 4 Overview of six included studies in which the association between platelet count and major bleeding is assessed (*continued*)

Author	Study type	Population	N total	N in groups	Study groups	Outcome	Type of bleed	Result of analysis	Risk/odds/hazard ratio (95% CI)	Adjusted for confounders
Studies in which the association between PLATELET TRANSFUSION and major bleeding is assessed										
Andrew 1993	RCT	GA < 33 weeks, BW 500-1500 g, platelet count < 150	152	78 74	A: tx if platelet count <150 B: tx if platelet count < 50 or bleeding	12 bleeds 5 bleeds	IVH	No separate analysis of major bleeds[8]	RR_c 2.3 (0.8-6.2)	NA
Sparger 2016	Cohort	BW < 1500 g	972	Not reported	A: transfused B: not transfused	62 bleeds	IVH	No association	HR 1.24 (0.6-2.6)	Yes
Usemann 2017	Case control	BW <1000	60	20 40	A: pulmonary hemorrhage B: no pulmonary hemorrhage	5 PLT tx 4 PLT tx	PH	No association	OR_c 3.0 (0.7-12.7)	No

Legend (1) IVH = intraventricular hemorrhage grade 3 or 4. PH = pulmonary hemorrhage. GI = gastro-intestinal hemorrhage, ALL = different types of major bleed combined. RCT = randomized controlled trial. GA = gestational age. BW = birth weight. NICU = neonatal intensive care unit. NA = not applicable. Platelet counts are represented as numbers only (e.g. <150) for clarity, but indicate counts per 10^9/L, (e.g. 150 x 10^9/L)

(2) These papers also included a near-term group, but because results for the near-term infants only could not be extracted from the results section, only the results for the GA<34 weeks group are presented here

(3) Platelet counts in first 12 hours of life. Median (25th – 75th percentile)

(4) Unclear which statistical test was used. The methods section mentions the student t-test and chi-square test with/without Yates correction, but as platelet count is presented as median, these tests are not applicable

(5) Ratios with underscore indicate that this ratio was not presented in the paper but was calculated for this review

(6)Analysis comparing thrombocytopenia (<150 x 10^9/L) vs no thrombocytopenia was also performed (9/10 in PH group and 21/69 in non-PH group, Odds ratio belongs to this analysis

(7) Platelet counts at birth compared between groups. Median (range)

(8) Number of IVH (all grades) in group A and B are compared in this study (no statistically significant difference), but analysis comparing IVH grade 3 and 4 to no IVH was not performed

Platelet transfusion and major bleeding

Three papers assessed the association between platelet transfusion and major bleeding, of which two focused on IVH and one on pulmonary hemorrhage.

A randomized controlled trial,(25) showed that platelet transfusions did not influence the presence or extent of any grade of IVH. In this study, 152 neonates were randomized between maintaining a platelet count >150 x 10^9/L versus a platelet count >50 x 10^9/L. Neonates with an initial platelet count <50 x 10^9/L were excluded, as were neonates who developed thrombocytopenia more than 72 hours after birth. It was difficult to calculate the number of new major IVHs from the study results, as it was unclear which major bleeds at end of study were already present at study entry. Based on the available data, at least 17 new cases of severe IVH must have occurred, of which 12 in the 150-threshold group (n=78) and 4 in the 50-threshold group (n=74), which would yield a risk ratio for severe IVH of 2.3 (95% CI 0.8 – 6.2).

A cohort study,(26) showed that platelet transfusions were not associated with reduced risk of severe IVH, with a hazard ratio of 1.24 (0.6 – 2.6). In this study, platelet transfusions were analyzed as a time-dependent variable in a Cox model with IVH grade III or IV within the next 7 days as outcome. The hazard ratio of 1.24 was adjusted for sex, gestational age <28 weeks, 5-minute Apgar score <7, antenatal corticosteroid treatment and pregnancy induced hypertension as an indication for delivery. Neonates with severe IVH on the first day of life were omitted from the analysis. The number of major bleeds in the model is not provided, and the number of patient days with platelet counts <50 x 10^9/L is low according to the authors (number not provided). The platelet transfusion thresholds at which transfusions were administered was >50 x 10^9/L in 65.4% of transfusions.

A case-control study,(28) showed no association between platelet transfusion and pulmonary hemorrhage. In this study, the proportion of neonates receiving a platelet transfusion prior to pulmonary hemorrhage (25%) was compared to the proportion of neonates receiving a platelet transfusion in the first 5 days of life (10%) in their matched controls without pulmonary hemorrhage. More than 20 potential prognostic factors for pulmonary hemorrhage were compared between cases and controls, and results were not adjusted for confounders. The thresholds for which platelet transfusions were administered were not recorded.

In short, three papers assessed the association between platelet transfusion and bleeding, and none of these showed that platelet transfusions were associated with reduced bleeding risk. No meta-analysis was performed due to the various limitations and heterogeneous study populations.

DISCUSSION

This extensive review does not demonstrate any convincing evidence for an association between major bleeding and platelet count or platelet transfusion in preterm neonates. Out of 36 eligible papers only six appropriately avoided temporal ambiguity, which is crucial when attempting to assess causality. The results of these six included papers are conflicting and inconclusive.

Platelet count and major bleeding

Two papers showed an association between pulmonary hemorrhage and thrombocytopenia, but two additional papers could not show an association between platelet count and all types of bleeding combined or pulmonary hemorrhage.

Unfortunately, all included studies had major limitations, as discussed in the results section. Platelet transfusion data were scarcely reported, therefore the effect of transfusions on platelet count could not be assessed. In addition, time-to-event analyses were not performed. This is important, because platelet counts can drop quickly but thrombocytopenia may go undetected for hours or even days, if the clinical status of the neonate does not warrant a platelet count measurement. The same applies to major intracranial bleeding, which is asymptomatic in a substantial number of neonates.(30) Furthermore, the two studies that found an association between pulmonary hemorrhage and thrombocytopenia defined thrombocytopenia as a platelet count $<100 \times 10^9$/L or $<150 \times 10^9$/L. Given the current prophylactic platelet transfusion thresholds of 20-50 x 10^9/L, these results are difficult to translate into clinical practice. In addition, it is possible that these studies found an association because they selected a relatively sick group of neonates and did not appropriately correct for confounding. Lastly, an additional complicating factor is platelet count measurement error in severe thrombocytopenia. This was demonstrated in a recent study, in which results of platelet count measurements varied widely between nine laboratories, resulting in significant potential under- and overtransfusion.(31) Previous studies have shown that measurement error in an exposure variable can significantly affect the estimation of an effect, resulting in false claims of no association, where in fact there is.(32)

In short, based on these studies, we cannot determine whether or not platelet counts are causally associated with major bleeding.

Platelet transfusion and major bleeding

Three studies were included, but none of these found an association between platelet transfusion and bleeding.

Unfortunately, these studies are also hampered by various serious limitations, as discussed in the results section. Therefore, no recommendations with regard to prophylactic platelet transfusions in preterm neonates can be made due to lack of evidence. This is in agreement with the conclusions of a structured review published in 2014, even though our study selection differs significantly from the selection in this review, due to different in- and exclusion criteria.(22)

Although the three studies assessing platelet transfusion in this review are hampered by severe limitations, the fact that no studies have yet found a protective effect of platelet transfusions on bleeding risk in preterm neonates is remarkable. In fact, the risk ratio of 2.3 in the randomized controlled trial, hazard ratio of 1.24 in the cohort study and odds ratio of 3.0 in the case-control study – though not statistically significant - suggest that transfusions could potentially be associated with increased risk of bleeding. This result might be due to confounding by indication, as neonates perceived to be at high risk of bleeding may receive more platelet transfusions. However, true adverse effects of platelet transfusions cannot be ruled out. Neonatal platelets are distinctly different from adult platelets, but platelet transfusions contain adult – not neonatal – platelets.(19, 33) Platelet transfusions are associated with higher risk of bacterial infection compared to other transfusions, because they are stored at room temperature. Also, platelet transfusions are given in relatively high volumes, which might disturb intracerebral blood pressure in neonates in a detrimental way.(30) It is thought that platelet transfusion related adverse events in neonates are underrecognized because of the high rate of morbidity and mortality in the NICU. Because of their immature immune and metabolic processes, and simultaneous rapid neurodevelopment, neonates are especially at risk for potential toxic and infective effect of transfusions.(21) Lastly, it is intriguing that a recent trial in adults with intracranial hemorrhage also showed increased risk of death or dependence in patients treated with platelet transfusions.(18)

It is extremely worrisome that no trials have yet conclusively shown a protective effect of platelet transfusions on bleeding risk in preterm neonates with severe thrombocytopenia, given the fact that over 75% of this population receives platelet transfusions. High-quality studies are urgently needed to accurately assess this association. A large randomized controlled trial has been performed in the UK, Ireland and the Netherlands, assessing major bleeding and mortality in neonates treated according to a 25 x 10^9/L versus 50 x 10^9/L prophylactic platelet transfusion threshold, and results have recently been published. The 50 x 10^9/L transfusion threshold was associated with increased risk of major bleeding or mortality compared with the 25 x 10^9/L threshold, within 28 days after randomization. This confirms our hypothesis regarding potentially adverse effects of platelet transfusions, and warrants reconsideration of our current platelet transfusion guidelines for neonates.(34, 35)

Strengths and limitations

Strengths of our review are the broad search strategy, and the fact that we included both randomized controlled trials and observational studies. Also, we have assessed the included papers very critically, including an estimation of whether or not they were subject to temporal ambiguity. Finally, we have provided specific and practical recommendations for future studies (see below).

Limitations of the study are the lack of randomized controlled trials, the fact that we did not search for grey literature, a small percentage of abstracts was screened by one author only, and no pooling or meta-analysis could be performed due to high risk of bias and heterogeneous study populations.

SUMMARY AND FUTURE DIRECTIONS

Areas for future research

Future studies assessing the association between platelet count or transfusions and major bleeding are advised to adhere to the following recommendations:

1. choose the appropriate study design. For platelet transfusion studies a randomized controlled trial is preferred. However, large high quality cohort studies can provide additional evidence. Large cohort studies are also the recommended study design for platelet count studies. Case control studies are not recommended, because time to event analyses are usually not performed and only relative risks are provided.
2. avoid temporal ambiguity by excluding major bleeds prior to thrombocytopenia/ platelet transfusions and using data from events prior to the major bleed, and a comparable time-period in the control group.
3. minimize missing data, by using a prospective design with strict platelet count measurement protocols and regular ultrasound scans, including measurements immediately after birth.
4. include a large number of neonates.
5. correct for confounders, and – in case of a platelet count study - think about ways to correct for the effect of platelet transfusions on platelet count.
6. use a time-to-event analysis, because informative censoring is likely to occur. Informative censoring happens when, for example, relatively healthy neonates are discharged or transferred, because they have lower risk of bleeding compared to non-discharged neonates.
7. correct for competing risk, as it is expected that a substantial number of neonates in these populations might die, and are therefore not at risk for developing a major bleed anymore.

Concluding remarks

In conclusion, there is insufficient evidence to assess whether platelet counts are causally related to major bleeding, or whether platelet transfusions reduce bleeding risk in thrombocytopenic preterm neonates. Several studies suggest that platelet transfusions may not reduce bleeding risk or may perhaps even increase it. High quality studies are urgently needed to assess these associations and provide a rationale for our platelet transfusion guidelines.

Practice points

- The severity of thrombocytopenia may not be directly related to risk of major bleeding in preterm neonates
- Prophylactic platelet transfusions may not reduce risk of bleeding in preterm neonates. Therefore, the need for a transfusion must be weighed against possible adverse effects.

Research agenda

- Develop prediction models for major bleeding and personalized platelet transfusion guidelines
- Perform randomized controlled trials comparing different transfusion thresholds
- Improve quality of observational studies (using recommendations in this review)

Supporting Information

- Additional Supporting Information may be found in the online version of this article:
- Table S1 Overview of all eligible papers (n=36)
- Table S2 assessment of temporal ambiguity for all eligible studies (n=36)

Funding

This research was supported by Grant PPOC-12-012027 from Sanquin Research, Amsterdam. The study sponsor had no involvement in the collection, analysis and interpretation of data and the writing of the manuscript.

Acknowledgements

We thank clinical librarians drs. ing. René Spijker and A.G.E. Leenders for their help in performing the literature search.

REFERENCES

1. Baer VL, Lambert DK, Henry E, Christensen RD. Severe Thrombocytopenia in the NICU. Pediatrics. 2009;124(6):e1095-100.
2. Stanworth SJ, Clarke P, Watts T, Ballard S, Choo L, Morris T, et al. Prospective, observational study of outcomes in neonates with severe thrombocytopenia. Pediatrics. 2009;124(5):e826-34.
3. von Lindern JS, Hulzebos CV, Bos AF, Brand A, Walther FJ, Lopriore E. Thrombocytopaenia and intraventricular haemorrhage in very premature infants: a tale of two cities. Arch Dis Child-Fetal. 2012;97(5):348-U105.
4. Horbar JD, Carpenter JH, Badger GJ, Kenny MJ, Soll RF, Morrow KA, Buzas JS. Mortality and Neonatal Morbidity Among Infants 501 to 1500 Grams From 2000 to 2009. Pediatrics. 2012;129(6):1019-26.
5. Adams M, Bassler D, Bucher HU, Roth-Kleiner M, Berger TM, Braun J, et al. Variability of Very Low Birth Weight Infant Outcome and Practice in Swiss and US Neonatal Units. Pediatrics. 2018;141(5).
6. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, et al. Neonatal Outcomes of Extremely Preterm Infants From the NICHD Neonatal Research Network. Pediatrics. 2010;126(3):443-56.
7. Ballabh P. Pathogenesis and Prevention of Intraventricular Hemorrhage. Clinics in Perinatology. 2014;41(1):47-67.
8. Ahmad KA, Bennett MM, Ahmad SF, Clark RH, Tolia VN. Morbidity and mortality with early pulmonary haemorrhage in preterm neonates. Arch Dis Child-Fetal. 2019;104(1):F63-F8.
9. Mukerji A, Shah V, Shah PS. Periventricular/Intraventricular Hemorrhage and Neurodevelopmental Outcomes: A Meta-analysis. Pediatrics. 2015;136(6):1132-43.
10. Bolat F, Kiliç SÇ, Oflaz MB, Gülhan E, Kaya A, Güven AS, et al. The Prevalence and Outcomes of Thrombocytopenia in a Neonatal Intensive Care Unit: A Three-Year Report. Pediatr Hemat Oncol. 2012;29(8):710-20.
11. Ulusoy E, Tüfekçi Ö, Duman N, Kumral A, Irken G, Ören H. Thrombocytopenia in neonates: causes and outcomes. Annals of Hematology. 2013;92(7):961-7.
12. Christensen RD, Henry E, Wiedmeier SE, Stoddard RA, Sola-Visner MC, Lambert DK, et al. Thrombocytopenia among extremely low birth weight neonates: data from a multihospital healthcare system. J Perinatol. 2006;26(6):348-53.
13. Beiner ME, Simchen MJ, Sivan E, Chetrit A, Kuint J, Schiff E. Risk factors for neonatal thrombocytopenia in preterm infants. Am J Perinat. 2003;20(1):49-54.
14. Gunnink SF, Vlug R, Fijnvandraat K, van der Bom JG, Stanworth SJ, Lopriore E. Neonatal thrombocytopenia: etiology, management and outcome. Expert Rev Hematol. 2014;7(3):387-95.
15. von Lindern JS, van den Bruele T, Lopriore E, Walther FJ. Thrombocytopenia in neonates and the risk of intraventricular hemorrhage: a retrospective cohort study. Bmc Pediatr. 2011;11.
16. Slichter SJ, Kaufman RM, Assmann SF, McCullough J, Triulzi DJ, Strauss RG, et al. Dose of Prophylactic Platelet Transfusions and Prevention of Hemorrhage. New Engl J Med. 2010;362(7):600-13.
17. Stanworth SJ, Estcourt LJ, Powter G, Kahan BC, Dyer C, Choo L, et al. A No-Prophylaxis Platelet-Transfusion Strategy for Hematologic Cancers. New Engl J Med. 2013;368(19):1771-80.
18. Baharoglu MI, Cordonnier C, Salman RAS, de Gans K, Koopman MM, Brand A, et al. Platelet transfusion versus standard care after acute stroke due to spontaneous cerebral haemorrhage associated with antiplatelet therapy (PATCH): a randomised, open-label, phase 3 trial. Lancet. 2016;387(10038):2605-13.

19. Ferrer-Marin F, Chavda C, Lampa M, Michelson AD, Frelinger AL, Sola-Visner M. Effects of -adult platelet transfusions on neonatal hemostasis. Journal of Thrombosis and Haemostasis. 2011;9:793-4.
20. Stainsby D, Jones H, Wells AW, Gibson B, Cohen H, Grp SS. Adverse outcomes of blood transfusion in children: analysis of UK reports to the serious hazards of transfusion scheme 1996-2005. Brit J Haematol. 2008;141(1):73-9.
21. New HV. Paediatric transfusion. Vox Sanguinis. 2006;90(1):1-9.
22. Lieberman L, Bercovitz RS, Sholapur NS, Heddle NM, Stanworth SJ, Arnold DM. Platelet transfusions for critically ill patients with thrombocytopenia. Blood. 2014;123(8):1146-51.
23. Moher D, Liberati A, Tetzlaff J, Altman DG, Grp P. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. Journal of Clinical Epidemiology. 2009;62(10):1006-12.
24. Venkatesh V, Curley A, Khan R, Clarke P, Watts T, Josephson C, et al. A novel approach to standardised recording of bleeding in a high risk neonatal population. Arch Dis Child-Fetal. 2013;98(3):F260-F3.
25. Andrew M, Vegh P, Caco C, Kirpalani H, Jefferies A, Ohlsson A, et al. A Randomized, Controlled Trial of Platelet Transfusions in Thrombocytopenic Premature-Infants. J Pediatr-Us. 1993;123(2):285-91.
26. Sparger KA, Assmann SF, Granger S, Winston A, Christensen RD, Widness JA, et al. Platelet Transfusion Practices Among Very-Low-Birth-Weight Infants. Jama Pediatr. 2016;170(7):687-94.
27. De Carolis RP, Romagnoli C, Cafforio C, Piersigilli F, Papacci P, Vento G, Tortorolo G. Pulmonary haemorrhage in infants with gestational age of less than 30 weeks. European Journal of Pediatrics. 1998;157(12):1037-8.
28. Usemann J, Garten L, Bührer C, Dame C, Cremer M. Fresh frozen plasma transfusion - a risk factor for pulmonary hemorrhage in extremely low birth weight infants? Journal of Perinatal Medicine. 2017;45(5):627-33.
29. Berger TM, Allred EN, Marter LJ Van. Antecedents of Clinically Significant Pulmonary Hemorrhage Among Newborn Infants. J Perinatol 2000;5:295–300.
30. Bassan H. Intracranial Hemorrhage in the Preterm Infant: Understanding It, Preventing It. Clinics in Perinatology. 2009;36(4):737-762.
31. Lozano M, Mahon A, van der Meer PF, Stanworth S, Cid J, Devine D, et al. Counting platelets at transfusion threshold levels: impact on the decision to transfuse. A BEST Collaborative - UK NEQAS(H) International Exercise. Vox Sanguinis. 2014;106(4):330-6.
32. Hutcheon JA, Chiolero A, Hanley JA. Random measurement error and regression dilution bias. Bmj-Brit Med J. 2010;340.
33. Sola-Visner M. Platelets in the neonatal period: developmental differences in platelet production, function, and hemostasis and the potential impact of therapies. Hematology-American Society Hematology Education Program. 2012:506-11.
34. Curley A, Venkatesh V, Stanworth S, Clarke P, Watts T, New H, et al. Platelets for Neonatal Transfusion - Study 2: A Randomised Controlled Trial to Compare Two Different Platelet Count Thresholds for Prophylactic Platelet Transfusion to Preterm Neonates. Neonatology. 2014;106(2):102-6.
35. Curley A, Stanworth SJ, Willoughby K, Fustolo-Gunnink SF, Venkatesh V, Hudson C, et al. Randomized Trial of Platelet-Transfusion Thresholds in Neonates. N Engl J Med. 2019;380(3):242-51.

Supplementary Table S1 Overview of all 36 eligible papers

RCTs in which the association between PLATELET TRANSFUSION PROTOCOL and major bleeding is assessed										
Author	**Year**	**Population**	**N total**	**N in groups**	**Study groups**	**No of bleeds**	**Type of bleed**	**Temporal ambiguity**	**Result of analysis**	**Adjusted for confounders**
Andrew	1993	GA < 33 weeks, BW 500-1500 g, platelet count < 150 during first 72 hours of life.	152	78 74	Group A: tx if platelet count <150 Group B: tx if platelet count < 50 or bleeding	12 5	IVH	No	No analysis[1] (RR_c 2.3 (0.8-6.2)	NA
Ozturk	2015	Platelet count <100 x 10^9/L	100	50 50 50 50 50 50	Group A: tx based on platelet mass Group B: tx based on platelet count Group A: tx based on platelet mass Group B: tx based on platelet count Group A: tx based on platelet mass Group B: tx based on platelet count	1 2 3 0 3 2	IVH GI PH	Yes	RR_c IVH 2.0 (0.2 – 21.4) RR_c GI 0.1 (0.0-2.7) RR_c PH 0.7 (0.1-3.8)	No
Zisk	2013	Platelet count <100	30	15 15 15 15	Group A: platelet mass tx protocol Group B: platelet count tx protocol Group A: platelet mass tx protocol Group B: platelet count tx protocol	2 1 1 0	IVH PH	Yes	RR_c IVH 2.0 (0.2-19.8) RR_c PH 3.0 (0.1 – 69.2)	No

Abbreviations ALL = different types of major bleed combined, BW = birth weight, GA = gestational age, GI = gastro-intestinal hemorrhage, IVH = intraventricular hemorrhage grade 3 or 4, MPV = mean platelet volume, NA = not applicable, NICU = neonatal intensive care unit, PH = pulmonary hemorrhage, RCT = randomized controlled trial, tx = transfusion

Legend Platelet counts are represented as numbers only (e.g. <150) for clarity, but indicate counts per 10^9/L, (e.g. 150 x 10^9/L)

[1] Number of IVH (all grades) in group A and B compared, but not IVH grade 3 and 4 on.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Cohort studies in which the association between PLATELET TRANSFUSION and major bleeding is assessed										
Author	**Year**	**Population**	**N total**	**N in groups**	**Study groups**	**No of bleeds**	**Type of bleed**	**Temporal ambiguity**	**Result of analysis**	**Adjusted for confounders**
Murray	2002	Platelet count < 50	44	25 19	A: transfused B: not transfused	0 0	IVH	NA (no events)	NA (no events)	NA
Stanworth	2009	Platelet count < 60	169	116 53	A: transfused B: not transfused	14 1	ALL	Yes	No analysis[1] (OR_c: 7.1 (0.9 to 55.8)	NA
Sparger	2016	Very low birth weight infants	972	Not reported	Transfusion analyzed as dichotomous time varying covariate (transfused / not transfused)	62	IVH	No	No association (HR 1.24 (0.6-2.6)	Yes
Von Lindern	2012	GA <32 weeks and thrombocytopenia.	653	141 147	A: liberal transfusion B: restrictive transfusion	16 12	IVH	Yes	RR_c IVH 1.3 (0.7-2.8)	Yes
				141 147	A: liberal transfusion B: restrictive transfusion	2 1	PH	Yes	No analysis RR_c PH 2.1 (0.2-22.7)	NA

[2] Only numbers are presented, no further analysis performed.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Case-control studies in which the association between PLATELET TRANSFUSION and major bleeding is assessed										
Author	**Year**	**Population**	**N total**	**N in groups**	**Study groups**	**N transfusions**	**Type of bleed**	**Temporal ambiguity**	**Result of analysis**	**Adjusted for confounders**
Baer[3]	2011	Neonates with IVH grade 1.	408	46 362	Cases: grade 1 IVH that progressed to severe IVH Controls: resolved grade 1 IVH	18 20	IVH	Yes	Platelet tx associated with increased bleeding risk. OR 5.4 (3.1-10.0)	Yes
Baer[1]	2011	VLBW (<1500 grams)	155	54 101	Cases: 1st ultrasound scan no major bleed, next scan IVH 3/4. Controls: no hemorrhage.	7 5	IVH	Yes	No association. OR_c: 2.9 (0.9-9.5)	No
Lee	2010	GA ≤ 34 weeks.	157	39 118	Cases: IVH grade 3 or 4 Controls: no IVH	20 21	IVH	Yes	No analysis.[2] OR_c: 4.9 (2.2-10.7)	No
Usemann	2017	BW < 1000 g	60	20 40	A: pulmonary hemorrhage B: no pulmonary hemorrhage	5 4	PH	No	OR_c 3.0 (0.7-12.7)	No

[3] Some overlap might exist between 3 papers by Baer et al. Baer 2011 includes 2 patients with VLBW and severe thrombocytopenia, that may have been included in Baer 2009. Baer 2011 includes an unknown number of neonates with severe thrombocytopenia that may have been included in Baer 2009, and an unknown number of neonates with VLBW, which may have been included in Baer 2011.

[4] Number of platelet transfusions compared between grade 2-4 and controls, not grade 3-4 and controls. Platelet transfusion associated with increased bleeding risk when grade 2-4 combined.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Cohort studies in which the association between PLATELET COUNT and major bleeding is assessed

Author	Year	Population	N total	N in groups	Study groups	N bleeding	Type of bleed	Temporal ambiguity	Result of analysis	Adjusted for confounders
Baer[3]	2009	Two platelet counts of ≤ 50	273	78 78 117	A: platelet count <20 B: platelet count 20-30 C: platelet count 31-50	13 12 22	IVH	Yes	No association between platelet count and bleeding[1]	No
				78 78 117	A: platelet count <20 B: platelet count 20-30 C: platelet count 31-50	6 6 7	PH			
				78 78 117	A: platelet count <20 B: platelet count 20-30 C: platelet count 31-50	4 1 2	GI			
Beiner	2003	GA 27-35 weeks.	305	93 212	A: platelet count ≤ 150 B: platelet count >150	9 7	IVH	Yes	RR_c 2.9 (1.1-7.6)	No
Bolat	2012	GA<37 weeks.	1246	185 1061	A: platelet count < 150 B: platelet count ≥ 150	4 12	PH	Yes	RR_c 1.9 (0.6 – 5.9) [2]	NA
Elmoneim	2015	GA < 32 weeks, NICU admission on DOL 1, survived > 7 days. Thrombocytopenia in DOL 1-7 excluded.	188	31 98 41 18	A: no thrombocytopenia, no drop (in platelet count) B: no thrombocytopenia, drop C: thrombocytopenia, no drop D: thrombocytopenia and drop	29 total	IVH	Yes	A is reference. OR B: 8.2 (1.1-63.6) OR C: no cases. OR D: 19.1 (2.1-173.4)	No
					Groups as in previous IVH analysis	10 total	Other	Yes	A is reference. OR B: 1.3 (0.1-11.9) OR C: 0.8 (0.1-12.5). OR D: 8.6 (0.9-83.9)	No

[5] Unclear what statistical analysis was performed to support this conclusion.
[6] Analysis in papers was performed for overall population, we have selected preterm neonates for this table. Results of overall analysis: no statistically significant difference between 2 groups with respect to pulmonary hemorrhage.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Cohort studies in which the association between PLATELET COUNT and major bleeding is assessed										
Author	**Year**	**Population**	**N total**	**N in groups**	**Study groups**	**N bleeding**	**Type of bleed**	**Temporal ambiguity**	**Result of analysis**	**Adjusted for confounders**
Kahn	2002	BW < 1500 g.	1283	145 1138	Group A: platelet count <100. Group B: platelet count ≥ 100.	30 73	IVH	Yes	Plt 50-99: OR 2.4 (1.2-4.6) Plt <50: OR 2.3 (0.5-9.7) Plt ≥100: reference	Yes[3]
Murray	2002	Platelet count < 50	44	17 27	Group A: platelet count nadir < 30 Group B: platelet count nadir 30-50	0 0	IVH	NA	No analysis	NA
Rastogi (1)	2011	GA ≤ 28 weeks and survived ≥ 7 days.[4]	286	74 73 17 122	A: no thrombocytopenia, no platelet count drop[5] B: no thrombocytopenia, ≥ 30% platelet count drop C: thrombocytopenia, no platelet count drop D: thrombocytopenia, ≥ 30% platelet count drop	Not reported[6] 0 Not reported[10] Not reported[10]	IVH	Yes	A: reference. B: OR 3.7 (0.6-24.1), D: OR 11.6 (2.6-52.3) No cases in group C	No
Rastogi (2)[7]	2011	GA ≤ 28 weeks and survived ≥ 7 days.	286	146 85 31 18 6	A: no thrombocytopenia B: platelet count 75-149 C: platelet count 50-74 D: platelet count 25-49 E: platelet count <25	Not reported[10]	IVH	Yes	A: reference OR B: 3.3(1.1-9.9) OR C: 6.4 (2-20.6) OR D: 12.6(3.4-47.5) OR E: 9.4(2.4-36.7)	No

[3] Adjusted for antenatal corticosteroids, maternal hypertension, presentation, Apgar score, neonatal hypotension, gender, race and site.
[4] Methods section states exclusion of thrombocytopenia prior to day 7, but results show number of neonates with thrombocytopenia prior to day 7. 2nd Rastogi paper suggests that thrombocytopenia < day 7 was not an exclusion criterion.
[5] All platelet counts were assessed on day of life 28.
[6] Only odds ratios and confidence intervals are provided.
[7] Note: this paper is based on the same cohort as the Rastogi(1) paper.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Cohort studies in which the association between PLATELET COUNT and major bleeding is assessed										
Author	**Year**	**Population**	**N total**	**N in groups**	**Study groups**	**N bleeding**	**Type of bleed**	**Temporal ambiguity**	**Result of analysis**	**Adjusted for confounders**
Ree	2017	Culture positive sepsis	460	86 353[8]	A: platelet count ≤50 B: platelet count >50	9 20	IVH	No	RR_c 1.8 (0.9-3.9)	No
Stanworth	2009	Platelet count < 60	169	15 123 31	Group A: major hemorrhage Group B: minor hemorrhage Group C: no hemorrhage	<50 in 13 neonates B,C in fig	ALL	No	No analysis, counts group A reported, counts B&C in figure.	NA
Von Lindern	2011	Platelet count <150	422[9]	69 67 164 122	A: platelet count <30 B: platelet count 30-49 C: platelet count 50-99 D: platelet count 100-149	4 1 15 9	IVH	Yes	Platelet count not associated with IVH	Unclear[10]
Von Lindern	2012	GA <32 weeks.	653	367 99 104 40 43	Group A: platelet count >150 Group B: platelet count 100-149 Group C: platelet count 50-99 Group D: platelet count 30-49 Group E: platelet count <30	14 7 12 3 6	IVH	Yes	Severity of thrombocytopenia not an independent predictor of IVH	Yes
				288 365[11]	Group A: platelet count <150 Group B: platelet count ≥150	14 28			Platelet count <150 independent predictor of IVH	

[8] Exposure of 13 cases without ultrasound scan and 8 with severe IVH prior to thrombocytopenia not reported in paper but provided by author via personal communication.
[9] Overlap with Von Lindern et al 2012. Neonates with GA<32 weeks, included between January 2007 and December 2008 were included in both studies. Number unclear, but rough estimate based on mean suggests that this involves 210 neonates.
[10] Logistic regression mentioned in text, but unclear whether this involved adjustment for confounders, and if so, which confounders was adjusted for. 1111111111
[11] Numbers don't seem to add up in paper. 50 patients with platelet count 50-100 in restrictive group in table 2 but 49 in table 3. And 20 patients with platelet count <30 in restrictive group in table 2 but 19 in table 3.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Case-control studies in which the association between PLATELET COUNT and major bleeding is assessed

Author	Year	Population	N total	N in groups	Study groups	Platelet counts	Type of bleed	Temporal ambiguity	Result of analysis	Adjusted for confounders
Amato	1988	GA < 35 weeks	21	15 6	A: IVH grade I-II B: IVH grade III-IV	255 ± 81 195 ± 55	IVH	Yes	Severe IVH associated with lower platelet count (p < 0.02)	No
Auerbach	2013	BW < 2000 g.	178	70 108	Cases: IVH grade 3 or 4 Controls: no IVH	16 <100 3 < 100	IVH	Yes	IVH associated with platelet count <100. OR 7.9 (1.9-33.3)	Yes
Baer[1]	2011	VLBW (<1500 grams) Initial normal ultrasound scan.	155	54 101	Cases: IVH grade 3 or 4 following normal ultrasound scan Controls: no hemorrhage.	1 <50 1 <50	IVH	Yes	IVH not associated with severe thrombo-cytopenia (p-value 0.457)	No
Baer[3]	2011	Neonates with initial IVH grade 1. Excluded: progression from grade 1 to 2.	408	46 362	Cases: grade 1 IVH that resolved Controls: grade 1 IVH that progressed to severe IVH	200 ± 58[2] 222 ± 68[17]	IVH	Yes	IVH progression associated with lower platelet count (p-value 0.047)	No

[1] Some overlap might exist between 3 papers by Baer et al. Baer 2011 includes 2 patients with VLBW and severe thrombocytopenia, that may have been included in Baer 2009. Baer 2011 includes an unknown number of neonates with severe thrombocytopenia that may have been included in Baer 2009., and an unknown number of neonates with VLBW, which may have been included in Baer 2011.

[2] First platelet count recorded during first 3 days after IVH grade 1. Mean (SD).

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Case-control studies in which the association between PLATELET COUNT and major bleeding is assessed

Author	Year	Population	N total	N in groups	Study groups	Platelet counts	Type of bleed	Temporal ambiguity	Result of analysis	Adjusted for con-founders
Baer[3]	2009	Platelet count ≤ 50	273	47 94	Cases: IVH grade 3 or 4 Controls: no IVH grade 3 or 4[3]	29±11[4] 125±83	IVH	Yes	IVH associated with lower platelet count (p-value <0.0001)	No
Berger	2000	GA <34 weeks.[5]	148	50 98	Cases: PH Controls: no PH	Not reported	PH	No	PH associated with platelet count <100 OR 4.0 (1.4-13.3)	Yes
Beverley	1984	GA ≤ 34 weeks.	101	81 5 15 5	Group A: no IVH Group B: grade 4 Group C: grade 2 and 3 Group D: grade 1	226 (56.3)[6] 149 (95.4) 222 (96.7) 232 (127.5)	IVH[7]	Yes	No association[8]	No
Braun	1999	GA <32 weeks.[9]	84	17 34	Cases: PH Controls: no PH	169 ± 74[10] 213 ± 84	PH	Yes	IVH not associated with platelet count (p-value > 0.05)	Unclear[11]

[3] Mean of lowest platelet count in this group: 125 ± 83. This suggests that control group did not have severe thrombocytopenia. Unclear how control group was selected.
[4] Lowest recorded platelet count. Mean±SD.
[5] This papers also included a near term / term group, but because results for the near-term infants could not be extracted in the results section, only the results for the GA<34 weeks group is presented here.
[6] Platelet count at birth. Mean (SD). Platelet counts were also measured 48 h after after birth (IVH associated with lower platelet count), but 17 bleeds (grade unknown) prior to 48 h, results therefore omitted from this table.
[7] IVH grade 4 only (not grade 3).
[8] No association at birth. IVH associated with lower platelet count at 48 hours, but 17 bleeds (grade unknown) occurred on day of life 1. These results have therefore been omitted from this table.
[9] Results for pre-surfactant era also reported in paper. Because these are not relevant for our current clinical practice, these results have been omitted from this table.
[10] Mean ± SD
[11] Multivariate analysis was performed, but unclear if platelet count was entered as variable.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Case-control studies in which the association between PLATELET COUNT and major bleeding is assessed

Author	Year	Population	N total	N in groups	Study groups	Platelet counts	Type of bleed	Temporal ambiguity	Result of analysis	Adjusted for confounders
Christensen	2014	GA ≤ 34 weeks, cord blood coagulation tests performed.	192	67 19 9 97	Group A: no IVH Group B: IVH grade 1 or 2 Group C: IVH grade 3 or 4 Group D: no ultrasound scan	221 ± 5525 230 ± 58 187 ± 59 249 ± 54	IVH	Yes	No association IVH (3 or 4 vs no IVH) and platelet count (p-value 0.097)	No
Dani	2011	GA < 30 weeks. Platelet count <50 excluded.	210	17 193	Cases: IVH grade 3-4 Controls: no IVH grade 3-4	173.0 ± 73.0[12] 206.0 ± 81.7	IVH	Yes	IVH associated with lower platelet count (p-value 0.020)	No
De Carolis	1998	GA < 30 weeks, included in one of 2 surfactant RCTs	79	10 69	Cases: PH. Controls: no PH	113 (82-120)[13] (9 <150) 183 (138-220) (21 <150)	PH	No	PH associated with lower platelet count	No
Jen	2006	BW < 1000 g.	106	12 12	Cases: IVH progression Controls: no IVH progression.	Not reported[14]	IVH	Yes	IVH associated with thrombocytopenia (<100). OR 6 (0.7-276.0)	No
Lim	2011	GA ≤ 26 weeks BW ≤ 1000 g	72	36 36	IVH grade III or IVH No IVH	207.0 ± 86.5 219.2 ± 77.3	IVH	Yes	No association between IVH and platelet count (p 0.530)	No
				36 36	IVH grade III or IVH No IVH	96.2 ± 72.8[15] 35.5 ± 39.330	IVH	Yes	IVH associated with higher delta platelet count (p < 0.001)	

[12] Platelet counts measured at birth. Platelet counts at 24-28 hours also reported, but whether bleeding occurred prior to 48 hours is not reported, results therefore omitted from table. Mean ± SD.
[13] Platelet count in first 12 hours of life. Median (25th – 75th percentile).
[14] Only presence of thrombocytopenia coinciding with infant's initial IVH diagnosis or time of IVH progression were recorded. Platelet counts not reported in paper, only odds ratios.
[15] Platelet counts are represented as numbers only (e.g. <150) for clarity, but indicate counts per 10^9/L, (e.g. 150 x 10^9/L). Difference between highest and lowest platelet count (not further specified)

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Case-control studies in which the association between PLATELET COUNT and major bleeding is assessed

Author	Year	Population	N total	N in groups	Study groups	Platelet counts	Type of bleed	Temporal ambiguity	Result of analysis	Adjusted for confounders
Linder	2003	BW <1500 g.	105	36 69	Cases: IVH grade 3 or 4. Controls: no IVH grade 3 or 4..	172.1 ± 77.77[16] 186.1 ± 67.27	IVH	Yes	No association (p-value 0.355)	No
Mitsiakos	2016	GA ≤ 28 weeks	130	31 99	Cases: IVH grade 3 or 4 Controls: IVH grade 1 or 2, or no IVH	208 ± 95 247 ± 83[17]	IVH	Yes	No association	No
Okur	2015	GA ≤ 32 weeks Platelet count <50 excluded.	330	133 197	Cases: IVH grade 3 or 4 Controls: no IVH	212 ± 74 (day 1) 228 ± 132 (3-7) 220 ± 85 (day 1) 264 ± 137 (3-7)	IVH	Yes	Platelet count between day 3-7 associated with increased bleeding risk. (p 0,02)	No
Usemann	2017	BW <1000 g	60	20 40	A: pulmonary hemorrhage B: no pulmonary hemorrhage	170 (61-446) 183 (45-443)[18]	PH	No	No association (p-value 0.51)	No
Van de Bor	1986	GA < 34 weeks	49	29 5 9 6	A: no IVH B: IVH grade I C: IVH grade II-III D: IVH grade IV	231 ± 15.0 242 ± 23 227 ± 23 139 ± 38	IVH	Yes	IVH grade IV associated with lower platelet count (group A vs D) (p < 0.01)	No
Zisk	2011	BW < 1500 g	408	43 365	Group A: severe IVH Group B: no severe IVH	190 ± 68[19] 196 ±71	IVH	Yes	No association (p-value 0.5)	No

Abbreviations ALL = different types of major bleed combined, BW = birth weight, CI = confidence interval, GA = gestational age, GI = gastro-intestinal hemorrhage, IVH = intraventricular hemorrhage grade 3 or 4,PH = pulmonary hemorrhage, MPV = mean platelet volume, NA = not applicable, NICU = neonatal intensive care unit, OR = odds ratio, RCT = randomized controlled trial

[16] Lowest platelet count in first 24 hours of life. Mean ± SD.
[17] Platelet counts (mean ± SD) were measured on the first day of life. Platelet counts on day 2, 3, 5 and 7 are also reported in the paper. None show an association between platelet count and bleeding.
[18] Platelet count at birth compared between groups. Median (range).
[19] Platelet count at the time of NICU admission. Mean ± SD.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Studies in which the association between PLATELET MASS and major bleeding is assessed										
Author	**Year**	**Population**	**N total**	**N in groups**	**Study groups**	**Platelet mass**	**Type of bleed**	**Temporal ambiguity**	**Result of analysis**	**Adjusted for confounders**
Mitsiakos	2016	GA ≤ 28 weeks	130	31 99	Cases: IVH grade 3 or 4 Controls: IVH grade 1 or 2, or no IVH	2073 ± 866 2395 ± 784[1]	IVH	Yes	No association between platelet mass and bleeding	No
Okur	2015	GA ≤ 32 weeks Platelet count <50 excluded.	330	133 197	Cases: IVH grade 3 or 4 Controls: no IVH	1706 ± 563[2] (day 1) 1987 ± 1136 (day 3-7) 1777 ± 668 (day 1) 2301 ± 1189 (day 3-7)	IVH	Yes	Lower platelet mass associated with increased bleeding risk between day 3-7 (p value 0.018)	No
Zisk	2011	BW < 1500 g	408	43 365	Cases: severe IVH Controls: no severe IVH	2060 ± 782[3] 2194 ± 719	IVH	Yes	IVH not associated with platelet mass at time of admission (p-value 0.28).	No
				39 ?[4]	Cases: severe IVH Controls: no severe IVH	Any mass <10%, mass <10% on admission, DOL 1 and 2. Numbers not reported.			IVH associated with any mass <10% (p-value < 0.01), mass <10% on DOL 1 (p-value 0.02) or 2 (p-value 0.01).	

Abbreviations ALL = different types of major bleed combined, BW = birth weight, CI = confidence interval, DOL = day of life, GA = gestational age, GI = gastro-intestinal hemorrhage, IVH = intraventricular hemorrhage grade 3 or 4,PH = pulmonary hemorrhage, MPV = mean platelet volume, NA = not applicable, NICU = neonatal intensive care unit, OR = odds ratio, RCT = randomized controlled trial

[1] Platelet counts are represented as numbers only (e.g. <150) for clarity, but indicate counts per 10^9/L, (e.g. 150 x 10^9/L).Platelet mass (mean ± SD) was measured on the first day of life. Platelet mass on day 2, 3, 5 and 7 are also reported in the paper. Only platelet mass on day 2 was associated with bleeding (1404 ± 562 vs 1874 ± 826, p 0,045). On all other days, no association between platelet mass and bleeding is found.

[2] Mean ± SD.

[3] Platelet mass at the time of NICU admission. Mean ± SD.

[4] Number of cases reduced from 43 to 39, probably due to missing data. Unclear what the number of controls for this analyses was.

Supplementary Table S1 Overview of all 36 eligible papers (*continued*)

Studies in which the association between MEAN PLATELET VOLUME and major bleeding is assessed										
Author	**Year**	**Population**	**N total**	**N in groups**	**Study groups**	**Mean platelet volume (fL)**	**Type of bleed**	**Temporal ambiguity**	**Result of analysis**	**Adjusted for confounders**
Dani	2011	GA < 30 weeks. Platelet count <50 excluded.	210	17 193	Cases: IVH grade 3-4 Controls: no IVH grade 3-4	10.7 ± 0.9[1] 10.7 ± 1.1	IVH	Yes	No association between MPV and bleeding (p-value 1.0)	
Mitsiakos	2016	GA ≤ 28 weeks	130	31 99	Cases: IVH grade 3 or 4 Controls: IVH grade 1 or 2, or no IVH	10.4 ± 1.8 9.9 ± 1.4[2]	IVH	Yes	No association between MPV and bleeding	No
Mosayebi	2017	GA < 34 weeks and respiratory distress syndrome.	100	5 10 10 25 50	Cases 1: IVH grade 4 Cases 2: IVH grade 3 Controls 1: IVH grade 2 Controls 2: IVH grade 1 Controls 3: no IVH	100% MPV > 11 60% MPV > 11 100% MPV > 11 64% MPV > 11 74% MPV ≥ 11	IVH	Yes[3]	No analysis performed	NA
Okur	2015	GA ≤ 32 weeks Platelet count <50 excluded.	330	133 197	Cases: IVH grade 3 or 4 Controls: no IVH	8.1 ± 0.9[4] 8.2 ± 1.0	IVH	Yes	No association between MPV and bleeding (p value 0.554)	No

Abbreviations ALL = different types of major bleed combined, BW = birth weight, CI = confidence interval, DOL = day of life, GA = gestational age, GI = gastro-intestinal hemorrhage, IVH = intraventricular hemorrhage grade 3 or 4,PH = pulmonary hemorrhage, MPV = mean platelet volume, NA = not applicable, NICU = neonatal intensive care unit, OR = odds ratio, RCT = randomized controlled trial

[1] Platelet counts are represented as numbers only (e.g. <150) for clarity, but indicate counts per 109/L, (e.g. 150 x 109/L). Mean platelet volume (mean ± SD) was measured at birth. MPV was also measured at 24-48 hours of life (no significant difference), but unclear whether any bleeding occurred prior to 48 h (not reported), results are therefore omitted from this table.
[2] Mean platelet volume (mean ± SD) was measured on the first day of life. Mean platelet volume on day 2, 3, 5 and 7 are also reported in the paper, none show an association between mean platelet volume and bleeding.
[3] First ultrasound scan on day 3 of life, MPV measured on day 1 and 3.
[4] MPV (mean ± SD) was also measured between day 3 and 7 of life, no significant association between MPV and bleeding was found.

Supplementary Table S2 Assessment of temporal ambiguity for all 36 eligible studies

N	Author, year	Population	Groups	Study entry	Exposure	Bleeding measurements	Temporal ambiguity?
1	Amato	GA < 35 weeks	A: IVH grade I-II B: IVH grade III-IV	At admission	Platelet count after parental consent (APTT measured at mean postnatal age 3.6 h (range 0.5 – 60h, unclear if this applies to platelet count also)	On admission, daily during DOL 1-7 and weekly until discharge	Yes. Timing exposure unknown. Bleeds occurred at median of 36 hours (range 9-63) after delivery. Unclear whether all neonates were admitted right after birth.
2	Andrew 1993	GA < 33 weeks, BW 500-1500 g, platelet count < 150 during DOL 1-3.	Group A: tx if platelet count <150 Group B: tx if platelet count < 50 or bleeding	Platelet count <150 within DOL 1-3.	Tx between entry and DOL 7. Postnatal age at entry: 1.6 – 1.9 days.	At entry and between DOL 7-10.	No
3	Auerbach 2013	BW < 2000 g.	Cases: IVH grade 3 or 4 Controls: no IVH	At birth	Thrombocytopenia categorized as background characteristic, no info about timing.	Between DOL 1-3 and 5-10, and subsequent US as clinically indicated.	Yes. Timing of exposure unknown.
4	Baer 2011	VLBW (<1500 g) Initial normal ultrasound scan.	Cases: 1st ultrasound scan normal, next IVH grade 3 or 4. Controls: no hemorrhage.	At birth	Tx in DOL 1-2. 88% given prior to IVH (12% after). Thrombocytopenia in DOL 1-2.	Not reported	Yes. 12% of tx given after IVH. Bleeding status at study entry unknown.
5	Baer 2011	Neonates with initial IVH grade 1.	Cases: grade 1 IVH that resolved Controls: grade 1 IVH that progressed to severe IVH	At birth	Thrombocytopenia in DOL 1-3.	Not reported, only that 4.7 days between grade 1 and grade 3 or 4	Yes. Bleeding status at study entry unknown. Exact timing of exposure unknown. Timing of bleeds is unknown.
6	Baer 2009	Two platelet counts of ≤ 50	A: platelet count <20 B: platelet count 20-30 C: platelet count 31-50 Plus case control analysis.	At birth	Onset Thrombocytopenia between DOL 1 and >150.	No screening protocol described.	Yes. Timing of exposure and bleeds unknown.

Supplementary Table S2 Assessment of temporal ambiguity for all 36 eligible studies (*continued*)

N	Author, year	Population	Groups	Study entry	Exposure	Bleeding measurements	Temporal ambiguity?
7	Beiner 2003	GA 27-35 weeks.	A: platelet count ≤ 150 B: platelet count >150	At birth	Platelet count at admission (= at birth)	Screening protocol not described.	Yes. Bleeding status at study entry unknown, timing of bleeds unknown.
8	Berger 2000	GA <34 weeks.[1]	Cases: PH. Controls: no PH	At birth	Thrombocytopenia prior to bleed and in similar time period for controls.	NA (pulmonary hemorrhage)	No. But time-interval between platelet count and PH unclear?
9	Beverley 1984	GA ≤ 34 weeks.	Group A: no IVH Group B: grade 4 Group C: grade 2 and 3 Group D: grade 1	At birth	Thrombocytopenia at birth (cord blood) and at age 48 h.	DOL 1: 8 hour intervals. DOL 2: 12 hour intervals. Weekly thereafter. 17 bleeds on DOL 1.	Yes. Bleeding status at study entry unknown. Overlap between exposure and outcome assessment: unclear if corrected for in analysis.
10	Bolat 2012	GA<37 weeks.	A: platelet count < 150 B: platelet count ≥ 150	At birth	Onset Thrombocytopenia between DOL 1 and >29.	NA (pulmonary hemorrhage). No information about timing.	Yes. Timing of exposure and bleeds unknown.
11	Braun 1999	GA <32 weeks.[2]	Cases: PH Controls: no PH	At birth	No information on timing.	NA (PH). Average age at onset of PH: 3 days. (and 14/17 within 7 days	Yes. Timing of exposure unknown.
12	Christensen 2014	GA ≤ 34 weeks, cord blood coagulation tests performed.	Group A: no IVH Group B: IVH grade 1 / 2 Group C: IVH grade 3 / 4 Group D: no screening	At birth	Platelet count at birth (cord blood)	At discretion of clinician	Yes. Bleeding status at study entry unknown and timing of bleeds unknown.

[1] This papers also included a near term / term group, but because results for the near term infants could not be extracted in the results section, only the results for the GA<34 weeks group is presented here.
[2] Results for pre-surfactant era also reported in paper. Because these are not relevant for our current clinical practice, these results have been omitted from this table.

Supplementary Table S2 Assessment of temporal ambiguity for all 36 eligible studies (*continued*)

N	Author, year	Population	Groups	Study entry	Exposure	Bleeding measurements	Temporal ambiguity?
13	Dani 2011	GA < 30 weeks. Platelet count <50 excluded.	Cases: IVH grade 3-4 Controls: no IVH grade 3-4	At birth	Platelet counts and MPV at birth and at 24-48 hours.	No screening protocol described	Yes. Bleeding status at study entry unknown, timing of bleeding unknown.
14	De Carolis 1998	GA < 30 weeks, included in one of 2 surfactant RCTs	Cases: PH. Controls: no PH.	At birth	Platelet counts within 12 h after birth	Pulmonary hemorrhage in first 3 DOL. PH occurred at least 24 h after birth	No
15	Elmoneim 2015	GA < 32 weeks, admission on DOL 1,survived > 7 days. Thrombocytopenia DOL 1-7 excluded	A: no thrombocytopenia, no drop (in platelet count) B: no thrombocytopenia, drop C: thrombocytopenia, no drop D: thrombocytopenia and drop	At birth	Thrombocytopenia and/or drop in platelet count during DOL 1-7.	No screening protocol described.	Yes. Bleeding status at study entry unknown. Timing of bleeding unknown.
16	Jen 2006	BW < 1000 g.	Cases: IVH progression Controls: no IVH progression.	At birth	Thrombocytopenia coinciding with infant's initial IVH diagnosis or time of IVH progression	Before DOL 4, at DOL 7, 14, 28 and then monthly until discharge. Clinical suspicion of bleeding: additional scans.	Yes. Exposure assessed only at time of bleed (not prior to it)
17	Kahn 2002	BW < 1500 g.	Group A: platelet count <100. Group B: platelet count ≥ 100.	At admission (within 24 h of birth)	Lowest platelet count on study day 1 and 3. Subset analyses for each day.	No screening protocol described	Yes. Timing of bleeding unknown.

Supplementary Table S2 Assessment of temporal ambiguity for all 36 eligible studies (*continued*)

N	Author, year	Population	Groups	Study entry	Exposure	Bleeding measurements	Temporal ambiguity?
18	Lee 2010	GA ≤ 34 weeks.	Cases: IVH grade 3 or 4 Controls: no IVH	At admission (if within 3 days of birth)	Transfusions prior to first day of bleeding in cases, similar time period in controls.	During first 3 days, on day 7 and 21. Unclear if this refers to DOL or days after admission.	Yes. Bleeding status at study entry unknown. Number of neonates admitted within DOL 1-3 unknown.
19	Lim	GA ≤ 26 weeks BW ≤ 1000 g	IVH grade III or IVH No IVH	At admission	Platelet count 'at birth'	Within DOL 1-3, exact timing not reported	Yes. Bleeding status at study entry and timing of bleeding unknown.
20	Linder 2003	BW <1500 g.	Cases: IVH grade 3 or 4. Controls: no IVH grade 3 or 4.	At birth	Lowest platelet count during DOL1.	On DOL 3, 14 28 and monthly until discharge. Clinical suspicion of bleeding: additional scans 70% of IVH < DOL 3.	Yes. Bleeding status at study entry unknown.
21	Mitsiakos 2016	GA ≤ 28 weeks	Cases: IVH grade 3 or 4 Controls: IVH grade 1 or 2, or no IVH	At admission (number of postnatal transfers unknown)	Platelet counts / MPV / mass on DOL 1, 2, 3, 5, 7.	Scans on DOL 1, 2, 3, 5, 7.	Yes. Overlap between timing of exposure and bleeding, unclear whether corrected for in analysis.
22	Mosayebi 2017	GA < 34 weeks and respiratory distress syndrome..	Cases 1: IVH grade 4 Cases 2: IVH grade 3 Controls 1: IVH grade 2 Controls 2: IVH grade 1 Controls 3: no IVH	At admission if soon (exact timing not specified) after birth.	Platelet count on admission and day 3. Congenital thrombocytopenia (<150 x 10^9/L) excluded.	DOL 3. For very sick infants or birth weight ≤ 1000 g on DOL 1. Serial scans according to grading of IVH.	Yes. Bleeding status at study entry unknown. Overlap between timing of exposure and bleeding, unclear whether corrected for in analysis.
23	Murray	Platelet count < 50	A: platelet count nadir < 30 B: platelet count nadir 30-50	At admission	All platelet transfusions and counts recorded, timing not reported	No new major bleeds occurred	No. But excluded because no major bleeds occurred.

Supplementary Table S2 Assessment of temporal ambiguity for all 36 eligible studies (*continued*)

N	Author, year	Population	Groups	Study entry	Exposure	Bleeding measurements	Temporal ambiguity?
24	Okur 2015	GA ≤ 32 weeks. Platelet count <50 excluded.	Cases: IVH grade 3 or 4 Controls: no IVH	At birth	Cord blood (within 2 h after birth), plus complete blood count at day 1 and between day 3-7	Screening protocol not described.	Yes. Cord blood sample not reported. Timing of bleeds unknown.
25	Ozturk 2015	Platelet count <100 x 10^9/L	A: tx based on platelet mass B: tx based on platelet count	When platelet count < 100 x 10^9/L	Transfusions according to platelet mass or count, duration study unknown.	Screening protocol not described. NA for GI and pulmonary bleeds.	Yes. IVH bleeding status at study entry unknown. Timing of bleeding unknown.
26	Rastogi (1) 2011	GA ≤ 28 weeks and survived ≥ 7 days.	A: no thrombocytopenia, no platelet count drop B: no thrombocytopenia, ≥ 30% platelet count drop C: thrombocytopenia, no platelet count drop D: thrombocytopenia, ≥ 30% platelet count drop	At birth	Platelet count data collected between day 1-7 and 7-28.	Screening protocol not described.	Yes. Timing of bleeds unknown.
27	Rastogi (2) 2011	GA ≤ 28 weeks and survived ≥ 7 days. (overlap Rastogi (1) cohort)	A: no thrombocytopenia B: platelet count 75-149 C: platelet count 50-74 D: platelet count 25-49 E: platelet count <25	At birth	Platelet counts measured at discretion of treating neonatologist.	Screening protocol not described	Yes. Timing of bleeds and exposure unknown.
28	Ree 2017	Culture proven sepsis	A: platelet count ≤50 B: platelet count >50	At birth	Lowest platelet count during sepsis	Not reported but IVH prior to thrombocytopenia excluded.	Yes. Timing PH and IVH unknown. Timing of lowest platelet count relative to bleed unknown.
29	Sparger 2016	Very low birth weight infants	A: transfused B: not transfused	At birth	Platelet transfusion during DOL 1-7 analyzed as dichotomous time-varying covariate.	Screening protocol not described, but correct analysis.	No.

Supplementary Table S2 Assessment of temporal ambiguity for all 36 eligible studies (*continued*)

N	Author, year	Population	Groups	Study entry	Exposure	Bleeding measurements	Temporal ambiguity?
30	Stanworth 2009	Platelet count < 60	A: transfused B: not transfused	When platelet count <60	Platelet count prior to bleed and lowest platelet count in neonates without bleed. 3% of transfusions therapeutic	Screening protocol not described	Not for platelet count. Therapeutic transfusions included (given after bleed).
31	Usemann	BW <1000 g	A: pulmonary hemorrhage B: no pulmonary hemorrhage	At admission	Platelet transfusions prior to PH or within first 96 h for controls. Platelet count at birth. PH occurred at median 3 days (range 2-5)	NA (PH)	No
32	Van de Bor	GA < 34 weeks	A: no IVH B: IVH grade I C: IVH grade II-III D: IVH grade IV	At birth or admission if within 8 h after birth	Platelet count immediately after birth, on DOL 3 and 5.	Immediately after birth/ admission, every 8 h during DOL 1-3, every 12 hours during DOL 4-7.	Yes. 30% of IVH detected at first ultrasound after birth, unclear if prior to or after platelet count measurement, and N major bleeds within 8 hours prior to admission unknown (at study entry).
33	Von Lindern 2011	Platelet count <150	A: platelet count <30 B: platelet count 30-49 C: platelet count 50-99 D: platelet count 100-149	At birth	Platelet count from birth	Screening protocol not described but number IVH grade 2-4 prior to/ simultaneously with / after thrombocytopenia.	Yes. Timing of grade 3 or 4 IVH in relation to thrombocytopenia not described.
34	Von Lindern 2012	GA <32 weeks and thrombocytopenia	A: liberal transfusion B: restrictive transfusion	At birth	Transfusions and platelet count from birth	Scans on DOL 1, 3 and 7. If abnormal: repeat scan within a week. Follow up scans until stabilization, additional as clinically indicated.	Yes. Therapeutic transfusions (after bleeding) included. In 77-78% of infants with thrombocytopenia, IVH (grade not reported) preceded thrombocytopenia.

Supplementary Table S2 Assessment of temporal ambiguity for all 36 eligible studies (*continued*)

N	Author, year	Population	Groups	Study entry	Exposure	Bleeding measurements	Temporal ambiguity?
35	Zisk 2013	Platelet count <100	Group A: platelet mass tx protocol Group B: platelet count tx protocol	When platelet count < 100	Transfusion from study entry.	Screening protocol not described	Yes. Bleeding status at study entry and timing of bleeds unknown. Therapeutic transfusions included.
36	Zisk 2011	BW < 1500 g	Group A: severe IVH Group B: no severe IVH	At birth, ±90% inbound	Complete blood counts on admission and daily for next 2 days.	Scan on DOL 4.	Yes. Bleeding status at study entry unknown. Postnatal age at admission not reported.

Abbreviations ALL = different types of major bleed combined, BW = birth weight, CI = confidence interval, DOL = day of life, GA = gestational age, GI = gastro-intestinal hemorrhage, IVH = intraventricular hemorrhage grade 3 or 4,PH = pulmonary hemorrhage, MPV = mean platelet volume, NA = not applicable, NICU = neonatal intensive care unit, OR = odds ratio, RCT = randomized controlled trial, tx = transfusion

Measure what can be measured, and make measurable what cannot be measured

Galileo Galilei (1564 – 1642 AD)

11 Desmopressin response in children with platelet disorders may depend on the presence of a genetic cause when response is measured with Von Willebrand activity and Platelet Function Analyzer

E.J. Huisman
A. Janssen
L. van de Watering
M. Kruip
M. de Haas
M.H. Cnossen

Haemophilia 2025;31:1340-1344

ABSTRACT

Introduction

Desmopressin can be used to prevent or treat bleeding in children with platelet disorders (PDs). However, in contrast to Von Willebrand disease, data is lacking on effect measurement.

Aim

Evaluate *in vitro* desmopressin response.

Methods

In a single center cohort study in children with PDs receiving intravenous desmopressin, response was categorized as good, partial or poor based on von Willebrand factor activity (VWF:Act) levels and/or Platelet Function Analyzer (PFA) normalization.

Results

We included 53 children (mean age 12.2 years; 33 females). Diagnosis was based on genetic mutations in 31 (58.5%), or abnormal aggregometry in 18 (34.0%), and PD was suspected in four (7.6%). Responses were good in 32 (60.4%), of which 13 had a genetic cause; were partial in 13 (24.5%), of which 10 had a genetic cause. Response was poor in 8 (15.1%), all with a genetic cause (100%). In nine children with concomitant low VWF desmopressin response was good.

Conclusion

When VWF rise and PFA normalization are taken as effect measurements for *in vitro* desmopressin response, we demonstrate that desmopressin response is adequate in the majority of children without a detected monogenetic cause. However, in a substantial proportion of children with a proven genetic cause of their PD, desmopressin response was partial or poor. In line with Von Willebrand disease, desmopressin response in children with PDs needs more research in order to define what is a good response, as well as to identify potential subgroups and correlation with clinical *in vivo* effect on bleeding.

Keywords

Blood Platelet Disorders [MeSH]; Blood Coagulation Disorders, Inherited [MeSH]; Child [MeSH]; Deamino Arginine Vasopressin [MeSH]; Medication Therapy Management [MeSH]

LETTER TO THE EDITOR

Platelet disorders (PDs) are a heterogeneous entity as they involve platelet count, function or both. They may result from monogenetic causes resulting in an inherited PD (IPD) or be part of a distinct syndrome (syndrome associated PD; SAPD) or have no identified cause.(1) Patients with PDs may have an increased bleeding tendency requiring treatment.(2) Currently, general treatment options consist of transfusions, antifibrinolytic agents or desmopressin.(3-5) Desmopressin (1-deamino-8-D-arginine vasopressin) is a synthetic analog of L-arginine vasopressin. By activating alpha granules and cyclic adenosine monophosphate, that in return leads to exocytosis of von Willebrand Factor (VWF) from the Weibel Palade bodies in the vascular endothelium, primary hemostasis is enhanced.(3, 6) Knowledge of its working mechanisms is derived from von Willebrand disease (VWD) and mild hemophilia A as only one double-blinded randomized, crossover trial in 18 PD-patients has been performed,(7) besides some small case-series.(3, 8-15) In most of these reports, desmopressin response in PD was quantified by reduction of bleeding time (3, 7-11, 15-17); a test no longer applied.(18) More recent reports use a rise in VWF antigen (VWF:Ag), VWF activity (VWF:Act) and/or FVIII.(7, 11-13, 19) Unfortunately, none of these tests directly measure the effect on platelet function. Platelet function tests such as light transmission aggregometry (LTA), flow cytometry, thrombin generation and intracellular Na+/Ca2+ flux or ATP release, are not suitable for repetitive measurement for various reasons.(6, 13, 20, 21) Therefore, normalization of the PFA closure time (CT) is used as an alternative.(12, 13) However, PFA as an instrument to measure bleeding tendency and diagnose PDs or Von Willebrand disease (VWD,(22)) is not without debate.(23, 24)

In this background, we conducted an observational cohort study from January 1st 2017 to May 1st 2022 to evaluate the desmopressin response in children of 4-18 years with various types of PDs using repeated measurements of VWF:Act and PFA as effect measurements. The study was subject to the Medical Research Involving Human Subjects Act (MEC-2016-218 and MEC-2013-026).

Study inclusion was based on the probability of PD, categorized as follows:

(1) 'proven' in the presence of a (likely) pathogenic variant or chromosomal abnormality associated with PD (Table 1). This group includes both patients with an IPD leading an isolated PD or PD with predisposition to malignancies, as well as SAPDs.
(2) 'probable' when LTA was repeatedly abnormal according to international guidelines,(20) or a congenital (macro)thrombocytopenia was presence, that was not explained by category (1).
(3) 'possible' when PFA was persistently prolonged PFA >200 seconds and an increased bleeding tendency in the absence of VWD and thrombocytopenia. These last two groups will be referred to as PD of unknown cause.

Exclusion criteria were acquired thrombocytopenia, severe type 1 VWD (VWF:Act <0.3 U/mL), hematocrit <0.25L/L, NSAIDs or platelet transfusions in last seven days. Mild type 1 VWD (VWF:Act 0.3-0.6 U/mL) was no exclusion criteria. Data was collected from electronic patient files, bleeding symptoms scored by the validated ISTH-bleeding assessment tool with cut-off values of ≥3 for abnormal bleeding.(25) Desmopressin (0.3 µg/kg) was infused intravenously. Blood sampling was repeated at t=1-2, 3-4 and 5-6 hours. At least one follow-up blood sample was required. If results for two time-points were available (e.g. t=1 and t=2) the 'best' response was taken for analysis.

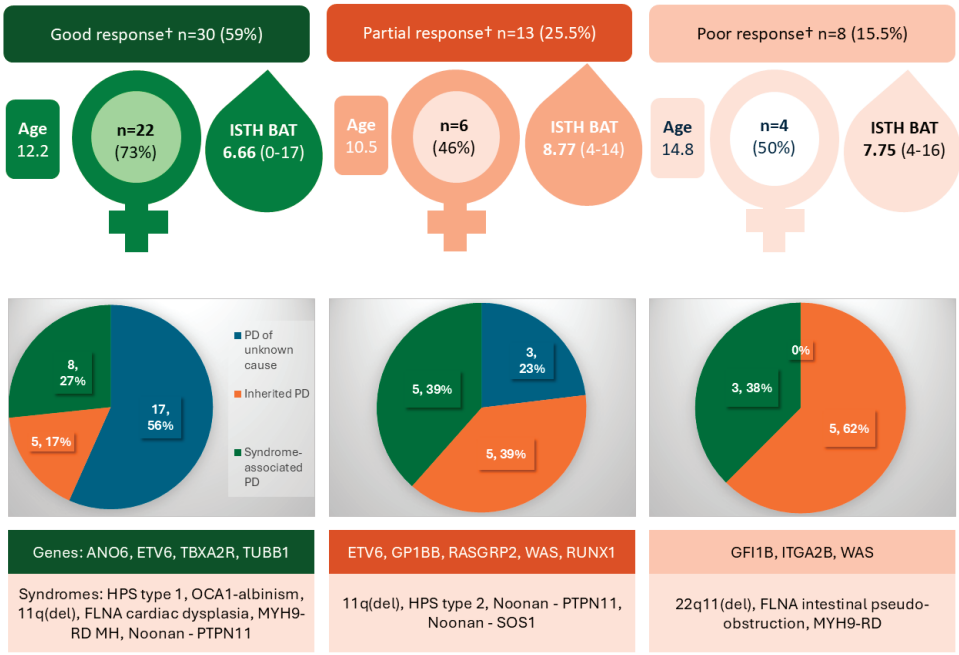
As there is no internationally available definition for adequate desmopressin response in PD patients, we have adapted the definition applied to manage VWD as documented in recent guidelines and publications in children.(11-13, 26, 27), defining response as follows:

- G*ood*: at least a two-fold rise of VWF:Act from baseline (t=0) and a normal or normalized PFA, defined as closing time (CT) <150 seconds at least once between t=1 and t=6.
- P*artial:* at least a two-fold rise of VWF:Act from baseline or a normal or normalized PFA at least once between t=1 and t=6.
- P*oor:* less than a two-fold rise of VWF:Act from baseline and no normal or normalization of PFA at least once between t=1 and t=6.

We analyzed blood count (Sysmex XN-9100), PFA epinephrine (PFA-EPI, INNOVANCE PFA-200®) closure time regardless of platelet count, VWF:Act by a platelet independent assay (Sysmex CS-5100). Descriptive statistics (SPSS version 21.0, IBM, Armonk, NY) and multiple line graphics (Julia programming language version 1.8.3) were used.

We included 51 children with a mean age of 12.1 years (range 4-18) and 32 (62.7%) were females. Mean bleeding score was 7.4 (range 0-17). Only five (9.8%) had a normal score of ≤2 (Figure 1). Categorization of PD was *proven* with known genetic cause in 31 (60.8%) of whom 15 had an IPD and 16 an SAPD. In the remaining patients, PD was categorized as *probable* in 18 (35.3%) and *possible* in 2 (3.9%). Platelet en VWF results at inclusion showed thrombocytopenia in 13 (25.5%, mean 71.3 x 10^9/L, range 27-132), of whom 10 <90 x 10^9/L. Seven patients (13.7%) had mild type 1 VWD as an additional risk factor (mean VWF:Act 0.45 U/mL, range 0.34-0.57), of whom five between 0.30-0.50.

Figure 1 Patient characteristics per response group



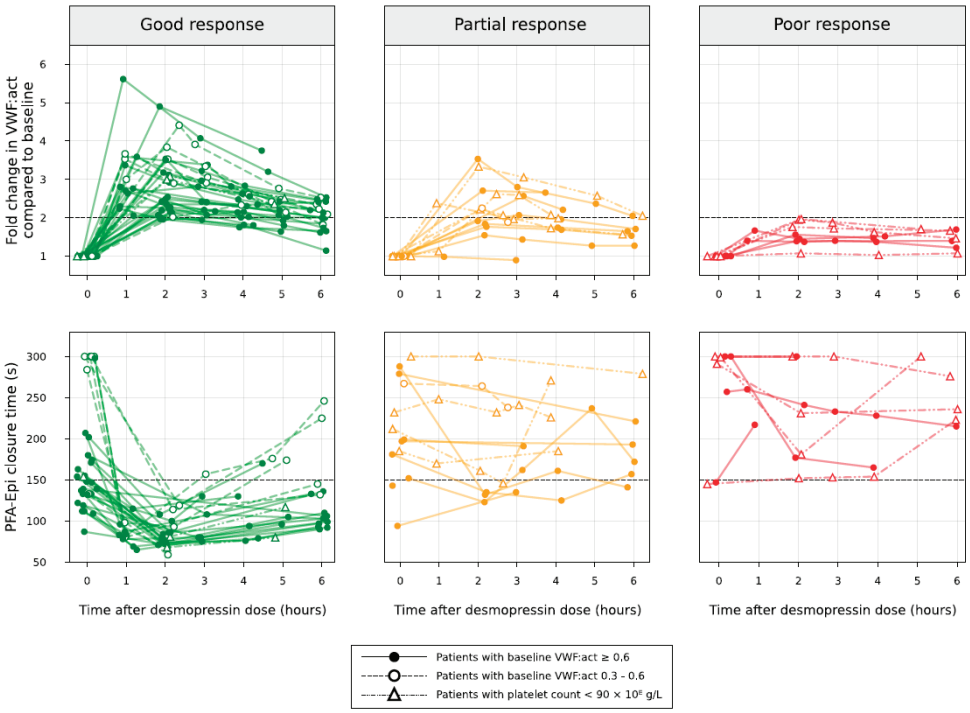
Abbreviations del = deletion, EPI = epinephrin, FLNA = Filamin A, HPS = Hermansky-Pudlak syndrome, ISTH BAT = bleeding assessment tool of the International Society of Thrombosis and Hemostasis, MYH9-RD = MYH9 related disorder, n = number
Legend †Good, partial and poor response is defined as both normalization of PFA and ≥2-fold rise in VWF activity from baseline, one or none of these criteria

The desmopressin responses (Figure 2) were good in 30/51 (58.8%) of whom 17 (56.7%) had a PD of unknown cause. This group represents the majority of the PDs of unknown cause (17/20, 85%). Response were partial in 13 children (25.5%). As the partial response was defined as either <2 two-fold rise in VWF:Act or no PFA normalization, this group consists of two subgroups: seven (53.8%) demonstrated an insufficient rise of VWF:Act, and six showed no PFA normalization. Responses were poor in 8/51 (15.7%) children, all with an IPD or SAPD (100%).

For the two effect measurements VWF rise and PFA normalization, a pre-existent thrombocytopenia <90 x 10^9/L or mild type 1 VWD may have influenced results. However, pre-existent thrombocytopenia was seen in all response groups: two children in the good response, four in the partial and four in the poor response group. Especially in this last group, pre-existent thrombocytopenia influences PFA results but not the absence of VWF rise. For the mild VWD, 6 out of 7 children with mild type 1 VWD were good responders, only one responded partially with PFA ≥150. Two of these seven had a proven PD (both

SAPD with Noonan syndrome and interstitial 11q deletion syndrome). Of the remaining, five had a probable PD with abnormalities on LTA analysis at diagnosis.

Figure 2 Desmopressin response per response group using PFA and VWF activity as effect measurements



Abbreviations act = activity; EPI = epinephrinL = liter, PFA = Platelet Function Analyzer, s = seconds VWF = Von Willebrand Factor

Legend Good, partial and poor response is defined as both normalization of PFA and ≥2-fold rise in VWF activity from baseline, one or none of these criteria; - - - = threshold of good response, defined as at least once ≥2 fold rise from baseline VWF:Act in t=1-6 hours and normal or normalization of PFA <150 seconds

In summary, we are the first to demonstrate *in vitro* desmopressin response in correlation to genetic PD cause using normalization of PFA and VWF rise as effect measurements. Response quantified as good in the majority (30/51, 59%) of children, most of whom had PDs of unknown cause and were female. In contrast, responses were partial in 13 or even poor in 8 children, most with IPDs or SAPDs. Overall, our results confirm earlier studies in smaller cohorts (3 ,8-11, 15, 16), using bleeding time as an effect measurement. Only Tauer et al,(13) and Hanebutt et al,(12) also applied PFA and VWF, albeit VWF:Ag without clear definitions of VWF rise. Both studies describe one patient with an insufficient response based on PFA results remaining >150 seconds. In our cohort we would classify this only as a partial response. Pre-existent thrombocytopenia nor mild type 1 VWD could

not fully explain the lack of response. Hypothetically, it may therefore be possible that certain genes or genetic variants may negatively influence platelet function in certain IPDs or SAPDs (10, 15, 17), or influence VWF secretion, as such is seen in subgroups with type 1 and type 2 VWDs with a certain genetic variants.(28, 29)

But, besides the knowledge gap on the variable outcomes, our study also shows that there is a need to classify and define good outcome in PD patients, as cut-offs for desmopressin response are extrapolated from evidence of desmopressin use in VWD patients and only to a small extent, from case-series of PD patients. So far, there are no alternative, easy-accessible effect measurements available, and in order to compare results to previous publications, VWF and PFA have been previously used. However, PFA normalization as a measurement of effect is debatable, as the sensitivity of PFA to screen for the diagnosis of PDs is low, especially in mild PDs.(30) We are however of the opinion that the repetitive measurement of PFA within one individual (intraindividual comparison) after desmopressin infusion may make it a valuable parameter in this specific context and for now, make comparison of case-series possible.

Our study demonstrated that the heterogeneity of the PD entity as a whole and the current lack of classification makes interpretation of the study results for the individual patient challenging. We have tried to overcome this by proposing a classification based on certainty of the diagnosis with proven (genetic), probable (functional) and possible (medical) subgroups. But even then, the clinical phenotype can still vary, as genetic causes may be related to platelets only (IPD) or be part of a larger syndrome (SAPD), or the underlying gene not yet unraveled. Finally, it remains unclear if an absence of *in vitro* effects as applied in our study, coincides with an *in vivo* absence of effects on hemostasis. (21, 31) Future research correlating laboratory tests and clinical outcomes are warranted.

REFERENCES

1. Lentaigne, C., et al., *Inherited platelet disorders: toward DNA-based diagnosis.* Blood, 2016. 127(23): p. 2814-23.
2. Blaauwgeers, M.W., et al., *Bleeding phenotype and diagnostic characterization of patients with congenital platelet defects.* Am J Hematol, 2020.
3. Coppola, A. and G. Minno, *Desmopressin in inherited disorders of platelet function.* Haemophilia, 2008. 14: p. 31-39.
4. Bolton-Maggs, P.H., et al., *A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO.* Br J Haematol, 2006. 135(5): p. 603-33.
5. Greinacher, A. and J.J.M. Eekels, *Simplifying the diagnosis of inherited platelet disorders? The new tools do not make it any easier.* Blood, 2019. 133(23): p. 2478-2483.
6. Colucci, G., et al., *The effect of desmopressin on platelet function: a selective enhancement of procoagulant COAT platelets in patients with primary platelet function defects.* Blood, 2014. 123(12): p. 1905-16.
7. Rao, A.K., et al., *Mechanisms of Platelet Dysfunction and Response to Ddavp in Patients with Congenital Platelet-Function Defects - a Double-Blind Placebo-Controlled Trial.* Thrombosis and Haemostasis, 1995. 74(4): p. 1071-1078.
8. Kobrinsky, N.L., et al., *Shortening of Bleeding-Time by 1-Deamino-8-D-Arginine Vasopressin in Various Bleeding Disorders.* Lancet, 1984. 1(8387): p. 1145-1148.
9. Schulman, S., et al., *Ddavp-Induced Correction of Prolonged Bleeding-Time in Patients with Congenital Platelet-Function Defects.* Thrombosis Research, 1987. 45(2): p. 165-174.
10. Dimichele, D.M. and W.E. Hathaway, *Use of Ddavp in Inherited and Acquired Platelet Dysfunction.* American Journal of Hematology, 1990. 33(1): p. 39-45.
11. Nolan, B., et al., *Desmopressin: therapeutic limitations in children and adults with inherited coagulation disorders.* British Journal of Haematology, 2000. 109(4): p. 865-869.
12. Hanebutt, F.L., et al., *Evaluation of desmopressin effects on haemostasis in children with congenital bleeding disorders.* Haemophilia, 2008. 14(3): p. 524-530.
13. Tauer, J.T., et al., *Evaluation of desmopressin effect on primary haemostasis in pediatric patients with aspirin-like defect as hereditary thrombocytopathy.* Klin Padiatr, 2011. 223(3): p. 169-72.
14. Zatik, J., et al., *Variable response of Hermansky-Pudlak syndrome to prophylactic administration of 1-desamino 8D-arginine in subsequent pregnancies.* Eur J Obstet Gynecol Reprod Biol, 2002. 104(2): p. 165-6.
15. Lethagen, S. and I.M. Nilsson, *DDAVP-induced enhancement of platelet retention: its dependence on platelet-von Willebrand factor and the platelet receptor GP IIb/IIIa.* Eur J Haematol, 1992. 49(1): p. 7-13.
16. Sutor, A.H., *DDAVP is not a panacea for children with bleeding disorders.* Br J Haematol, 2000. 108(2): p. 217-27.
17. Cordova, A., et al., *Poor response to desmopressin acetate (DDAVP) in children with Hermansky-Pudlak syndrome.* Pediatr Blood Cancer, 2005. 44(1): p. 51-4.
18. Peterson, P., et al., *The preoperative bleeding time test lacks clinical benefit - College of American Pathologists' and American Society of Clinical Pathologists' Position Article.* Archives of Surgery, 1998. 133(2): p. 134-139.
19. Lethagen, S. and I.M. Nilsson, *Ddavp-Induced Enhancement of Platelet Retention - Its Dependence on Platelet-Vonwillebrand Factor and the Platelet Receptor Gp-Iib/Iiia.* European Journal of Haematology, 1992. 49(1): p. 7-13.

20. Cattaneo, M., et al., *Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH.* Journal of Thrombosis and Haemostasis, 2013. 11(6): p. 1183-1189.
21. Swieringa, F., et al., *Desmopressin treatment improves platelet function under flow in patients with postoperative bleeding.* J Thromb Haemost, 2015. 13(8): p. 1503-13.
22. https://doclib.siemens-healthineers.com/rest/v1/view?document-id=850635
23. Kaufmann, J., et al., *Utility of the Platelet Function Analyzer in Patients with Suspected Platelet Function Disorders: Diagnostic Accuracy Study.* TH Open, 2020. 4(4): p. e427-e436.
24. Sladky, J.L., et al., *The PFA-100® does not predict delta-granule platelet storage pool deficiencies.* Haemophilia, 2012. 18(4): p. 626-629.
25. Elbatarny, M., et al., *Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project.* Haemophilia, 2014. 20(6): p. 831-5.
26. Connell, N.T., et al., *ASH ISTH NHF WFH 2021 guidelines on the management of von Willebrand disease.* Blood Adv, 2021. 5(1): p. 301-325.
27. Revel-Vilk, S., et al., *Desmopressin (DDAVP) responsiveness in children with von Willebrand disease.* J Pediatr Hematol Oncol, 2003. 25(11): p. 874-9.
28. Atiq, F., et al., *Desmopressin response depends on the presence and type of genetic variants in patients with type 1 and type 2 von Willebrand disease.* Blood Adv, 2022. 6(18): p. 5317-5326.
29. Heijdra, J.M., et al., *Desmopressin testing in von Willebrand disease: Lowering the burden.* Res Pract Thromb Haemost, 2022. 6(6): p. e12784.
30. Brown, M.C. and G. Woods, *Platelet Function Analyzer-100 Shows Poor Predictive Value for Children with Low VWF and Mild Platelet Function Defects.* Blood, 2020. 136.
31. Desborough, M.J., et al., *Desmopressin use for minimising perioperative blood transfusion.* Cochrane Database Syst Rev, 2017. 7: p. CD001884.

Supplementary Table S1 Molecular diagnosis of children with inherited or syndrome-associated platelet disorders

Patient	Gene	Nucleotide	Protein	Class †	Syndrome name
Inherited PD with monogenetic variants, n=15					
Transcription factor					
1	*RBM8A*	?		5	Thrombopenia with absent radius
31-32	*GFI1B*	c.814+1G>A	p.?	5	
40-41	*ETV6*	c.1105C>T	p.(Arg369Trp)	4	
42	*RUNX1*	c.319C>A	p.(Arg107Ser)	4	Familial PD with associated myeloid malignancy
Pro-platelet formation					
2	*TUBB1*	c.751C>A	p.(Arg251Ser)	4	
35-36	*TUBB1*	c.751C>A	p.(Arg251Ser)	4	
43-44	*WAS*	c.360+3A>T	p.?	4	X-linked thrombocytopenia
45	*WAS*	c.137C>A	p.(Pro58His)	4	X-linked thrombocytopenia
Platelet glycoprotein or receptor signaling					
37	*ITGA2B*	c.2875G>C	p.(Ala959Pro)	5	Glanzmann Thrombasthenia
38	*RASGRP2*	c.706C>T	p.(Gln236*)	5	
39	*GP1BB*	c.281A>G	p.(Asp94Gly)	5	Bernard-Soulier syndrome
Platelet dysfunction					
24	*TBXA2R*	c.343-345del	p.(Phe115del)	4	
46	*ANO6*	c.1318C>T	p.(Arg440*)	4	Scott syndrome
Syndrome-associated PD, monogenetic variants, n=12					
20	*PTPN11*†	-	-	4	Noonan syndrome
21	*PTPN11*	c.179G>C	p.(Gly60Ala)	4	Noonan syndrome
22	*PTPN11*†	-	-	4	Noonan syndrome
23	*SOS1*	c.1656G>T	p.(Arg552Ser)	4	Noonan syndrome
25-26	*MYH9*	c.5797C>T	p.(Arg1933*)	5	May-Hegglin anomaly (MYH9-RD))
27	*MYH9*	c.2104C>T	p.(Arg702Cys)	5	Fechtner syndrome (MYH9-RD)
28	*OCA1*	c.898dupG	p.(Val300fs)	4	Oculocutaneous albinism type 1
29	*HPS1*	c.972dup	p.(Met325Hisfs*128)	5	Hermansky-Pudlak syndrome type 1
30	*HPS2*†	-	-	4	Hermansky-Pudlak syndrome type 2
33	*FLNA*	c.7941_7942del	p.(*2648Serext*100)	5	Filamin A - Intestinal pseudo-obstruction
34	*FLNA*	c.7078delG	p.(Ala2360fs)	4	Filamin A - Cardiac dysplasia

Supplementary Table S1 Molecular diagnosis of children with inherited or syndrome-associated platelet disorders (*continued*)

Inherited PD with monogenetic variants, n=15		
Syndrome-associated PD, chromosomal abnormality, n=4		
Patient	**Chromosome**	**Syndrome**
47	11q14.2-q22.1(del) 1x	Interstitial 11q deletion
48	11q14.1-q23.3(del) 1x	Interstitial 11q deletion
49	11q23.3-qt(del) 1x	Jacobsen syndrome
50	22q11(del) 1x	Velocardiofacial or DiGeorge syndrome

Abbreviations del = deletion, dup =duplication, fs = frame shift, MYH9-RD = MYH9 related diseases, n = number, PD = platelet disorder

Legend †Classification according to American College of Medical Genetics (ACMG) guidelines with 4 = likely pathogenic, 5 = pathogenic. [†]Test results performed in another center, no genomic data available, but conclusions in correspondence of clinical geneticist

There are far, far better things ahead than any we leave behind

C.S. Lewis (1898-1963 AD)

GENERAL DISCUSSION & SUMMARY

To see, we must forget the name of the thing we are looking at

Claude Monet (1840-1926 AD)

12 GENERAL DISCUSSION

BACKGROUND AND STUDY OBJECTIVES

In the last decade, platelet disorders (PD) have become a growing area of interest due to advances in molecular genetic diagnostic testing, leading to more diagnoses and increasing knowledge.(1) These tests have revealed that many genes are involved in PD, but also that the genetic origin cannot always be identified yet. Finally, we have discarded the oversimplification of PD as either a shortage of platelets (thrombocytopenia) or the presence of dysfunctional platelets (thrombocytopathy). Importantly, despite these increasing insights, the clinical heterogeneity observed in PD is large. Therefore, data on clinical phenotypes in relation to the outcomes of basic laboratory tests for platelet characteristics and function, and their association with molecular genetic tests remain important.

Our overall goal was to investigate the clinical spectrum of children with a proven PD or suspected PD focusing on the clinical and diagnostic challenges as well as the therapeutic dilemmas to gain a better understanding of PD as a whole. We investigated the impact of a PD diagnosis on children and caregivers by reviewing the literature on health-related quality of life (HRQoL) in children with any form of bleeding disorder. Subsequently, we assessed a large cohort of children with PD, evaluating the clinical symptoms and diagnostic utility of ready-available tests for platelet count, indices and function, but also studying specific PD subgroups. Furthermore, we evaluated the role of basic laboratory testing in the effect-measurement of current therapies for PD, as well as treatment indications if and how to transfuse specific PD groups. Lastly, we evaluated the potential diagnostic utility of mass spectrometry-based analysis and gained more insight in pathophysiological mechanisms behind PD subgroups by in vitro generated cellular models.

MAIN FINDINGS

We observed that only the use of currently available laboratory tests such as platelet count and function is too limited for PD diagnosis. We demonstrated that addition of molecular genetic testing to specify PD diagnosis is useful, resulting in better PD classification and supporting individualization of screening and follow-up requirements. We also showed that in a proportion of children no genetic diagnosis is found, and that in this group, platelet count and functional platelet testing still remains the main pilar for diagnosis. In addition, we investigated mass spectrometry (MS)-based analysis of the platelet proteome as an alternative diagnostic tool for PD Unfortunately, we were not able to find sufficient basis to structurally implement this technique for PD diagnostics in the current setting. Therefore, the clinician must still depend on medical history, clinical symptoms and basic laboratory tests. Our studies have added to the knowledge of how to use this information for diagnosis and counseling of children with PD, potentially

impacting disease control and quality of life. This is important, as studies on patient or caregiver reported health-related quality of life (HRQoL) in PD are lacking.(2, 3)

PD are a disease entity that has long been relatively neglected. This neglect is most certainly due to the complexity of the disease group itself as well as the long-term absence of adequate diagnostic tools to classify PD. As a result, clinicians were forced to treat and monitor patients according to a one-size-fits-all approach. With our studies, we believe to have contributed not only to the current knowledge on PD, but also more specifically to individualization of treatment, future classification, and a better understanding of underlying pathophysiological mechanisms, improving disease diagnosis, clinical management, prognosis and health-related quality of life.

In **PART I**, we present studies on the unmet needs in diagnosis and clinical management of PD in children. We will discuss three knowledge gaps that should be addressed in future research:

- Lack of HRQoL research and appropriate support
- Relevance of a classification of PD
- Urgent need for adequate diagnostic tests for PD, specifically in children

In **PART II**, we describe therapeutic dilemmas and offer management strategies, discussing two important knowledge gaps:

- Importance of individualized treatment
- Need for practical (inter)national guidelines

LACK OF HRQOL RESEARCH AND APPROPRIATE SUPPORT

The absence of HRQoL research in children with PD, leaves their voices and those of their caregivers unheard. We therefore support extending HRQoL research to all bleeding disorders, including PD. A first step is the 'Thrombocytopathy in the Netherlands kids/young study' (TIN kids and TINy study) as they have included measurement of HRQoL, using legacy questionnaires as a study endpoint. These results will be of great importance. Future steps should improve patient reported outcome measures (PROMs) specifically for PD. We believe that HRQoL research in PD should lead to development of a more disease-specific questionnaire as disease specific hemophilia-related HRQoL questionnaires have been shown to be insufficient due to different bleeding symptoms as well as the absence of female-related bleeding symptoms.(3)

Additionally, we observed that when investigating HRQoL with standardized questionnaires, reporting the mean or median HRQoL for a study population is less helpful. Future HRQoL research should include standard deviations or interquartile ranges; or even

better, add the minimum clinically important difference (MCID) along with analysis of the outliers, as these may represent the genuine impact of the disease. As the top 10 of "happy kids and caregivers" may guide clinicians to strong coping skills or disease and treatment related factors that positively influence QoL, the bottom 10 of "unhappy kids and caregivers'" may be the group that needs additional support or better disease education for coping. Or these children have a subgroup of PD that leads to a higher disease burden, indicating the need to intensify treatment as was demonstrated recently in adults and caregivers of children with Glanzmann Thrombasthenia.(4)

Disease acceptance and control also rely on recognition of the disease burden as well as reliable and patient-oriented disease information to empower patients and caregivers. During this thesis, a Cyberpoli was developed by "Artsen voor kinderen". This Cyberpoli "Bloedplaatjesaandoeningen" is for children and adolescents with PD, both inherited and acquired.(5) Through this digital platform, patients or caregivers can digitally consult a panel of medical and laboratory specialists, enabling them to receive information from experts.

RELEVANCE OF A CLASSIFICATION OF PD

We strongly promote the relevance of a better classification of PD. Unlike hemophilia and Von Willebrand disease (VWD), PD cannot be categorized as mild, moderate or severe based on residual Factor VIII, IX or Von Willebrand Factor (VWF) activity levels. Also, the differentiation between PD patients based on thrombocytopenia and thrombocytopathy is no longer sufficient. In this thesis, we therefore propose a classification based on phenotype and genotype. This differentiation of PD into subgroups is essential to avoid unjust pooling of mild and severe phenotypes and to ultimately better understand the multicolor spectrum of genetic mutations and the various clinical symptoms. This refinement is needed, as reporting overall bleeding in a heterozygous disease entity like PD does not do justice to the variation in bleeding tendency seen. In our DoReMi cohort study, we presented an overview of children with PD and we showed that bleeding symptoms are similar to other primary hemostasis disorders, such as VWD.(6, 7) However, bleeding phenotype is influenced by sex, underlying pathophysiological mechanism and genetic mutation, as well as the presence of comorbidities or additional risks.

To start with sex-related differences, as bleeding around circumcision in males or heavy menstrual bleeding in females can be a presenting symptom for PD.(8, 9) Hemostatic analysis should include platelet count and function testing, especially when VWD cannot be diagnosed. Importantly, PD and mild VWD type 1 coincide in 5-10% of children with proven or suspected PD, so clinicians should be aware that presence of VWD alone may not be a sufficient explanation. (Chapter 3,(10)). Furthermore, certain inherited PD are

caused by x-linked aberrations, therefore, a family history with male dominance may hint already to specific PD cause.

Secondly, specific or excessive bleeding symptoms may also hint to PD subgroups as bleeding phenotype relies on the underlying pathophysiological mechanism of PD. For example, in our cohort study, we confirmed that combinations of PD with additional coagulation factor deficiencies occurred mainly in patients with Noonan syndrome as described by others.(11) Also, our observation that central nervous system (CNS) bleedings may occur in children with *WAS*-related PD may be in line with the existing literature on this topic. We found an overall incidence on CNS bleedings of 3% in our cohort, which resembles incidences of 1.3-2% found in other studies with pooled PD subgroups.(7, 12) But in one of these three patients, an unprovoked CNS bleeding occurred twice in the patient with *WAS*-related PD. The potentially higher incidence in this subgroup, is in line with a large international cohort of 173 patients that reported CNS bleeding in 10% of patients with *WAS*-related PD, in majority before the age of 10 years.(13) Contrastingly, there are also PD with mild thrombocytopenia and a mild bleeding such as the majority of *TUBB1*-related PD. Such a specified diagnosis will lead to individualized counseling and follow-up consisting of follow-up with low frequency and only on demand care.

Bleeding phenotype also relies on the underlying pathophysiological mechanism of PD. Currently >100 genes have been identified that influence either platelet production or function. In these so called inherited PD (or IPD), defects in production and function of hematopoietic cells are the ultimate (likely) *proven* PD cause.(1, 14, 15) Therefore, we propose to use the term **inherited PD** when a specific molecular genetic cause is found that leads to a PD influencing megakaryopoiesis or platelet function.

In order to identify the subgroups that are represented in the main category of IPD, we propose to add the specific gene involved, e.g. *ETV6*-related IPD. We advocate for this extension, as clustering and investigating PD subgroups may uncover the need for specific care and counseling in certain subgroups. An illustrative example is the increased risk of hematological malignancies in children with e.g. *ETV6, ANKRD26* or *RUNX1*-related IPD; or the increased risk of auto-immunity in *WAS*-related IPD. Adding the genetic specification, quickly reveals if and what additional screening and surveillance is needed.(14, 16, 17)

However, in the examples of *ETV6* and *WAS*-related PD, these IPDs come with other blood cell-related complications, but no with other physical tracts impairments. In other words, the sequalae of the mutations are isolated to the hematopoietic tract. This is not the case for all IPD. In contrast to this so to say, isolated IPD (ISPD), PD can be part of a wider, underlying syndrome. Children with these **syndrome-associated PD** (SAPD) also have an identified molecular genetic mutation as the inherited cause for the PD, but their mutation will result in more than PD only.(1, 14, 15) As we reported, in tertiary setting,

40% of children with PD report comorbidities in other tracts. We therefore believe that this specific group should be classified as a separate entity as these children may require additional medical screening at PD diagnosis, and should undergo platelet function screening at syndrome diagnosis and certainly before surgery, as surgery may be indicated more frequently and at a younger age due to defects of other organs or tissues associated with the syndrome. This increases the risk of serious bleeding complications due to the concomitant PD. Additionally, we found, that children that suffered from atypical bleeding events such as intramuscular bleeding after vaccination, vitreous bleeding with vision loss or gastro-intestinal bleeding, all had a SAPD. Maybe rare bleeding symptoms occur more frequently in this SAPD group due to the nature of the PD or due to the association with other syndromic morbidity, but more research is needed to confirm this finding in future clinical reports.

How valuable clinical reports of subgroups are, is illustrated by the detailed reports on the clinical phenotype and laboratory results of patients with a variety of 11q disorders. We were the first to report that patients with non-terminal 11q deletions and terminal 11q duplications have a similar phenotype with an increased bleeding tendency and platelet abnormalities in basic laboratory tests. This was confirmed by MS analysis as well as by selective electron imaging. Remarkably, light transmission aggregometry (LTA) showed that abnormal responses to TRAP, a thrombin receptor-activating peptide mimicking the action of thrombin and heavily involved in granules function, was present in the majority.(18, 19) Moreover, we demonstrated that patients with 11q disorders, may also present with T cell dysfunction and, sometimes with granulocyte dysfunction as well. More research is needed to analyze if platelet granule defects are also associated with granulocyte granule defects, especially in PD patients with Hermansky-Pudlak syndrome type 2,(20) *WAS*-related PD,(13, 21) and *NBEAL* or *GFI1B*-related grey platelet PD.(22, 23)

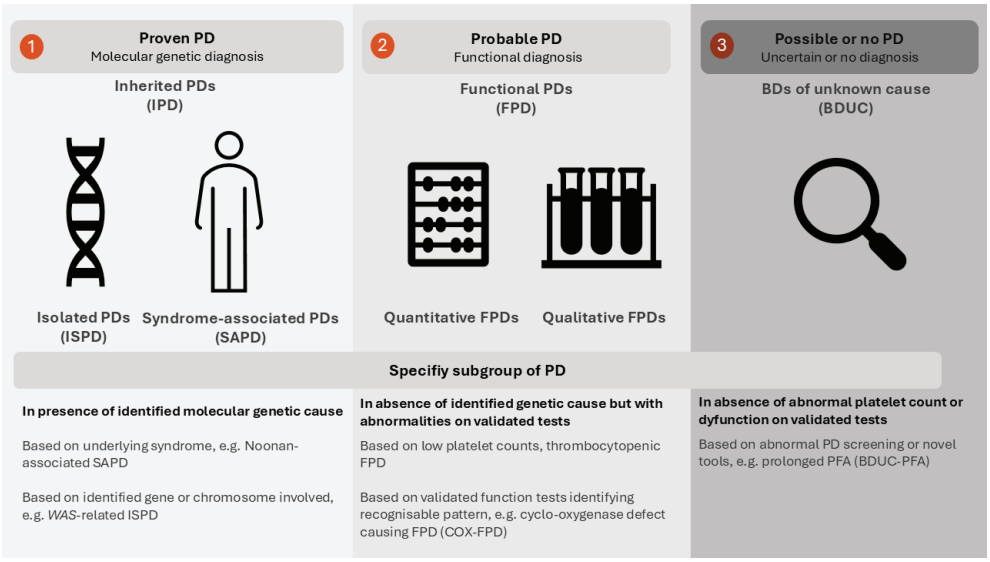
The second PD classification group are the *probable* PD and involves children with either a congenital low platelet count, or aberrant function tests in validated assays such as light transmission or lumi-aggregometry but without an identified molecular genetic cause. We propose to classify this group as functional PD, using standardized functional tests, as LTA for example, as key diagnostic tool. In this group of FPD, two subgroups can be identified: the **thrombocytopenic PD** showing a quantitative defect, sometimes combined with platelet dysfunction. Ant the pure **functional PD** with a qualitative defect. We therefore advocate to, when possible, add an addendum to subcategorize these qualitative FPD as well, based on recognizable patters on functional tests. For example, when response on arachidonic acid and U46619 are absent or abnormal, fitting a cyclooxygenase defect, this can be named and grouped as COX-FPD.(24, 25) Or when additional tests such as ATP-ADP ratios and mepacrine concentrations point to a storage pool defect, it can be named a SPD-FPD. These terms are not new and already widely used throughout literature, but it has so far not been proposed to use them routinely. By doing

this more consequently, future research will make it easier to compare and pool results of these specific FPD subgroups.(18, 19, 26)

The third PD classification was categorized as *possible* in our DoReMi cohort study. Classification was based on prolonged PFA that could not be explained otherwise or medical history suspect for PD. However, we now consider the controverse around the role of PFA as well as the bleeding symptoms in the medical history as only suggestion for PD too meager to classify this patient group as PD. Till more information is known about these two entities, we propose to reclassify this group to bleeding disorders of unknown cause (BDUC). Optional, with the addition of 'PFA' to indicate the aberrant Platelet Function Analyzer (PFA) test results, so future research may pool results.

We have illustrated the above in Figure 1, where we propose a PD classification, combining molecular genetic tests and functional analysis results, in order to direct clinical impact, surveillance and when possible, individualized treatment.

Figure 1 Proposed classification based on molecular genetic analysis and functional tests



Legend BDUC = bleeding disorder of unknown cause, COX = cyclo-oxygenase defects, FPD = functional platelet disorder, HMP = hematopoietic malignancy predisposition, IPD = inherited platelet disorder, ISPD = isolated platelet disorder, PD = platelet disorder, PFA = platelet function analyzer®, SAPD = syndrome-associated platelet disorder

We consider this classification a starting point for discussion, as more expert opinion is crucial to develop a widely applicable classification. We underline once more that the current categorization based on thrombocytopenia or thrombocytopathy is insufficient, and so far, no clear classification has been proposed. By taking this classification as a starting

point, terminology will improve PD identification, ultimately, revealing specific key-points for hemostatic and general care of different PD subgroups. We are convinced that future knowledge of clinical symptoms, as well as discovery of new genes involved in PD or new insights in pathophysiology will lead to further adjustments and refinement.

URGENT NEED FOR ADEQUATE DIAGNOSTIC TESTS FOR PD, SPECIFICALLY IN CHILDREN

To improve diagnostic testing in children with suspected PD, two major goals are important:

- Develop and validate low-volume functional tests for use in children
- Identify key points/ biomarkers that support diagnosis and classification of PD subgroups

In the PD classification as proposed in Figure 1, the *probable* PD group is a diagnosis that still remains based on low platelet count and/or platelet function only as identification of PD. Unfortunately, where a platelet count is easily measured, platelet function with the current 'golden standard' test, LTA is challenging.(24, 27) Critical pre-analytical variables, a substantial volume of whole blood, which is even more when low platelet counts are present, a skilled team, difficulties in obtaining intravenous access due to young age or needle fear, are just a few of its challenges. We described that LTA as diagnostic tool is limited and often leads to non-specific results. For example, LTA abnormalities observed for a weak agonist such as epinephrin and ADP suggest storage pool defects (SPD), but can also be the due to other causes, or may even be part of normal variation. Also, LTA can even be completely normal in patients with SPD.(26, 28) The alternative in these cases is to perform ATP-ADP ratios, a test that is not widely available and has not been renewed since the 70's of the last century.(18, 19) Future efforts should focus on implementation of already available but not widely implemented tools to diagnose SPD, as well as develop novel tools to measure SPD using easy-accessible tools.

Another shortcoming of LTA is the restriction of its use in thrombocytopenic patients. In a proportion of thrombocytopenic patients in our cohort study, PRP was insufficient to meet quality standards. We demonstrated that in all three patients LTA was responsive to all agonists, but in lower amplitudes. A recent publication demonstrated that LTA can still be performed when PRPs have low platelet numbers, applying a cut-off of $<25 \times 10^9$/L platelets in the PRP,(29) which is far lower than the 150×10^9/L that is mentioned in the current guideline. Future studies should focus on the validation of lower PRPs in LTA or even better, develop novel functional platelet tests that do not require minimal platelet counts.(27, 30)

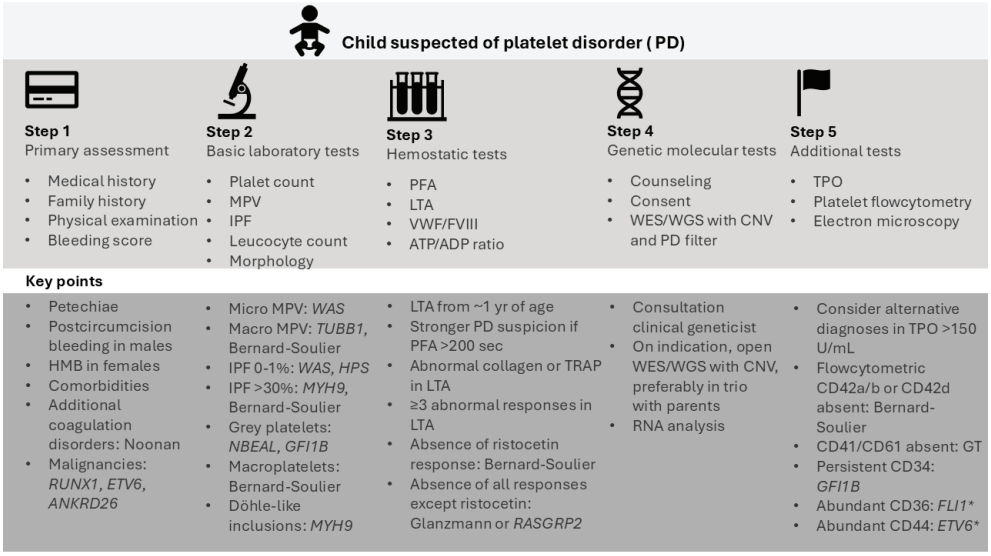
We investigated if the use of MS to analyze the platelet proteome of circulating platelets may be valuable in these situations, but concluded that in its present form it is not yet suitable as a general diagnostic tool.(31) Currently, its role is restricted to patients where a variant of unknown significance (VUS) is found in a single gene leading to a change in a single, identifiable protein present in a platelet in the circulation, for example WASp. But, in genes involving transcription factors which regulate the megakaryopoiesis on a progenitor level, MS analysis performed in circulating peripheral platelets showed multiple alteration in the generated proteome profiles resembling the downstream effect of the dysregulated megakaryopoiesis but no detection of the transcription factor itself. Detecting and quantifying upregulated or downregulated proteins only, is not sufficient for disease identification in a diagnostic setting.(32) It is possible though, that profiling these signal transduction pathways as a whole maybe of additional value. This is, however, an opportunity for future research where involvement of bio-informaticians skilled to analyze big-data and apply artificial intelligence techniques is vital to train systems using the input of sufficient platelet proteomes of healthy controls and well-defined patients.

So far, for the use of MS in diagnostic setting, studies investigating platelet proteome profiles should ideally focus on unique, identifiable 'detector' proteins or 'detector proteome combinations' that are landmarks for specific IPD and do not overlap with other IPD entities. Illustrative is our work showing that an increase of IGFBP2p in patients with *ETV6*-related PD occurred and persistence of CD34 markers on peripheral platelets was detectable in *GFI1B*-related PD.(31) Other future opportunities are measurement of the proteomes of inactivated and activated platelets as a new functional tests, (32, 33) as well as unraveling the pathophysiology of specific PD subgroups based on platelet proteomes.

Based on current evidence and results of our studies, we propose in Figure 2 a diagnostic flowchart with a stepwise approach based on clinical assessment and both basic and novel diagnostic tests. As megakaryocytes and platelets arise from hematopoietic stem and progenitor cells through a tightly regulated process involving various transcription factors, various cell pathways are involved. Combining the efforts of fundamental researchers with clinical expertise may lead to more insights in the pathophysiology of these pathways in case of pathogenic variants causing either ISPD or SAPD.

Fundamental researchers investigated the effect of a de novo heterozygous nonsense mutation, *FLI1* p.Met100* (c.297del) on megakaryopoiesis by comparing wild-type (WT) FLI1p and FLI1 p.Met100* in Meg01 cells on RNA and proteome level. This confirmed that the truncated FLI1 p.Met100* protein expression in cell line Meg01 cells stimulates both megakaryopoiesis as well as formation of erythroblasts, and at the same time impairs megakaryocyte maturation in a dominant-negative manner contributing to the inherited thrombocytopenia. *In vitro,* CD36 expression was elevated in the patient platelets, suggesting that it may serve as a potential biomarker for *FLI1*-related thrombocytopenia.

Figure 2 Diagnostic flowcharts for children with suspected PD



Abbreviations BSS = Bernard-Soulier syndrome, CD = cluster of differentiation, CNV = copy number variation, FVIII = Factor VIII, GT = Glanzmann Thrombasthenia, HMB = heavy menstrual bleeding, IPF = immature platelet fraction, LTA = light transmission aggregometry, MPV = mean platelet volume, PFA = platelet function analyzer®, TPO = thrombopoietin, VWF = Von Willebrand Factor, WES = whole exome sequencing, WGS = whole genome sequencing

Legend *this thesis, needs further confirmation

In addition, another transcription factor that tightly regulates megakaryopoiesis, is ETV6 (ETV6/TEL). ETV6 is a member of the ETS transcription factor family. Pathogenic variants in *ETV6* lead to autosomal dominant thrombocytopenia. ETV6 mainly acts as a transcriptional repressor, forming dimers through its pointed (PNT) domain, interacting with cofactors via its central domain, and binding DNA through its conserved ETS domain, where most pathogenic variants are found. We described two patients with inherited thrombocytopenia carrying a likely pathogenic variant in *ETV6* (NM_001987.4(ETV6): c.1105C>T, p.(Arg369Trp, in short ETV6-R369W). In the laboratory, we found that 424 genes were upregulated and 203 downregulated in this variant when compared to wild-type cells, consistent with ETV6's repressive function in normal conditions. In $CD34^+$ cultures, ETV6-R369W accelerated megakaryocyte differentiation but did not lead to a balanced maturation. In vitro, CD44 expression was also elevated on the patient platelets, also suggesting it may serve as a potential biomarker for ETV6-related thrombocytopenia.

IMPORTANCE OF INDIVIDUALIZED TREATMENT

The data described in our studies are also meaningful to individualize treatment in PD. Currently ≥10 drugs are registered as antiplatelet medication in cardiovascular disease ,(34) but when replacement or platelet enhancement in numbers or function is needed, there are only limited therapeutic options. Treatment options consist of platelet transfusions, tranexamic acid, desmopressin, and activated recombinant Factor VIIa (rFVIIa) which is registered only for Glanzmann Thrombasthenia (both *ITGA2B* and *ITGB3*-related IPD).

We demonstrated that HLA matched platelets as prophylactical transfusions for patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome is advised in various reviews and practice is implemented already by a majority of Dutch clinicians treating patients with various PD (survey).(35) Despite a clear theoretical hypothesis, current reviews and guidelines that are referred to are of low-quality.(35) Case-series and case-reports available mainly cover HLA and rarely HPA antibody formation and lack clear overviews of related risk factors for HLA and HPA antibody formation. Unfortunately, the impact of the formed HLA and HPA antibodies on treatment options and potential pregnancy outcomes is high. Therefore, we concluded that for now the benefit of HLA matched platelet transfusion in elective settings outweighs the extra logistic efforts and costs till more knowledge on antibody formation incidence and risk factors is gained. Due to the low incidence of Glanzmann Thrombasthenia and Bernard-Soulier syndrome (~1:1,000,000) as well as the fact that only type 1 or type 2 patients seem to be at risk for bleeding, research should be initiated in international collaborations, such as the Harvard-based "Biomedical Excellence of Safer Transfusion" (BEST) consortium.(36) In the meantime, future research should focus on alternative treatment options to further avoid the use of prophylactic, donor-derived transfusions in these particular PD subgroups.(37)

Furthermore, we have demonstrated that the practice of transfusing prophylactic platelet transfusions is also implemented in (inter)national guidelines for extremely premature neonates born before 30 weeks of gestational age, as low platelet counts or prophylactic platelet transfusions have long been associated with major bleedings, such as intraventricular hemorrhage, or prevention of bleeding in case of prophylaxis. We demonstrated that only few publications have investigated the time between a bleeding event and prior platelet counts or transfusions. Due to temporal ambiguity, the cause-and-effect relationship remains uncertain in these cross-sectional studies.(38) This study underlines that the field of neonatal transfusion needs more attention. First of all, the complex medical setting of a neonatal intensive care may not be suitable for a simplification of a "one risk factor leads to one problem" approach. Moreover, the debate on the utility of prophylactic transfusions derived from adult donors and transfused to premature neonates is growing, with studies relating prophylactic platelet transfusions (Planet2/MATISSE

trial,(39)) and red blood cell transfusions (Born trial,(40)) to an adverse outcome. Several groups are now working on the possibility to derive platelet and red blood cell transfusions from the cord blood of healthy, term neonates.(40, 41) Until this meets quality and safety standards, a restrictive transfusion policy with minimal thresholds for transfusions is required.(42) Future research should focus on even lower thresholds and investigate the effect of dose and transfusion velocity in these patients, as well as the specific risks of adult-derived blood products and application of TPO agonists for endogenous platelet production.(43)

To avoid transfusions in children, desmopressin (1-deamino-8-D-arginine vasopressin) may be an alternative as it enhances hemostasis.(44, 45) Knowledge of its working mechanisms is mainly derived from studies in patients with VWD and mild hemophilia A. We demonstrated that not all PD patients respond adequately to desmopressin. This is partly in line with previous publications in smaller cohorts (range: 11-37 patients).(44, 46-53) Unfortunately, all but two studies used bleeding time as an effect measurement, making the comparison of results difficult between these studies. Only Tauer et al.(50) and Hanebutt et al.(52) also applied VWF and screening with PFA as effect measurements. In both studies, one of the study patients was categorized as having an insufficient response based on a PFA that was persistently >150 seconds after desmopressin, which in our cohort would have only been classified as a partial response. The possibility that certain genetic variants causing ISPD or SAPD may negatively influence VWF secretion or influence VWF clearance.(48, 49, 54-56)

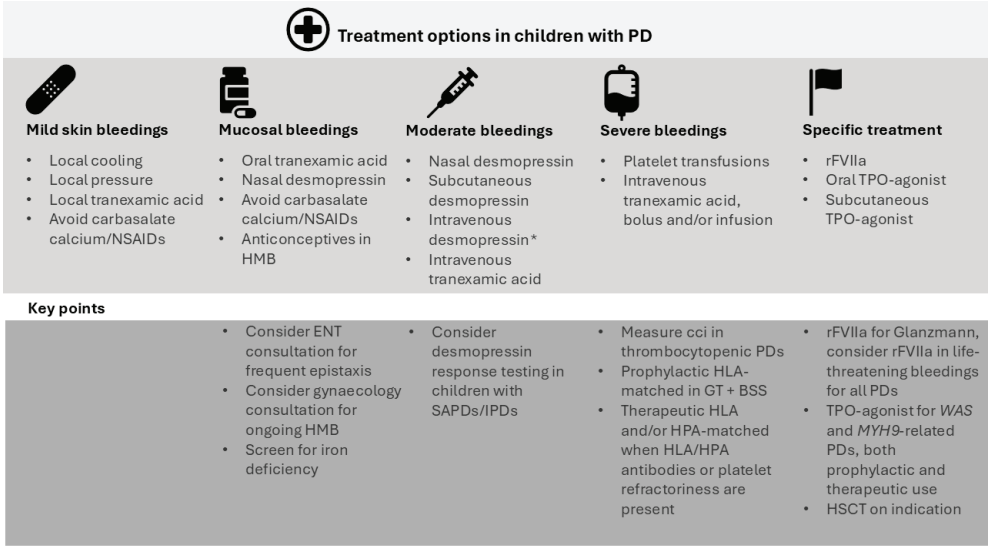
Unfortunately, we reported that a widely accepted definition of a good desmopressin response for use in PD is lacking. We therefore proposed a categorization of responses that consist of *good, partial* or *poor* responses. The relatively arbitrary cut-off for PFA and VWF rise we have now chosen, although based on practice in VWD, requires further research to understand the value of PFA in this specific context. Is absence of PFA normalization PD patients with normal platelet counts and normal VWF levels at baseline, a true indicator of desmopressin failure? And why does the PFA normalize in some patients with a low platelet count while in others it remains abnormal? Is the platelet dysfunction in this group more severe? And most striking, why do VWF levels not rise significantly in the poor response group after desmopressin? Importantly, are these laboratory results representatives of real-world effects on hemostasis?(57, 58) Future research correlating laboratory tests with clinical outcomes may lead to conclusions on which effect measurements should be targeted for desmopressin.

The above underlines the fact that more knowledge is needed and future research should focus on more pharmacological and pharmacokinetic knowledge of currently used treatment options but also focus on development of specific treatment options:

1. Analyzing and defining biochemical and clinical effect-measurements of desmopressin to identify responders and non-responders
2. Developing specific platelet transfusion products as well as evaluating the potential risk of transfusions in pediatric risk groups such as premature neonates, or specific subgroups
3. Investigating novel applications of TPO agonists beyond use in *WAS* and *MYH9*-related PD. Phase II trials for TPO agonists in new PD subgroups (59)
4. Investigating the role of temporary use of TPO-agonists as bridging therapy towards surgical interventions (59)
5. Monitoring safety of TPO agonists in PD with defects of megakaryopoiesis or with a predisposition to malignancies, as long term side-effects are not known in these specific subgroups, such as *ANKRD26*-related PD (60)
6. Investigating novel applications of rFVIIa in other PD besides Glanzmann Thrombasthenia (59, 61)
7. Developing and testing novel drugs, such as monoclonal antibodies that overcome or bypass functional platelet defects, like HMB001 (Hemab Therapeutics, USA), a bispecific antibody that aims to provide prophylaxis for Glanzmann Thrombasthenia patients by targeting and stabilizing FVIIa.(37)
8. Develop flowcharts and risk-assessment tools as guidance for PD patients with a predisposition to malignancies that require (upfront) stem cell transplantation
9. Develop gene therapies for PD with increased disease burden due to excessive bleeding, concommitant immune deficiencies or predisposition to malignancies, like the recently FDA aproved Waskyra(©) for WAS-related PD.

With the current knowledge and the insights gained from this thesis, we depict therapeutic options in Figure 3.

Figure 3 Therapeutic options for children with platelet disorders



Abbreviations BSS = Bernard-Soulier syndrome, cci = corrected count increment, ENT = ear-nose-throat doctor, rFVIIa = recombinant activated Factor VII, GT = Glanzmann Thrombasthenia, HLA = human leukocyte antigen, HMB = heavy menstrual bleeding, HPA = human platelet antigen, IPD = inherited platelet disorder, HSCT = hematopoietic stem cell transplantation, NSAID = non-steroid anti-inflammatory drug, PD = platelet disorder, SAPD = syndrome-associated platelet disorder, TPO = thrombopoietin

Legend *desmopressin cannot be used in children <1 year of age, surveillance needed when used between 2-4 years of age

NEED FOR PRACTICAL (INTER)NATIONAL GUIDELINES

In order to implement novel insights, an (inter)national guideline is needed that addresses both diagnostic and therapeutic management. Currently, there are no high-quality international guidelines for PD, in contrast to other primary hemostasis defects, such as the ASH guidelines for acquired immune thrombocytopenia,(62) or heparin-induced thrombocytopenia,(63) as well and VWD.(64) To replace the national Dutch guideline from 2019,(65) which only consisted of one chapter on 'rare factor deficiencies and thrombocytopathy' combined,(66) a new protocol is currently under review covering novel developments, diagnostic and treatment options in PD patients. This guideline is a major step forward (Schutgens R, Huisman EJ et al., unpublished data).

STRENGTHS AND LIMITATIONS OF OUR STUDIES

Strengths

We analyzed a variety of clinical, diagnostic and therapeutic topics relevant for children with PD. We used various scientific methodologies and statistics, such as retrospective data analysis, a prospective cohort study, as well as systematic reviews and surveys. We performed a large cohort study including almost 100 children as well as their family members with a suspected or proven PD diagnosis. For a rare disease, this is exceptional and an important step forward to generate new insights. Moreover, we used the findings within this cohort study to propose a novel classification for PD to do justice to the heterogeneity of this disease group and give direction to surveillance and treatment individualization.

Lastly, to perform these studies multidisciplinary teams were formed consisting of stakeholders from various treatment centers, medical specialists such as pediatric hematologists, clinical geneticists and immunologists. But also, laboratory specialists and fundamental researchers. We believe that this is vital for discovering new techniques and to formulate new research questions.

Limitations

Our studies had various limitations, such as missing values in the cohort study. The main two reasons for this deviation were the omission of test validation when thrombocytopenia occurred or that relatively large blood volumes were necessary to perform all study requirements stated in the study protocol, and this was not possible in all younger children due to difficult venous access.

Furthermore, definitions of PD categories were adjusted during analysis after the study protocol was approved. At first, the categories *proven, probable* and *possible* PD were proposed. At the end of our study, due to our observations, we proposed another classification based on molecular genetic results, platelet count and functional tests.

Lastly, although we performed the largest pediatric PD cohort study yet published and our cohort was very heterogeneous, results may not be representative for all PD types. Results obtained from our cohort study are therefore not applicable to all individuals with a diagnosis of PD. In line with our recommendations, we promote a clear classification and performance of more or even larger cohort studies to cover as many PD and PD subgroups as possible.

FINAL CONCLUSIONS

With the studies presented in this thesis, important steps towards individualization of counseling and treatment of children with PD have been made. This is important as PD are a long-neglected disease entity, especially when compared to some other bleeding disorders, due to the complexity of clinical presentation and genetic heterogeneity and limitations of current diagnostic tests. There is an urgent need to improve clinical care, diagnosis and HRQoL in this patient group with PD. This can be done by using a PD classification and update this with ongoing data collection of clinical symptoms an bleeding, underlying genetic defects, as well as investments in novel laboratory techniques to test platelet function.

Based on a combination of genetic molecular analysis and basic laboratory tests, we propose a classification based on the two proven and probable PD groups, with subgroups per main category to identify the various subgroups. We also advocate and started individualization of treatment by proposing standardization of effect-measurements for desmopressin response and a risk-benefit approach for HLA matched platelet transfusions in specific PD subgroups.

We conclude that of all bleeding disorders, in our opinion PD currently presents the most challenges but also the most opportunities for clinicians, hemostasis specialists, fundamental scientists and most importantly, for the patients.

REFERENCES

1. Lentaigne C, Freson K, Laffan MA, Turro E, Ouwehand WH, Consortium B-B, the ThromboGenomics C. Inherited platelet disorders: toward DNA-based diagnosis. Blood. 2016;127(23):2814-23.
2. Mussert CMA, Kamminga NCW, van Hoorn ES, Eekelaar TM, Dehli SR, van der Velden-van't Hoff C, et al. Experiences and Needs Regarding Information Provision in Children With Haemophilia: A Qualitative Study on Caregivers' and Healthcare Providers' Perspectives. Haemophilia. 2025;31(4):657-67.
3. Huisman EJ, Mussert C, Bai G, Raat H, Cnossen MH. Knowledge gaps in health-related quality of life research performed in children with bleeding disorders - A scoping review. Haemophilia. 2024;30(2):295-305.
4. Khair K, Fletcher S, Boyton M, Holland M. Bleeding and quality of life in people with Glanzmann thrombasthenia-insights from the Glanzmann's 360 study. Res Pract Thromb Haemost. 2024;8(7):102586.
5. https://www.cyberpoli.nl/bloedplaatjesaandoeningen/
6. Sanders YV, Fijnvandraat K, Boender J, Mauser-Bunschoten EP, van der Bom JG, de Meris J, et al. Bleeding spectrum in children with moderate or severe von Willebrand disease: Relevance of pediatric-specific bleeding. Am J Hematol. 2015;90(12):1142-8.
7. Blaauwgeers MW, Kruip M, Beckers EAM, Coppens M, Eikenboom J, van Galen KPM, et al. Bleeding phenotype and diagnostic characterization of patients with congenital platelet defects. Am J Hematol. 2020.
8. Rajpurkar M, O'Brien SH, Haamid FW, Cooper DL, Gunawardena S, Chitlur M. Heavy Menstrual Bleeding as a Common Presenting Symptom of Rare Platelet Disorders: Illustrative Case Examples. J Pediatr Adol Gynec. 2016;29(6):537-41.
9. Comishen KJ, Bhatt M, Yeung K, Irfan J, Zia A, Sidonio RJr, James P. Etiology and diagnosis of heavy menstrual bleeding among adolescent and adult patients: a systematic review and meta-analysis of the literature. Journal of Thrombosis and Haemostasis. 2025;23(3):863-76.
10. Huisman EJ, Dalm V, Joosten M, Smiers FJ, Porcelijn L, Hoogendijk A, et al. Interstitial 11q Deletions and Terminal 11q Duplications Cause a Bleeding Tendency due to Platelet Dysfunction That Is Similar to 11q Deletions Causing Jacobsen Syndrome. Eur J Haematol. 2025.
11. Stoffman JM, Chodirker BN, Israels SJ. Coagulation abnormalities in patients with Noonan syndrome - a single centre case series. Blood. 2004;104(11):295a-6a.
12. Gresele P, Orsini S, Noris P, Falcinelli E, Alessi MC, Bury L, et al. Validation of the ISTH/SSC bleeding assessment tool for inherited platelet disorders: A communication from the Platelet Physiology SSC. J Thromb Haemost. 2020;18(3):732-9.
13. Albert MH, Bittner TC, Nonoyama S, Notarangelo LD, Burns S, Imai K, et al. X-linked thrombocytopenia (XLT) due to WAS mutations: clinical characteristics, long-term outcome, and treatment options. Blood. 2010;115(16):3231-8.
14. Di Paola J, Porter CC. ETV6-related thrombocytopenia and leukemia predisposition. Blood. 2019;134(8):663-7.
15. Stockley J, Morgan NV, Bem D, Lowe GC, Lordkipanidze M, Dawood B, et al. Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects. Blood. 2013;122(25):4090-3.
16. Wahlster L, Rowe JH, Weichert-Leahey N, Rowe RG, Brundige K, Godley L, et al. Expanding the Morphologic Spectrum of ANKRD26-Related Thrombocytopenia-2: A Case Series. Blood. 2024;144:5708-9.

17. Illango J, Nair AS, Gor R, Fernando RW, Malik M, Siddiqui NA, Hamid P. A Systematic Review of the Role of Runt-Related Transcription Factor 1 (RUNX1) in the Pathogenesis of Hematological Malignancies in Patients With Inherited Bone Marrow Failure Syndromes. Cureus J Med Science. 2022;14(5).
18. Weiss HJ, Witte LD, Kaplan KL, Lages BA, Chernoff A, Nossel HL, et al. Heterogeneity in storage pool deficiency: studies on granule-bound substances in 18 patients including variants deficient in alpha-granules, platelet factor 4, beta-thromboglobulin, and platelet-derived growth factor. Blood. 1979;54(6):1296-319.
19. Akkerman JWN, Nieuwenhuis HK, Mommersteegleautaud ME, Gorter G, Sixma JJ. Atp-Adp Compartmentation in Storage Pool Deficient Platelets - Correlation between Granule-Bound Adp and the Bleeding-Time. Brit J Haematol. 1983;55(1):135-43.
20. Fontana S, Parolini S, Vermi W, Booth S, Gallo F, Donini M, et al. Innate immunity defects in Hermansky-Pudlak type 2 syndrome. Blood. 2006;107(12):4857-64.
21. Ochs HD, Slichter SJ, Harker LA, Von Behrens WE, Clark RA, Wedgwood RJ. The Wiskott-Aldrich syndrome: studies of lymphocytes, granulocytes, and platelets. Blood. 1980;55(2):243-52.
22. Sims MC, Mayer L, Collins JH, Bariana TK, Megy K, Lavenu-Bombled C, et al. Novel manifestations of immune dysregulation and granule defects in gray platelet syndrome. Blood. 2020;136(17):1956-67.
23. Aarts CEM, Downes K, Hoogendijk AJ, Sprenkeler EGG, Gazendam RP, Favier R, et al. Neutrophil specific granule and NETosis defects in gray platelet syndrome. Blood Adv. 2021;5(2):549-64.
24. Alessi MC, Sie P, Payrastre B. Strengths and Weaknesses of Light Transmission Aggregometry in Diagnosing Hereditary Platelet Function Disorders. J Clin Med. 2020;9(3).
25. Bruno A, Tacconelli S, Contursi A, Ballerini P, Patrignani P. Cyclooxygenases and platelet functions. Adv Pharmacol. 2023;97:133-65.
26. Yoxtheimer L, Smith MR, Gunning WT. Platelet aggregation assays do not reliably diagnose platelet storage pool deficiency. Journal of Thrombosis and Haemostasis. 2015;13:47-.
27. Cattaneo M, Cerletti C, Harrison P, Hayward CPM, Kenny D, Nugent D, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. Journal of Thrombosis and Haemostasis. 2013;11(6):1183-9.
28. Brunet JG, Iyer JK, Badin MS, Graf L, Moffat KA, Timleck M, et al. Electron microscopy examination of platelet whole mount preparations to quantitate platelet dense granule numbers: Implications for diagnosing suspected platelet function disorders due to dense granule deficiency. Int J Lab Hematol. 2018;40(4):400-7.
29. Hayward CPM, Al Dawood R, Wice L, Moffat KA. How Trustworthy is Light Transmittance Platelet Aggregometry With Low Platelet Count Samples? Insights From Test Replicates and Retrospective Analysis of Several Decades of Diagnostic Samples. Int J Lab Hematol. 2025.
30. Vinholt PJ, Frederiksen H, Hvas AM, Sprogoe U, Nielsen C. Measurement of platelet aggregation, independently of patient platelet count: a flow-cytometric approach. Journal of Thrombosis and Haemostasis. 2017;15(6):1191-202.
31. Kreft IC, Huisman EJ, Cnossen MH, van Alphen FPJ, van der Zwaan C, van Leeuwen K, et al. Proteomic landscapes of inherited platelet disorders with different etiologies. J Thromb Haemost. 2023;21(2):359-72 e3.
32. Huang JN, Zhang PY, Solari FA, Sickmann A, Garcia A, Jurk K, Heemskerk JWM. Molecular Proteomics and Signalling of Human Platelets in Health and Disease. International Journal of Molecular Sciences. 2021;22(18).
33. Shevchuk O, Begonja AJ, Gambaryan S, Totzeck M, Rassaf T, Huber TB, et al. Proteomics: A Tool to Study Platelet Function. International Journal of Molecular Sciences. 2021;22(9).

34. https://www.nivel.nl/sites/default/files/bestanden/1004409.pdf
35. Huisman EJ, Holle N, Schipperus M, Cnossen MH, de Haas M, Porcelijn L, Zwaginga JJ. Should HLA and HPA-matched platelet transfusions for patients with Glanzmann Thrombasthenia or Bernard-Soulier syndrome be standardized care? A Dutch survey and recommendations. Transfusion. 2024;64(5):824-38.
36. https://www.bestcollaborative.org/
37. Sivapalaratnam S, Austin S, Lorch U, York T, Want A, Gosnell A, et al. A Phase 1/2, First-in-Human, Study to Investigate the Safety, Tolerability, Pharmacokinetics, Pharmacodynamics, and Efficacy of HMB-001 in Participants with Glanzmann Thrombasthenia. Blood. 2023;142.
38. Fustolo-Gunnink SF, Huisman EJ, van der Bom JG, van Hout FMA, Makineli S, Lopriore E, Fijnvandraat K. Are thrombocytopenia and platelet transfusions associated with major bleeding in preterm neonates? A systematic review. Blood Rev. 2019;36:1-9.
39. Curley A, Stanworth SJ, Willoughby K, Fustolo-Gunnink SF, Venkatesh V, Hudson C, et al. Randomized Trial of Platelet-Transfusion Thresholds in Neonates. N Engl J Med. 2019;380(3):242-51.
40. Teofili L, Papacci P, Orlando N, Bianchi M, Pasciuto T, Mozzetta I, et al. BORN study: a multicenter randomized trial investigating cord blood red blood cell transfusions to reduce the severity of retinopathy of prematurity in extremely low gestational age neonates. Trials. 2022;23(1):1010.
41. Cortesi V, Cavallaro G, Raffaeli G, Ghirardello S, Mosca F, Klei TRL, et al. Why might cord blood be a better source of platelets for transfusion to neonates? Blood Transfusion. 2024;22(4):292-302.
42. van der Staaij H, Stanworth SJ, Fustolo-Gunnink SF. Prophylactic Platelet Transfusions: Why Less Is More. Clinics in Perinatology. 2023;50(4):775-92.
43. Zeno RN, Awad S, Lesmana H. Utility and Safety of Romiplostim as an Alternative to Platelet Transfusion for Neonates. Pediatric Blood & Cancer. 2025;72(7).
44. Coppola A, Minno G. Desmopressin in inherited disorders of platelet function. Haemophilia. 2008;14:31-9.
45. Colucci G, Stutz M, Rochat S, Conte T, Pavicic M, Reusser M, et al. The effect of desmopressin on platelet function: a selective enhancement of procoagulant COAT platelets in patients with primary platelet function defects. Blood. 2014;123(12):1905-16.
46. Kobrinsky NL, Israels ED, Gerrard JM, Cheang MS, Watson CM, Bishop AJ, Schroeder ML. Shortening of Bleeding-Time by 1-Deamino-8-D-Arginine Vasopressin in Various Bleeding Disorders. Lancet. 1984;1(8387):1145-8.
47. Schulman S, Johnsson H, Egberg N, Blomback M. Ddavp-Induced Correction of Prolonged Bleeding-Time in Patients with Congenital Platelet-Function Defects. Thrombosis Research. 1987;45(2):165-74.
48. Dimichele DM, Hathaway WE. Use of Ddavp in Inherited and Acquired Platelet Dysfunction. American Journal of Hematology. 1990;33(1):39-45.
49. Lethagen S, Nilsson IM. DDAVP-induced enhancement of platelet retention: its dependence on platelet-von Willebrand factor and the platelet receptor GP IIb/IIIa. Eur J Haematol. 1992;49(1):7-13.
50. Tauer JT, Gneuss A, Lohse JE, Jürgens T, Knöfler R. Evaluation of desmopressin effect on primary haemostasis in pediatric patients with aspirin-like defect as hereditary thrombocytopathy. Klin Padiatr. 2011;223(3):169-72.
51. Nolan B, White B, Smith J, O'Reily C, McMahon C, Fitzpatrick B, Smith OP. Desmopressin: therapeutic limitations in children and adults with inherited coagulation disorders. Brit J Haematol. 2000;109(4):865-9.

52. Hanebutt FL, Rolf N, Loesel A, Kuhlisch E, Siegert G, Knoefler R. Evaluation of desmopressin effects on haemostasis in children with congenital bleeding disorders. Haemophilia. 2008;14(3):524-30.
53. Sutor AH. DDAVP is not a panacea for children with bleeding disorders. Br J Haematol. 2000;108(2):217-27.
54. Cordova A, Barrios NJ, Ortiz I, Rivera E, Cadilla C, Santiago-Borrero PJ. Poor response to desmopressin acetate (DDAVP) in children with Hermansky-Pudlak syndrome. Pediatr Blood Cancer. 2005;44(1):51-4.
55. Atiq F, Heijdra J, Snijders F, Boender J, Kempers E, van Heerde WL, et al. Desmopressin response depends on the presence and type of genetic variants in patients with type 1 and type 2 von Willebrand disease. Blood Adv. 2022;6(18):5317-26.
56. Heijdra JM, Atiq F, Al Arashi W, Kieboom Q, Wuijster E, Meijer K, et al. Desmopressin testing in von Willebrand disease: Lowering the burden. Res Pract Thromb Haemost. 2022;6(6):e12784.
57. Swieringa F, Lance MD, Fuchs B, Feijge MA, Solecka BA, Verheijen LP, et al. Desmopressin treatment improves platelet function under flow in patients with postoperative bleeding. J Thromb Haemost. 2015;13(8):1503-13.
58. Desborough MJ, Oakland K, Brierley C, Bennett S, Doree C, Trivella M, et al. Desmopressin use for minimising perioperative blood transfusion. Cochrane Database Syst Rev. 2017;7:CD001884.
59. Bastida JM, Gonzalez-Porras JR, Rivera J, Lozano ML. Role of Thrombopoietin Receptor Agonists in Inherited Thrombocytopenia. International Journal of Molecular Sciences. 2021;22(9).
60. Zaninetti C, Gresele P, Bertomoro A, Klersy C, De Candia E, Veneri D, et al. Eltrombopag for the treatment of inherited thrombocytopenias: a phase II clinical trial. Haematologica. 2020;105(3):820-8.
61. Lisman T, Adelmeijer J, Cauwenberghs S, Van Pampus ECM, Heemskerk JWM, De Groott PG. Recombinant factor VIIa enhances platelet adhesion and activation under flow conditions at normal and reduced platelet count. Journal of Thrombosis and Haemostasis. 2005;3(4):742-51.
62. Neunert C, Terrell DR, Arnold DM, Buchanan G, Cines DB, Cooper N, et al. American Society of Hematology 2019 guidelines for immune thrombocytopenia. Blood Adv. 2019;3(23):3829-66.
63. Cuker A, Arepally GM, Chong BH, Cines DB, Greinacher A, Gruel Y, et al. American Society of Hematology 2018 guidelines for management of venous thromboembolism: heparin-induced thrombocytopenia. Blood Adv. 2018;2(22):3360-92.
64. Connell NT, Flood VH, Brignardello-Petersen R, Abdul-Kadir R, Arapshian A, Couper S, et al. ASH ISTH NHF WFH 2021 guidelines on the management of von Willebrand disease. Blood Adv. 2021;5(1):301-25.
65. https://www.nvhp.nl/Files/Documentatie/NVHB%20richtlijn%202009%20Diagnostiek%20en%20behandeling%20van%20hemofilie%20en%20aanverwante%20hemostasestoornissen.pdf
66. https://www.nvhp.nl/Files/Documentatie/NVHB.pdf

A journey of a thousand miles must begin with a single step

Lao Tzu (571 BC)

13 SUMMARY & SAMENVATTING

SUMMARY

The focus of this thesis is on children with platelet disorders (PD). The aim is to improve the diagnostic algorithms identifying the causes and underlying mechanisms of PD in order to improve counseling and treatment of children suffering from a PD. Until recently, Glanzmann Thrombasthenia and Bernard-Soulier syndrome were the only clearly identifiable PD subgroups based on routinely available platelet function testing. The last years, the role of molecular genetic testing has increasingly become part of the diagnostic work up. This is important, as PD are heterogenous with regard to genetic background and clinical expression, but also associated with symptoms in various organs resulting in additional comorbidities. The basis of this work is the DoReMi cohort study, which was initiated to better define PD and advance the knowledge of PD in children. This study aims to move from the oversimplification of PD as a disease with either a shortage (thrombocytopenia) or a dysfunction of platelets (thrombocytopathy) towards a rare hereditary bleeding disorder classification based on complex underlying pathways. A scientific boost to better understand the multicolored spectrum of PD was therefore urgently needed. The studies in this thesis contribute to emergence of evidence-based differentiation of PD (**Chapter 1**).

In **Part I** of this thesis, we evaluate and review the current clinical and diagnostic landscape identifying where improvements can be made. In **Part II**, we investigated current dilemmas regarding therapeutic options for PD.

PART I

In **Chapter 2**, we present a scoping review on health-related quality of life (HRQoL) of children with PD and other rare bleeding disorders. We found that research investigating HRQoL in children with PD and their caregivers is lacking. Only one publication on this topic was identified, but used a questionnaire that was developed for children with immune mediated thrombocytopenia, an acquired PD. Extrapolation of disease-specific questionnaires applied in other rare bleeding disorders, such as the extensively studied hemophilia, is clearly not adequate for use in the multi-colored PD disease-entity, as female-related bleeding is not represented.

We also show that when HRQoL is investigated with questionnaires, only reporting the mean or median HRQoL of the study population is not sufficiently informative. We propose that future HRQoL research in children with bleeding disorders, such as PD, should focus on the outliers, as the children and caregivers scoring high on HRQoL as well as the those scoring low may guide clinicians to recognize valuable or ineffective coping skills or points to disease and treatment related factors that influence HRQoL positively or negatively. Furthermore, pooling of children with various disease severity should be avoided.

In **Chapter 3**, we describe the clinical symptoms and laboratory parameters in a cohort of 98 children with PD (DoReMi study). We found that 40% of children with PD reported other comorbidities. Overall, children with PD report mainly mucocutaneous and postoperative bleeding. Interestingly, only a small proportion reports the presence of petechiae. Sex-related differences were found in bleeding presentation and underlying etiology of the PD. In boys, the differential diagnosis of bleeding after circumcision should include PD. In girls, heavy menstrual bleeding occurred frequently and was often the presenting symptom of their PD. Besides sex-related differences, different subgroups within PD were identified. We confirmed that PD combined with specific coagulation factor deficiencies occurred mainly in patients with Noonan syndrome.

Based on our findings, we propose a classification with two main groups. These are based on the results of molecular genetics and functional analysis. When a molecular genetic cause is identified, we propose to use the term inherited PD (IPD). When no such molecular genetic cause is identified to explain the platelet function disorder leading to either a low platelet count (thrombocytopenia) or a platelet dysfunction (thrombocytopathy), the PD should be classified as a functional PD (FPD)

Within these two main groups of IPD and FPD, a subgroup can be identified. For IPD, we identify mutations that lead to isolated disorders in the hematopoietic tract. This so-called isolated PD (ISPD) will lead to a PD and may be accompanied by other red or white blood cell disorders with this not necessarily so. Or the PD is part of an underlying syndrome, a syndrome-associated PD (SAPD). Further specification can be given by adding the gene related to or the syndrome associated with the PD, e.g. Noonan-associated IPD.

For the second group, the FPD, the subgroup classification is based on the low platelet count (quantitative disorder), or functional defect (qualitative disorder), that can be further specified by adding the functional defect, e.g. COX-mediated FPD.

In **Chapter 4**, we investigated if mass-spectrometry (MS) analysis of the platelet proteome is able to better diagnose PD. As proteins are the functional derivatives of our genetic code, analyses of the platelet proteome may be more informative to identify the genetic disorders underlying IPD and SAPD. Moreover, these results may also help to unravel the modifying pathways in this complex disorder with a heterogeneous presentation. To test this hypothesis, the platelet proteome of 80 children and family members with a PD and an identified genetic defect was analyzed. We demonstrated that >4000 proteins are detectable in these platelets using the applied set-up and MS technique. These proteins can be grouped by either their location or functional role, such as 'granule-associated proteins' and 'hemostasis-involved proteins'. Variability and reproducibility was good or acceptable for almost all proteins. One important limitation of the technique is that gene

products involved in early megakaryopoiesis, like FLI1p and ETV6p, are not detectable in the peripheral platelets.

In **Chapter 5**, we investigated the clinical phenotype and laboratory results of 14 patients with a variety of 11q aberrations. Partial deletions or duplications of the long arm of chromosome 11 (11q), ranging from 1-20 Mb, are rare and considered a contiguous gene deletion or duplication syndrome. Terminal 11q deletions occur most frequently. Breakpoints arising within or distal to 11q23.3 with a deletion extending to the telomere lead to a clinical entity known as Jacobsen syndrome. The clinical phenotype of Jacobsen syndrome includes increased bleeding tendency due to low platelet counts and/or platelet dysfunction. Typically, giant alpha granules may be observed known as Paris-Trousseau syndrome, but abnormal dense granules and microkaryocytes have also been reported. Besides Jacobsen syndrome, interstitial deletions as well as interstitial and terminal duplications of 11q are also reported. Detailed data on bleeding phenotype, platelet numbers and platelet function in non-terminal 11q disorders is lacking.

We observed that patients with interstitial 11q deletions as well as terminal 11q duplications have a similar phenotype as patients with terminal 11q deletions (Jacobsen syndrome). Most frequently reported bleeding symptoms were hematomas, bleeding from small wounds, perioperative bleeding and heavy menstrual bleeding in females. We demonstrated that despite normal platelet number, platelet dysfunction was highly prevalent. Therefore, when 11q aberrations are present, platelet count and function must be tested by light transmission aggregometry (LTA). Remarkably, the abnormal responses to thrombin receptor-activating peptide (TRAP) that we observed may reflect impaired effect of thrombin *in vivo*. This may point to storage pool disease (SPD), a subgroup of PD with abnormal platelet granule function. This was supported in our report by abnormal ATP-ADP ratio and abundance of NUBP2p and ATP5Sp observed by MS-based analysis of the platelet proteome. Finally, abnormal alpha granules were seen by electron microscopy in two patients with an interstitial and a terminal 11q deletion.

There is evidence from other PD, like Hermansky-Pudlak syndrome type 2 or Grey platelet syndrome that granule function in the neutrophils may also be abnormal. As patients with 11q disorders, including Jacobsen syndrome, are reported to be susceptible to bacterial and prolonged viral and fungal infections, we investigated the function of neutrophils as well as of B and T cells in **Chapter 6**. We found that in 30% of patients' granulocyte function was abnormal, but this observation did not lead to a distinct clinical phenotype fitting granulocyte disorders. Importantly, T cell dysfunction was present in almost all patients. This supports the findings of a high burden of infections in patients, consisting of respiratory, ear-nose-throat infections or secondary bacterial infections of varicella skin lesions, despite normal immunoglobulin G levels. A large proportion of patients also reported chronic eczema, warts, and chronic fungal infections. Furthermore, decreased

numbers of B cells (57%), as well as T cells (36%) and abnormal T cell function (86%) were demonstrated in patients with 11q aberrations except for interstitial 11q duplications. We did not find a clear phenotype-genotype correlation with *FLI1* and *ETS1* involvement in the majority of patients. Therefore, we concluded that other candidate genes on 11q may play a role in both platelet and immune dysregulation. Fundamental research is needed to further understand the role of these genes coding for hematopoietic transcription factors, like *FLI1*.

In **Chapter 7** we investigated the role of the transcription factor FLI1, a member of the erythroblast transformation specific (ETS) family, on megakaryopoiesis and platelet production in a patient with a de novo heterozygous nonsense mutation in *FLI1* (NM_002017.5(FLI1): c.297del, p(Met100*)). Effect of this in potential truncating variant on megakaryocyte maturation was investigated by comparing wild-type (WT) FLI1p and FLI1 p.Met100* in Meg01 cells on RNA and proteome level. By doing so, we demonstrated that the truncated FLI1 p.Met100* proteins that were expressed in Meg01 cells, upregulated both megakaryoblasts and erythroblasts in the progenitor stage of differentiation, by advantaging *KLF1* impact. This led to a distinct pattern on flow cytometry with significantly elevated and prolonged expression of CD235a on erythroid cells and CD36 on megakaryocytes, as well as elevated KLF1p expression by FLI1^{Met100*}. However, after this megakaryocyte progenitor phase, maturation and differentiation to megakaryocytes were both accelerated and impaired in the FLI1^{Met100*} Meg01 cells. This was not seen in WT *FLI1*, as up-regulation of WT *FLI1* alone only led to accelerated differentiation but normal maturation. Our findings therefore suggest that FLI1 p.Met100* impairs megakaryocyte maturation through a dominant-negative manner contributing to the congenital thrombocytopenia. Potentially this is the result of upregulated erythroid features at the expense of megakaryocytic development.

In **Chapter 8,** we present two siblings with pathogenic variants in another transcription factor gene that tightly regulates megakaryopoiesis, the *ETV6* (formerly *TEL*), which is also a member of the ETS family. Pathogenic variants in *ETV6* lead to autosomal dominant thrombocytopenia. ETV6 acts mainly as a transcriptional repressor, forming dimers through its pointed (PNT) domain, interacting with cofactors via its central domain, and binding DNA through its conserved ETS domain. It is in this domain that most pathogenic variants are found. In the two patients with an inherited thrombocytopenia due to a class 4 variant in *ETV6* (NM_001987.4(ETV6): c.1105C>T, p.(Arg369Trp, in short ETV6-R369W), we found that 424 genes were upregulated and 203 were downregulated in comparison to identical genes in the wild-type (WT) cells. This is consistent with ETV6's repressive function in normal conditions. Products from erythroid genes (*GYPA*, *CD36*, and *KLF1*) were reduced, while products from genes associated with hematopoietic lineage and platelet activation pathways (*CD44*, *KIT*, *GP6*, and *FLI1*) were increased. Overexpression of ETV6-R369W in wild-type Meg01 cells produced a similar pattern, suggesting a dom-

inant-negative mechanism. In $CD34^+$ cultures, ETV6-R369W accelerated megakaryocyte differentiation. CD44 was also elevated in both patient's platelets, suggesting its potential as a biomarker for ETV6-related thrombocytopenia.

PART II

Platelet transfusions are indicated in case of acute and/ or severe bleeding (therapeutic transfusion) or to prevent bleeding (prophylactic transfusion) to resolve thrombocytopenia or thrombocytopathy. The development of HLA or HPA antibodies can of course complicate treatment, as platelets will not increase sufficiently, therefore forming a potentially life-threatening complication. We demonstrate in **Chapter 9** that prophylactic matching for HLA is not only regarded as good clinical practice in literature reviews but, according to the survey performed, is also already widely applied by a majority of Dutch clinicians treating PD patients. Literature and guidelines reviewed unfortunately lack essential information on non-transfusion and transfusion related risk factors contributing to HLA or HPA antibody formation, to estimate incidence estimation. We conclude that regarding HLA antibody prevention, prevention of HLA formation is the key leading to preservation of random platelet transfusions in acute setting for patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome. This benefit outweighs the effort of timely-ordered and more costly HLA matched platelets in elective setting, until more knowledge is gained on risk groups and incidence of HLA antibody formation. For HPA antibody prevention, we demonstrated that screening for HPA antibodies may be helpful in very specific clinical management and risk assessment, such as pregnancy. Assessment can be based on a risk model based on HPA genotyping. For pregnant mothers with Glanzmann Thrombasthenia or Bernard-Soulier syndrome, we recommend fetal monitoring during pregnancy when maternal HLA, HPA or isoantibodies are present, as well as postnatal platelet count measurements in the neonate.

In **Chapter 10,** we present a systematic review to provide the essential insights on the association between thrombocytopenia or prophylactic platelet transfusions on major bleedings in premature neonates. This is important information to establish what platelet counts in premature neonates triggers prophylactic platelet transfusions. We screened 8734 publications and included 36 publications that met established study inclusion criteria. Of these 36 publications, only seven were of sufficient scientific quality to include in the final analysis. The other 29 were excluded due to inexact information on timeframe between major bleeding and platelet count or major bleeding and platelet transfusion. This so-called temporal ambiguity, led to cause-and-effect relationships influencing the outcome. In the seven papers, n=3 addressed the association between prophylactic platelet transfusions and bleeding, and n=4 platelet count and bleeding (n=4). No association was seen between prophylactic platelet transfusions or platelet count with major bleeding in n=5, but in n=2, an associated between thrombocytopenia and pulmonary hemorrhage was seen. Overall, we concluded that the causal relationship between

thrombocytopenia, platelet transfusions and major bleeding in extreme premature neonates can neither be proven nor rejected in literature. Therefore, the clinical need for prophylactic platelet transfusions in neonates with an asymptomatic thrombocytopenia could not be resolved.

Chapter 11 describes the use of desmopressin (1-deamino-8-D-arginine vasopressin) in children with PD. Desmopressin enhances primary hemostasis by activating alpha granules and cyclic adenosine monophosphate, that in return leads to exocytosis of von Willebrand Factor (VWF) in the vascular endothelium. Mode of action is derived from its use in von Willebrand disease (VWD) and mild hemophilia A patients. Previously, only one double-blind randomized crossover trial in 18 PD patients was performed, besides few case-series describing small numbers of (pooled) adult and pediatric PD patients. In most reports, desmopressin response was quantified by reduction of bleeding time, a test that is no longer applied. We, therefore, conducted an observational cohort study to evaluate desmopressin response in 51 children, who were aged between 4-18 years, and had various subtypes of PD. We evaluated repeated measurements of von Willebrand factor (VWF activity) and Platelet Function Analyzer (PFA) results. We defined the response as '*good*, *partial* or '*poor*' based on a combination of VWF rise and/or PFA normalization. We found that desmopressin response was '*good*' in 30, '*partial*' in 13 and '*poor*' in eight of these children. We concluded that not all PD patients respond adequately to desmopressin when the response is quantified by rise in VWF activity or PFA normalization, and that this this was especially true for children classified as inherited PD (IPD) or Syndrome-associated PD (SAPD).

Lastly in **Chapter 12**, the most relevant outcomes of the studies are placed in a broader perspective. We discuss the diagnostic and therapeutic approaches that we have investigated and describe the knowledge gaps identified by our studies, suggesting future research aims.

SAMENVATTING

Het doel van het onderzoek in dit proefschrift is het verbeteren van de manier waarop we een diagnose stellen bij en hoe we kinderen behandelen met een aangeboren bloedplaatjesaandoening (PD, afkorting voor 'platelet disorder'). Door de diagnostiek te verbeteren krijgen we meer inzicht in de onderliggende mechanismen waardoor een PD ontstaat en kunnen we het veelkleurige spectrum van deze ziekte beter begrijpen. Hierdoor kunnen we de zorg, toekomstperspectieven en leefstijladviezen beter op het individuele kind aanpassen.

Lange tijd waren Glanzmann Thrombasthenia en het Bernhard-Soulier syndroom de enige PD subgroepen die met de huidige bloedplaatjesfunctietesten goed onderscheiden konden worden. De afgelopen jaren is door de toegenomen mogelijkheden van het inzetten van moleculair-genetisch onderzoek deze vorm van diagnostiek van steeds groter belang geworden voor de diagnosestelling van PD bij kinderen. Vooral omdat PD als ziektegroep een grote variatie aan betrokken genen kent. Dit verklaart ook de variatie in symptomen en complicaties die kinderen met een PD kunnen ervaren. Daarnaast kunnen bepaalde genen naast een PD, ook tot afwijkingen in andere organen leiden.

De basis van dit onderzoek wordt gevormd door de DoReMi cohortstudie. Deze is gestart om een beter inzicht te krijgen in de verschillende soorten PD, maar ook om meer kennis te verwerven over symptomen en complicaties van PD bij kinderen rondom diagnose en op langere termijn, en over de waarde van moleculair genetisch onderzoek bij kinderen die verdacht worden van een PD. De DoReMi studie bevestigt dat het eenvoudige beeld dat PD een aandoening is die alleen bestaat uit een tekort aan bloedplaatjes (trombocytopenie) of een verstoorde werking van bloedplaatjes (trombocytopathie) niet langer voldoende is. Met deze studie hebben we data verzameld die gebruikt kan worden voor een wetenschappelijk onderbouwde classificatie waarbij PD in verschillende hoofdgroepen en subgroepen wordt ingedeeld. Hierdoor worden onderliggende mechanismen en de gevolgen van PD voor diagnostiek keuze, follow-up en prognose voor de individuele patiënt inzichtelijker. Ook biedt het de mogelijkheid om patiënten te kunnen groeperen in specifieke subgroepen (**Hoofdstuk 1)**. De resultaten van het onderzoek zijn beschreven in dit proefschrift en dragen zo bij aan nieuwe inzichten over PD bij kinderen.

In **Deel I** van dit proefschrift evalueren we het huidige klinische en diagnostische spectrum van PD. We stellen vast waar er ruimte is voor verbetering. In **Deel II** onderzoeken we dilemma's tussen en mogelijkheden voor behandelingen die momenteel beschikbaar zijn voor kinderen met PD.

DEEL I

In **Hoofdstuk 2** presenteren we een overzicht van het onderzoek dat verricht is naar de gezondheid-gerelateerde kwaliteit van leven (HRQoL, afkorting voor 'Health-Related Quality of Life') in kinderen met PD. We vergelijken dit met HRQoL onderzoek dat verricht is bij kinderen met andere stollingsstoornissen. Belangrijke conclusies waren ten eerste dat er weinig tot geen onderzoek is verricht naar de HRQoL bij kinderen met PD noch bij hun ouders of wettelijke vertegenwoordigers (vanaf nu 'ouders'). De enige publicatie die we vonden, analyseerde het gebruik van een HRQoL-vragenlijst die was ontwikkeld voor kinderen met een verworven, immuungemedieerde trombocytopenie. Deze bleek echter niet goed bruikbaar voor kinderen met een aangeboren, congenitale PD. Vragenlijsten die ontwikkeld waren voor andere stollingsstoornissen, bleken ook niet één op één gebruikt te kunnen worden voor HRQoL onderzoek bij kinderen met PD. Ter illustratie, vragenlijsten die ontwikkeld waren voor hemofilie, een zeldzame stollingsstoornis die met name bij jongens voorkomt, bevatten geen menstruatie-gerelateerde bloedingen waardoor een belangrijke risicofactor voor bloedverlies bij meisjes ontbreekt.

Ten tweede observeerden we dat, indien de HRQoL wordt onderzocht met behulp van klassieke vragenlijsten, het rapporteren van het gemiddelde of de mediaan van de studiepopulatie onvoldoende informatie geeft. We stellen voor om in toekomstig HRQoL onderzoek bij kinderen met stollingsstoornissen zoals PD ook de uitschieters te vermelden, dat wil zeggen de datapunten die significant afwijken van de overige waarden in de dataset. Juist de kinderen en ouders die hoog of laag scoren op de HRQoL vragenlijsten, kunnen artsen en behandelaren meer inzicht bieden over gunstige, bijdragende coping strategieën of juist ineffectieve, minder goede strategieën identificeren. Daarnaast kunnen deze uitschieters inzicht geven in mogelijke ziekte- of behandelgerelateerde factoren die de HRQoL voor deze kinderen en hun ouders verbeteren of juist doen afnemen.

In **Hoofdstuk 3** beschrijven we de klinische symptomen en laboratoriumparameters voor een cohort van 98 kinderen met PD (DoReMi studie). We rapporteren dat 40% van de kinderen met PD naast hun bloedplaatjesaandoening, één of meerdere andere problemen als co-morbiditeit vermeldt. Ten aanzien van de bloedingssymptomen, melden kinderen vooral mucocutane en postoperatieve bloedingen. Slechts een deel van de kinderen rapporteert de aanwezigheid van zogenaamde petechiën, kleine rood-blauwe puntjes die kenmerkend zijn voor PD. We vinden sekse-gerelateerde verschillen in de bloedingssymptomen en in de etiologie van de PD. Bijvoorbeeld, bij jongens dient PD opgenomen te worden in de differentiaaldiagnose voor een bloedingscomplicatie tijdens of na een besnijdenis. Bij meisjes komt hevig menstrueel bloedverlies frequent voor, en dit was ook relatief vaak de klacht waarmee zij zich bij een arts presenteerden. Naast het onderscheid naar sekse werden ook andere subgroepen onderscheiden. We bevestigen dat de combinatie van een PD met stollingsfactor-deficiënties voornamelijk voorkwam bij patiënten met het Noonan syndroom.

Op basis van onze observaties stellen we een classificatie met twee hoofdgroepen voor. Hierbij wordt het ziektebeeld PD ingedeeld op basis van moleculair genetisch onderzoek en functionele analyse van de bloedplaatjes. Wanneer er een onderliggend gen of chromosomale afwijking wordt geïdentificeerd, dan is er sprake van een erfelijke PD (IPD, afkorting van 'inherited platelet disorder'). Wanneer er geen moleculair genetische verklaring wordt gevonden, maar wel een functiestoornis van het bloedplaatje die of leidt tot een verlaagd aantal bloedplaatjes (trombocytopenie), of leidt tot een verslechtering in de bloedplaatjesfunctie (trombocytopathie), dan is er sprake van een functionele PD (FPD).

Binnen deze twee hoofdindelingen is er de mogelijkheid om aan te geven tot welke subklasse een IPD of FPD behoort. Voor de IPD, is er allereerst een onderverdeling mogelijk voor genetische afwijkingen die leiden tot een geïsoleerde hematopoïetische afwijking, waar in ieder geval de bloedplaatjes één van de afwijkende bloedceltypes zijn, een zogenaamd geïsoleerd PD (ISPD, afkorting voor 'isolated PD'). Daarnaast kan het voorkomen dat de onderliggende genafwijking leidt tot een syndroom waarbij er naast een PD, problemen in meerdere organen en weefsels kunnen optreden. Deze subgroep wordt geclassificeerd als een syndromale PD (SAPD, afkorting voor 'syndrome-associated PD'). Een verdere specificatie kan dan plaatsvinden door de naam van het aangedane gen of het met PD-geassocieerde syndroom toe te voegen, bijvoorbeeld Noonan-geassocieerde PD.

Voor de tweede hoofdgroep, de FPD, vormt een laag aantal bloedplaatjes (kwantitatief defect) of een disfunctie (kwalitatief defect) de basis voor de verdere onderverdeling, bijvoorbeeld door een kenmerkend functiedefect aan de term FPD toe te voegen, zoals COX-gemedieerde FPD.

In **Hoofdstuk 4** onderzoeken we met behulp van een massaspectrometrische analyse (MS, afkorting voor 'mass-spectromtry') of we de diagnose PD kunnen stellen door het bloedplaatjes proteoom te onderzoeken. Aangezien proteïnes de functionele afgeleiden zijn van onze genetische code, kunnen analyses van het bloedplaatjes proteoom informatie geven over de genetische afwijking die leidt tot een IPD. Omgekeerd, kunnen deze analyses ook een hulpmiddel zijn in het ontrafelen van de onderliggende interactiepatronen in complexe en heterogene ziektes zoals PD. Om deze hypothese te testen analyseerden we het bloedplaatjes proteoom van 80 kinderen en hun familieleden waarbij de PD het gevolg was van een moleculair genetisch defect. We tonen aan dat met de gebruikte methode >4000 proteïnen in de bloedplaatjes worden gedetecteerd met behulp van MS. Deze proteïnen kunnen worden gegroepeerd naar hun positie en specifieke functie, zoals bijvoorbeeld de 'granulen-gerelateerde proteïnen' of de 'hemostase-actieve' proteïnen. Variabiliteit en reproduceerbaarheid waren goed of tenminste acceptabel voor vrijwel alle proteïnen. Echter, een belangrijke beperking van de methode is dat de

eiwitten van genen die actief zijn in de vroege megakaryopoïese, zoals FLI1p en ETV6p, niet detecteerbaar zijn in perifere bloedplaatjes.

In **Hoofdstuk 5** onderzoeken we het klinisch fenotype en de laboratoriumresultaten van 14 patiënten met verschillende 11q afwijkingen. Gedeeltelijke deleties of duplicaties van de lange arm van chromosoom 11 (11q) variëren in grootte van 1 tot 20 Mb. Deze 11q afwijkingen zijn zeldzaam en worden beschouwd als een zogenaamd 'aaneengesloten gendeletie of duplicatiesyndroom'. Terminale 11q deleties komen hierbij het meest voor. Breekpunten die optreden binnen of distaal van 11q23.3 met een deletie die reikt tot aan het telomeer, leiden tot een klinische conditie die bekend staat als het Jacobsen syndroom. Bij het klinisch fenotype van het Jacobsen syndroom hoort een bloedingsneiging vanwege de lage aantallen en/of een disfunctie van de bloedplaatjes. Kenmerkend hierbij is het voorkomen van reuze alfa-granules in de bloedplaatjes, dit wordt het Paris-Trousseau syndroom genoemd. Daarnaast zijn er ook publicaties van afwijkende dense-granules en microkaryocyten. Deleties van chromosoom 11 kunnen echter ook op niet-terminale, interstitiële 11q loci voorkomen, of er kan sprake zijn van interstitiële of terminale duplicaties van 11q. Voor deze interstitiële 11q deleties en 11q duplicaties ontbrak gedetailleerde data voor wat betreft bloedingsfenotype en bloedplaatjesaantal en -functie. Wij observeerden dat patiënten met een interstitiële 11q deleties of een terminale 11q duplicatie hetzelfde fenotype laten zien als patiënten met terminale 11q deleties (het Jacobsen syndroom).

Ook laten we zien dat patiënten met interstitiële 11q deleties en terminale l11q duplicaties een vergelijkbaar fenotype hebben als patiënten met het terminale 11q deletiesyndroom (Jacobsen syndroom). Hierbij zijn de meest gemelde bloedingssymptomen: hematomen, langdurig bloedingen uit kleine wondjes, perioperatieve bloedingen en, bij vrouwen, hevig menstrueel bloedverlies. We tonen aan dat vooral bij een normaal aantal bloedplaatjes, een verstoorde bloedplaatjesfunctie veel voorkwam. In de functietest die verricht wordt met een licht-transmissie aggregometer (LTA, afkorting voor 'light transmission aggregometry') zijn er opvallend vaak abnormale reacties op de agonist van 'Trombine receptor-activerende peptide (TRAP)' gevonden, die een *in vivo* verstoorde werking van trombine weerspiegelt. Dit kan wijzen op een storage pool ziekte (SPD, afkorting voor 'storage pool disease'), een subgroep van PD met een abnormale werking van de granules in de bloedplaatjes. De abnormale ATP-ADP ratio's en een overvloed van NUBP2p en ATP5Sp in de MS-analyse van het bloedplaatjes proteoom ondersteunen deze hypothese. De aanwezigheid van abnormale alfa-granules werd ook door middel van elektronenmicroscopie (EM) geobserveerd bij twee patiënten, waarvan één een interstitiële 11q deletie had en de andere de 'klassieke' terminale 11q deletie.

Er zijn aanwijzingen vanuit andere SPD-PD subgroepen, zoals het Hermansky-Pudlak type 2 syndroom of het grijze bloedplaatjes syndroom dat de functie van neutrofielen

granules ook afwijkend kan zijn bij SPD-PD. Aangezien patiënten met het Jacobsen syndroom, veel infecties hebben of een ernstiger beloop van infecties kunnen hebben, onderzochten wij de functie van deze neutrofielen. Tevens analyseerden we de B- en T-celfunctie. Deze studie beschrijven wij in **Hoofdstuk 6**. We vinden dat in 30% van de patiënten de granulocytenfunctie abnormaal is, maar dat dit niet leidt tot een onderscheidend klinisch fenotype passend bij granulocytenfunctiestoornissen. Wat wel opvalt is dat vrijwel alle patiënten een disfunctie van de T-cellen hebben. Dit verklaart mogelijk onze observatie dat de virale infectiedruk ook verhoogd was in patiënten met normale concentraties van immunoglobuline G. Infecties die frequent voorkomen in deze groep zijn bovenste en onderste luchtweginfecties, keel-neus-oor-infecties en huidinfecties, waarbij er opvallend vaak eczeem is gerapporteerd als ook wratten en geïnfecteerde waterpokken. Verder zien we verlaagde aantallen B-cellen (57%) en T-cellen (36%), als ook afwijkende T-cel functies (86%) bij patiënten met verschillende 11q afwijkingen, met als enige uitzondering de resultaten van een patiënt met een interstitiële 11q duplicatie. We vinden geen duidelijke correlatie tussen het fenotype en het genotype voor de genen *FLI1* en *ETS1*. Mogelijk zijn er andere kandidaatgenen op 11q die geassocieerd zijn met de ontregeling van de bloedplaatjes en het immuunsysteem. Verder fundamenteel onderzoek is noodzakelijk om een beter begrip te krijgen van de rol van de genen die de hematopoïetische transcriptiefactoren beïnvloeden, zoals de reeds genoemde *FLI1*.

In **Hoofdstuk 7** beschrijven de resultaten van ons onderzoek naar de rol van de transcriptiefactor FLI1 op de megakaryopoïese en de bloedplaatjesproductie in een patiënt met een de novo heterozygote nonsens mutatie in *FLI1 (*NM_002017.5(*FLI1*): c.297del, p.(Met100*)). Het effect van deze mogelijk trunkerende variant op de rijping van megakaryocyten analyseerden we door het wilde-type (WT) FLI1p met de FLI1 p.Met100* in Meg01-cellen te vergelijken op RNA en proteoomniveau. Hiermee laten we zien dat het getrunkeerde FLI1 p.Met100* eiwit in Meg01-cellen leidt tot een versnelde megakaryopoïese en erythroblastvorming. Hierbij valt ook op dat de vorming van erytroblasten op voorloperniveau versneld plaats vindt door een toegenomen *KLF1*-activiteit als neveneffect van de verhoogde *FLI1*-activiteit. Dit leidt tot een identificeerbaar patroon in de flowcytometrie met een significant verhoogde en langer aantoonbare expressie van CD235a op erythroïde voorlopercellen en uitgerijpte erytrocyten, als ook een verhoogde en langer aantoonbare expressie van CD36 op megakaryocyten in combinatie met een verhoogde KLF1p expressie in de FLI1^{Met100*} Meg01-cellen.

Naast deze versnelde megakaryoblastaire voorloperfase, neemt ook latere maturatie en differentiatie sterk af in de laatste fase van de megakaryopoïese in de *FLI1*^{Met100*} Meg01-cellen. Dit gecombineerde effect komt niet voor in de WT *FLI1*-cellen. Hier is bij toename van de activatie (in het Engels, 'upregulation') van de WT *FLI1*-cellen weliswaar ook een versnelde differentiatie gezien, maar deze gaat niet gepaard met de gelijktijdige abnormale maturatie van megakaryocyten. Onze resultaten suggereren dat de Met100*-variant

in *FLI1* de uiteindelijke maturatie van megakaryoblasten tot werkzame megakaryocyten verhindert op een dominant negatieve manier. Dit kan de congenitale trombocytopenie verklaren in de patiënten met deze aandoening. Het is ook mogelijk dat de parallel optredende en versneld erythroïde differentiatie en rijping ten koste gaat van megakaryocytaire differentiatie en maturatie.

In **Hoofdstuk 8** presenteren we twee 1e lijns familieleden met een variant in *ETV6* (voorheen *TEL* genaamd). *ETV6* is, net als *FLI1*, lid van de ETS-familie. *ETV6* codeert voor de ETV6 transcriptiefactor die de megakaryopoïese reguleert. Bekende pathogene varianten in *ETV6* leiden tot een autosomaal dominante trombocytopenie. In de megakaryopoïese fungeert ETV6 als een transcriptieonderdrukker door dimeren te vormen via een zogenaamd 'puntig domein' (in het Engels, pointed domain, PNT). Tevens vormt ETV6 een interactie met cofactoren via het centrale domein, en bindt het zich aan DNA via een sterk geconserveerd ETS-domein. In dit ETS-domein, worden de meeste pathogene varianten gevonden die leiden tot een congenitale trombocytopenie. We onderzoeken de Meg01-cellen van twee patiënten met een congenitale trombocytopenie al gevolg van een klasse 4 variant in *ETV6* (NM_001987.4(ETV6): c.1105C>T, p.(Arg369Trp), afgekort tot ETV6-R369W). Hierdoor zien we dat, vergeleken met het wild type (WT), dat er 424 genen een toegenomen activiteit (in het Engels, upregulation) vertonen, en dat 203 genen een verminderde activiteit (in het Engels, downregulation) vertonen. Op zich past dit bij de controlerende functie van *ETV6* in normale omstandigheden., maar we vonden duidelijk minder proteïnes van erythroïde genen (zoals *GYPA, CD36* en *KLF1*) dan in de WT *ETV6*, en juist meer proteïnes van genen die geassocieerd zijn met de algemene hematopoëse of juist specifiek waren voor bloedplaatjesactivatiepaden (zoals *CD44, KIT, GP6* en *FLI1*). De overexpressie van ETV6-R369W in de Meg01-cellen tonen een vergelijkbaar patroon met de hiervoor beschreven *FLI1* Meg100* cellen, waarbij ondanks aanvankelijke activatie van megablastocytaire voorlopers de uiteindelijke differentiatie en maturatie tot bloedplaatjesvormende megakaryocyten minder goed verloopt. Hierbij treedt ook een opvallend vroege bloedplaatjesactivatie op. CD44 komt verhoogd tot expressie op rijpe bloedplaatjes van beide patiënten, zodat dit wellicht een biomarker zou kunnen zijn voor aan *ETV6*-gemedieerde trombocytopenie.

DEEL II

Bloedplaatjestransfusies worden voorgeschreven bij een acute of ernstige bloeding (therapeutisch) of om een bloeding te voorkomen (profylactisch). De onderliggende indicatie kan zowel een trombocytopenie als een trombocytopathie zijn. De aanwezigheid van HLA- of HPA-antistoffen kunnen deze bloedplaatjestransfusies minder effectief maken, doordat de bloedplaatjesaantallen onvoldoende toenemen. De onderliggende trombocytopenie of trombocytopathie is dan onvoldoende gecorrigeerd om een bloeding of een potentiële bloeding te behandelen of te voorkomen. Om deze antistoffen te voorkomen, kan er preventief gematcht worden op HLA-en/of HPA-antigenen van

de patiënt. In **Hoofdstuk 9** laten we door middel van literatuuronderzoek en een vragenlijst, die gestuurd is aan Nederlandse experts, zien dat het profylactisch matchen op HLA-antigenen voor een transfusie in de literatuur wordt beschouwd als de aanbevolen medische handeling voor patiënten met Glanzmann Thrombasthenia en Bernard-Soulier syndroom. Tevens blijkt dit reeds breed toegepast door een meerderheid van de artsen die PD-patiënten behandelen in Nederland.

Echter, de literatuur en richtlijnen die wij systematisch onderzochten, bevatten helaas onvoldoende informatie over transfusie en non-transfusiegerelateerde risicofactoren die bijdragen aan de vorming van HLA-antistoffen. Hierdoor was het niet mogelijk om de incidentie van het voorkomen van HLA-antistoffen of selectieve risicogroepen te bepalen. Totdat meer bekend is over het vóórkomen van HLA-antistoffen bij risicogroepen, is het nodig om regelmatig op vaste momenten de aanwezigheid van HLA- of HPA-antistoffen te testen. We adviseren daarom op praktische gronden, om voor patiënten met Glanzmann Thrombasthenia en Bernard-Soulier syndroom in electieve situaties HLA-gematchte bloedplaatjestransfusies te selecteren ondanks de hogere kosten en ingewikkeldere logistiek, met als doel om de standaard transfusieproducten voor acute transfusie situaties beschikbaar en effectief te houden.

Ter voorkoming van de vorming van HPA-antistoffen, tonen we aan dat het screenen op HPA-antistoffen behulpzaam kan zijn voor een risico-inschatting bij onder andere zwangerschap. De noodzaak tot preventief matchen voor HPA-antigenen kan met behulp van een risico-inschatting gebaseerd worden op de HPA-genotypering van de patiënt. Bij zwangeren met Glanzmann Thrombasthenia of Bernard-Soulier syndroom adviseren we foetale monitoring gedurende de zwangerschap wanneer er bij de zwangere HLA-HPA- of iso-antilichamen aanwezig zijn, in combinatie met postnatale bepaling van het bloedplaatjesaantal bij de pasgeborene.

In **Hoofdstuk 10** presenteren we een systematische beoordeling (in het Engels, 'systematic review') die zowel de mogelijke verbanden onderzoekt tussen het ontstaan van ernstige bloedingen bij te vroeg geboren baby's en de aanwezigheid van een trombocytopenie, als ook het verband tussen het ontstaan van ernstige bloedingen en het geven van profylactische trombocytentransfusie bij een trombocytopenie. We hoopten met dit onderzoek duidelijk te krijgen bij welk bloedplaatjesaantal te vroeg geboren baby's een trombocytentransfusie nodig hebben. We screenden 8734 publicaties op basis van titel en abstract, en selecteerden uiteindelijk 36 publicaties die voldeden aan de vooraf bepaalde inclusiecriteria. Van deze 36 publicaties, waren slechts zeven van voldoende wetenschappelijke kwaliteit om in de uiteindelijke analyse te worden meegenomen. De overige 29 publicaties werden uitgesloten vanwege onnauwkeurige informatie over het tijdsbeloop tussen de bloeding en de bepaling van het bloedplaatjesaantal, of het tijdsbeloop tussen de bloeding en de bloedplaatjestransfusie. Deze zogenaamde

tijdsambiguïteit (in het Engels, 'temporal ambiguity') leidt tot een onjuiste oorzaak-gevolg relatie in de geëxcludeerde artikelen. In vijf van de zeven geïncludeerde artikelen leidde het profylactisch geven van een transfusie (n=3) of een ernstige trombocytopenie (n=2) niet tot een bloeding, in twee werd wel een verband beschreven tussen een ernstige trombocytopenie en een longbloeding. Samenvattend, concluderen we dat op basis van de beschikbare literatuur het causale verband tussen trombocytopenie of bloedplaatjestransfusies en ernstige bloeding bij te vroeg geboren baby's als uitkomst niet kan worden aangetoond dan wel afgewezen. Op basis van de geïncludeerde literatuur is het niet mogelijk om een minimale drempelwaarde vast te stellen voor het profylactisch toedienen van bloedplaatjestransfusies bij te vroeg geboren baby's met een trombocytopenie

Hoofdstuk 11 beschrijft het gebruik van desmopressine (1-deamino-8-D-arginine vasopressine) bij kinderen met PD. Desmopressine versterkt de primaire hemostase door het activeren de alfa-granules en het cyclisch adenosine monofosfaat. Dit leidt vervolgens tot exocytose van von Willebrand Factor (VWF) vanuit het vasculair endotheel. De kennis over de effectiviteit van desmopressine tijdens een acute bloeding bij PD patiënten is hoofdzakelijk afgeleid van het gebruik van desmopressine bij patiënten met von Willebrand ziekte (VWD, afkorting van 'von Willebrand Disease') en bij patiënten met milde hemofilie A. Er is slechts één dubbelblind gerandomiseerde cross-over studie uitgevoerd bij 18 kinderen met PD. Daarnaast is er een beperkt aantal case-series die kleine aantallen PD patiënten beschrijven die met desmopressine zijn behandeld. Bij het merendeel van de publicaties werd de desmopressine respons gemeten door het bepalen van de bloedingstijd, een sterk verouderde test.

In een observationeel cohortonderzoek evalueerden wij de desmopressine-respons in 51 kinderen met een leeftijd tussen de 4 en 18 jaar en met verschillende PD-subtypen. Dit deden we door middel van het herhaald bepalen van de VWF-activiteit en door bloedplaatjesfunctie te screenen met behulp van een PFA (afkorting voor 'Platelet Function Analyzer'). We deelden de respons op in 'goed', 'deels' of 'slecht' op basis van een combinatie van tenminste een verdubbeling van VWF-activiteit en/of de normalisatie van de PFA t.o.v. de uitgangswaarde. Resultaten laten zien dat de desmopressine-respons 'goed' is in 30 kinderen, maar 13 kinderen scoren 'deels' en 8 kinderen zelfs 'slecht'. We concluderen daarom dat, als er gekeken wordt naar de toename in VWF-activiteit en de normalisatie van de PFA na het toedienen van desmopressine, niet alle PD-patiënten adequaat op desmopressine reageerden, en dat dit in het bijzonder het geval was bij kinderen met een onderliggend gendefect als basis voor de PD, een zogenaamde erfelijke PD (IPD, afkorting voor 'inherited PD') of waarbij PD als onderdeel van een syndroom aanwezig was (SAPD, afkorting voor 'syndrome-associated PD').

Tenslotte bespreken we in **Hoofdstuk 12** de relevante uitkomsten van onze studies in breder perspectief. We gaan dieper in op de diagnostische en therapeutische benaderingen die we hebben onderzocht en we beschrijven de kennishiaten die in onze studies zijn blootgelegd, om vervolgens suggesties te geven voor toekomstige studies.

It does not matter how slowly you go, as long as you do not stop

Confucius (551 – 479 BC)

A

LIST OF AUTHORS AND AFFILIATIONS IN THIS THESIS
LIST OF PUBLICATIONS IN THIS THESIS
FULL LIST OF AUTHOR'S PUBLICATIONS
LIST OF ABBREVIATIONS
PHD PORTFOLIO
ABOUT THE AUTHOR
DANKWOORD

LIST OF AUTHORS AND AFFILIATIONS IN THIS THESIS

Floris PJ van Alphen, Msc
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Emile van den Akker, MSc PhD
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Guannan Bai, Msc, PhD
Department of Public Health, Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands
Department of Child Health Care, Zhejiang University School of Medicine - Children's Hospital, National Clinical Research Center for Child Health, Hangzhou, Zhejiang, China

Maartje van den Biggelaar, MSc PhD
Sanquin Research and Landsteiner Laboratory, Sanquin research, Amsterdam, The Netherlands

Anske JG van der Bom, MSc Prof
Department of Clinical Statistics, Leiden University Medical Centrum, Leiden, The Netherlands

Louis de Bont, MD PhD
Department of Pediatric Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

A Rick Brooimans, MSc PhD
Laboratory Medical Immunological, Department of Immunology, Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands

Chantal C Clark
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Marjon H Cnossen, MD PhD Prof
Department of Pediatric Hematology and Oncology, Erasmus MC Sophia Children's Hospital, University Medical Center Rotterdam, Rotterdam, The Netherlands

Virgil ASH Dalm, MD PhD
Laboratory Medical Immunological, Department of Immunology, Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands
Department of Internal Medicine, division of Allergy & Clinical Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Mariëlle Dekker, MD
Department of Pediatrics, Albert Schweitzer hospital, Dordrecht, The Netherlands

Karin Fijnvandraat, MD PhD Prof
Department of Pediatric Hematology, Amsterdam University Medical Center, Amsterdam, The Netherlands

Suzanne F. Fustolo-Gunnink, MD PhD
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Nynke Gillemans
Department of Developmental Biology, Erasmus University Medical Centre, Rotterdam, The Netherlands

Masja de Haas, MD PhD Prof
Laboratory of Immunohematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, the Netherlands
Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands
Department of Clinical Transfusion Research, Sanquin Research, Amsterdam, The Netherlands

P Martin van Hagen, MD PhD Prof
Laboratory Medical Immunological, Department of Immunology, Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands
Department of Internal Medicine, division of Allergy & Clinical Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Arie J Hoogendijk, MSc PhD
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Nory Holle, MD
Department of Medical Affairs, Unit of Transfusion Medicine, Sanquin Blood Supply, Amsterdam, The Netherlands

Elise J Huisman, MD
Department of Pediatric Hematology and Oncology, Erasmus MC Sophia Children's Hospital, University Medical Centre Rotterdam, Rotterdam, The Netherlands
Department of Medical Affairs, Unit of Transfusion Medicine, Sanquin Blood Supply, Amsterdam, The Netherlands
Laboratory of Blood Transfusion, Department of Clinical Chemistry, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands

Alexander Janssen, MD
Department of Clinical Pharmacology, Hospital Pharmacy, Amsterdam University Medical Center, Amsterdam, The Netherlands

Hans J Janssen, MSc
Department of Electron Microscopy, Dutch Cancer Institute – Nederlands Kankerinstituut, Amsterdam, The Netherlands

A. Marieke S. Joosten, MD PhD
Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands

Iris C Kreft, Msc PhD
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands
Department of Clinical Chemistry, Maasstadziekenhuis, Rotterdam, The Netherlands

Idske CL Kremer Hovinga, MD PhD
Department of Pediatric hematology, Center for Benign Hematology, Thrombosis and Hemostasis,
van Creveldkliniek, University Medical Centre Utrecht, Utrecht University, Utrecht, The Netherlands

Marieke J.H.A. Kruip, MD PhD Prof
Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands

Lucas Kuijpers
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Karin van Leeuwen, Msc
Sanquin Research and Landsteiner Laboratory, Sanquin research, Amsterdam, The Netherlands

Enrico Lopriore, MD PhD Prof
Department of Neonatology, Leiden University Medical Center, Leiden, The Netherlands

Samone Mayer, MD
Department of Pediatric Hematology and Oncology, Erasmus MC Sophia Children's Hospital, University Medical Centre Rotterdam, Rotterdam, The Netherlands

Alexander B Meijer, Msc PhD Prof
Department of molecular hematology, Sanquin research, Amsterdam, The Netherlands
Utrecht institute for pharmaceutical sciences, Utrecht University, Utrecht, The Netherlands

Caroline Mussert, MD
Department of Pediatric Hematology, Erasmus MC Sophia Children's Hospital, University Medical Centre Rotterdam, The Netherlands

Sjaak NJ Philipsen, MSc, PhD Prof
Department of Developmental Biology, Erasmus University Medical Centre, Rotterdam, The Netherlands

Leendert Porcelijn, MD PhD
Laboratory of Immunohematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, The Netherlands
Laboratory of Platelet and Leucocyte Serology, Sanquin Diagnostic Services, Sanquin Research, Amsterdam, The Netherlands

Hein Raat, Msc PhD Prof
Department of Public Health, Erasmus MC, University Medical Centre Rotterdam, Rotterdam, The Netherlands.

Elisabeth LM Rammeloo, MD
Department of Pediatrics, Bernhoven Hospital, Uden, the Netherlands

R. van Rhijn
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Simon Rouschop
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Martin Schipperus, MD PhD
Department of Medical Affairs, Unit of Transfusion Medicine, Sanquin Blood bank, Amsterdam, The Netherlands

Frans J Smiers, MD PhD
Department of Pediatric Hematology, Leiden University Medical Center, Leiden, the Netherlands.

Rosalina van Spaendonk, Msc, PhD
Laboratory of Immunohematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, The Netherlands
Department of Human Genetics, Amsterdam university medical center, Amsterdam, The Netherlands

Arthur F Svendesen, MSc PhD
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Caroline SB Veen, MD PhD
Department of hematology, Erasmus MC, University Medical Center Rotterdam, The Netherlands
Department of Elderly Care, Woonzorg Flevoland, Lelystad, The Netherlands

Leo van de Watering, MD PhD
Department of Medical Affairs, Unit of Transfusion Medicine, Sanquin Blood Supply, Amsterdam, The Netherlands

Nicole N. van der Wel, MD PhD
Department of Electron Microscopy, Amsterdam UMC, Amsterdam, The Netherlands

Wilfred FJ van IJcken, MSc
Department of Core Facility Genomics, Erasmus University Medical Centre, Rotterdam, The Netherlands

C Michel Zwaan, MD PhD Prof
Department of Pediatric Hematology and Oncology, Erasmus MC Sophia Children's Hospital, University Medical Centre Rotterdam, Rotterdam, The Netherlands
Department of Pediatric Oncology, Princess Máxima Center, Utrecht, The Netherlands

Carmen van der Zwaan, Msc
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Huan Zhang, MSc
Sanquin Research and Landsteiner Laboratory, Sanquin Research, Amsterdam, The Netherlands

Jaap-Jan Zwaginga, MD PhD Prof
Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands

LIST OF PUBLICATIONS IN THIS THESIS

E.J. Huisman, Ferdows Atiq, Caroline S.B. Veen, Leo van de Watering, Marieke Joosten, Karin van Leeuwen, Leendert Porcelijn, Marieke J.H.A. Kruip, Masja de Haas, Marjon H. Cnossen:

Bleeding symptoms, laboratory and molecular genetic results in a cohort of children with platelet disorders. Under review European Journal of Hematology April 2026

H Zhang, **EJ Huisman**, R van Rhijn, AF Svendsen, J Janssen, NN van der Wel, C van der Zwaan, N Gillemans, WFJ van Ijcken, M Hansen, MH Cnossen, S Philipsen, AJ Hoogendijk, E van den Akker: *Heterozygous nonsense FLI1 p.Met100* variant results in thrombocytopenia and increased erythroid gene expression*. RPTH 2025 Nov; online ahead of print

H Zhang, **EJ Huisman** S Rouschop, L Kuijpers, CC Clark, AF Svendsen, C van de Zwaan, N Gillemans, WFJ van Ijcken, MH Cnossen, S Philipsen, AJ Hoogendijk, E van den Akker: *ETV6/TEL knockout leads to impaired megakaryoid gene regulation, which is phenocopied by its ETS-domain mutant ETV6-R369W via dominant negative actions.* Under review in JTH 2026

EJ Huisman, A Janssen, L van de Watering, M Kruip, M de Haas, MH Cnossen: *Desmopressin response in children with platelet disorders may depend on the presence of a genetic cause when response is measured with Von Willebrand activity and Platelet Function Analyzer*. Haemophilia;31:1340-1344

EJ Huisman, VASH Dalm, M. Joosten, FJ Smiers, L. Porcelijn, A. Hoogendijk, H. Janssen, NN. Van der Wel, ICL Kremer Hovinga, M. de Haas, MH Cnossen: *Interstitial 11q deletions and terminal 11q duplications cause a bleeding tendency due to platelet dysfunction that is similar to 11q deletions causing Jacobsen syndrome.* European Journal of Haematology 2025 Jul 22; online ahead of print

Elise J Huisman, Nory Holle, Martin Schipperus, Marjon H. Cnossen, Masja de Haas, Leendert Porcelijn, Jaap-Jan Zwaginga: *Implementing HLA and HPA-matched platelet transfusions for patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome as standardized care? A Dutch survey and recommendations*. Transfusion 2024;64(5):824-838

Huisman EJ, Mussert C, Bai G, Cnossen M, Raat H, Cnossen M: *Knowledge gaps in health-related quality of life research performed in children with bleeding disorders – A scoping review*. Haemophilia 2024;30(2):295-305

Kreft C, **Huisman EJ** (shared 1st authors), Cnossen MH, Alphen va, FPJ, Zwaan van der, C, Leeuwen van, K, Spaendonk van R, Porcelijn, L, Veen CSB, Biggelaar van den, M, Haas de, M, A.B. Meijer, A.J. Hoogedijk. *Proteomic landscapes of inherited platelet disorders with different etiologies*. JTH 2023; 21(2):359-372

Huisman EJ, Brooimans AR, Mayer S, Joosten M, De Bont L, Dekker M, Rammeloo ELM, Smiers FJ, Van Hagen PM, Zwaan CM, De Haas M, Cnossen MC, Dalm VASH*: Patients with chromosome 11q deletions are characterized by inborn errors of immunity involving both B- and T-lymphocytes*. J Clin Immunol 2022;42:1521-1534.

Fustolo-Gunnink SF, **Huisman EJ,** van der Bom JG, van Hout FMA, Makineli S, Lopriore E, Fijnvandraat K: *Are thrombocytopenia and platelet transfusions associated with major bleeding in preterm neonates? A systematic review*. Blood Rev 2019;36:1-9

FULL LIST OF AUTHOR'S PUBLICATIONS

Satyam Arora, Ruchika Goel, Jip van Daelen, **Elise J. Huisman**, Cassandra D Josephson, Luciana Teofili: *Umbilical Cord Red Blood Cell Transfusions in Very Premature Neonates: A Physiologically Aligned Transfusion Strategy.* Under review Transfusion May 2026

Jip H. van Daelen, Valeria Cortesi, Lianne I. Verbeek, Sophie J.E. Cramer, **Elise J. Huisman**, Thomas R.L. Klei, Irwin K. M. Reiss, Pauline M. Snijder, Enrico Lopriore. *Comparing the volume of umbilical cord blood collection using different techniques*. Under review Vox Sanguinis April 2026

Jip H. van Daelen, Norani H. Gangaram-Panday, **Elise J. Huisman**; H. Rob Taal; Thomas R. L. Klei, Enrico Lopriore, Irwin K. M. Reiss, Andrei N. Tintu, Pauline M. Snijder. *Gestational age references and trajectory of fetal haemoglobin in relation to outcome in a cohort of neonates born before 30 weeks of gestation*. Under review in The Lancet eClinical Medicine, April 2026

Sven Crama, **Elise J. Huisman**, Peter te Boekhorst, Michaela van Bohemen, René Kornelissen, Sander Galjaard, Hans J. Duvekot, Anouk van der Knijff, Jenny Kissler, Maarten Ter Horst, Tjebbe Hagenaars, Gert van Dijk, mr. Marcel Voskamp: *Uitgangspunten bij het weigeren van een behandeling met bloedproducten bij patiënten in acute medische situaties*. Accepted for publication in EMPACT April 2026

E.J. Huisman, Ferdows Atiq, Caroline S.B. Veen, Leo van de Watering, Marieke Joosten, Karin van Leeuwen, Leendert Porcelijn, Marieke J.H.A. Kruip, Masja de Haas, Marjon H. Cnossen: *Bleeding symptoms, laboratory and molecular genetic results in a cohort of children with platelet disorders*. Under review European Journal of Hematology April 2026

E.J. Huisman, K. Le Poole, L.F.A. Visser, C.R. van der Torren, C.S. Ootjers, J.L. Kerkhoffs , C.L. Eckhardt *Toelichting op de landelijke richtlijn Erytrocytaferese bij kinderen met sikkelcelziekte*. Accepted for publication in Ned Tijdschr Hematol. March 2026

Houben NAM, Cardigan R, de Korte D, Caram-Deelder C, Fazli L, Wikman A, Larsson S, Radovčić MK, Vinkovic M, Fustolo-Gunnink S, Roehr CC, Dame C, Stanworth S, Lopriore E, New HV, **on behalf of the Neonatal Transfusion Network, for the SCONE collaborators***:*The specifications of blood components for neonatal transfusion - A European study.* Transfusion Medicine Reviews, Under review 2025 Dec

Muñoz-Pujol G, **Huisman EJ**, Joosten M, Francisco Mesa J, Fernandez-Burriel M, Ugarteburu O, Segur-Bailach E, Gea-Sorlí S, Moliner S, Farré-Tarrats L, Guitart-Mampel M, Villarreal L, Arauz-Garofalo G, Gay M, Villamor N, Martín G, Calvo M, Garrabou G, Gort L, García-

Villoria J, Macias M, Vilaseca M, Fillat C, Tort F, Ribes A: *TfR1 deficiency beyond combined immunodeficiency. Insights into iron-sulfur cluster biogenesis and mitochondrial function.* Genes and Diseases; accepted 2025 Dec

Van Daelen J, Besteboer J, Korsten H, Reiss IKM, Lopriore E, Snijder PM, **Huisman EJ**, Klei TRL: *Development and production of an allogeneic cord blood-derived red blood cell concentrate for transfusion to extremely preterm neonates.* Transfusion 2025 Nov; online ahead of print

H Zhang, **EJ Huisman**, R van Rhijn, AF Svendsen, J Janssen, NN van der Wel, C van der Zwaan, N Gillemans, WFJ van Ijcken, M Hansen, MH Cnossen, S Philipsen, AJ Hoogendijk, E van den Akker: *Heterozygous nonsense FLI1 p.Met100* variant results in thrombocytopenia and increased erythroid gene expression*. RPTH 2025 Nov; online ahead of print

H Zhang, **EJ Huisman** S Rouschop, L Kuijpers, CC Clark, AF Svendsen, C van de Zwaan, N Gillemans, WFJ van IJcken, MH Cnossen, S Philipsen, AJ Hoogendijk, E van den Akker: *ETV6/TEL knockout leads to impaired megakaryoid gene regulation, which is phenocopied by its ETS-domain mutant ETV6-R369W via dominant negative actions.* Under review in JTH 2025 Oct

Verbeek LI, Cortesi V, Van Daelen J, Klei T, Houden NAM, **Huisman EJ**, Snijder P, Cramer SJE, Reiss IK, Lopriore E: *Volume of umbilical cord blood collection in the era of delayed cord clamping: a multi-center, prospective, feasibility study*. Arch of Dis 2025 Jun 5; online ahead of print

EJ Huisman, A Janssen, L van de Watering, M Kruip, M de Haas, MH Cnossen: *Desmopressin response in children with platelet disorders may depend on the presence of a genetic cause when response is measured with Von Willebrand activity and Platelet Function Analyzer.* Haemophilia 2025;31:1340-1344

EJ Huisman, VASH Dalm, M. Joosten, FJ Smiers, L. Porcelijn, A. Hoogendijk, H. Janssen, NN. Van der Wel, ICL Kremer Hovinga, M. de Haas, MH Cnossen: *Interstitial 11q deletions and terminal 11q duplications cause a bleeding tendency due to platelet dysfunction that is similar to 11q deletions causing Jacobsen syndrome.* European Journal of Haematology 2025 Jul 22; online ahead of print

Cain L, Lafrance C, Morton S, Latour C, Girard M, Tiberghien P, de la Taille V, Datta SS, Virk MS, Andrews J, Yahalom V, Pugliese AM, Alba R, Charlewood R, Kirwan S, Yokoyama APH, Kutner JM, Nogues EA, Martinez I Llonch N, Daly J, Irving DO, Schulze TJ, **Huisman E**, Le Poole K, Vrielink H, Saifee NH, Pagano MB, Stanworth S: *Differences in the product*

characteristics and clinical use of granulocytes for transfusion: The BEST Collaborative study. Transfusion 2025;65(6):1111-1123

Huisman EJ, Holle N, Schipperus M, Cnossen MH, De Haas M, Porcelijn L, Zwaginga JJ: *Implementing HLA and HPA-matched platelet transfusions for patients with Glanzmann Thrombasthenia and Bernard-Soulier syndrome as standardized care? A Dutch survey and recommendations*. Transfusion 2024;64(5):824-838

Huisman EJ, Mussert C, Bai G, Cnossen M, Raat H, Cnossen M: *Knowledge gaps in health-related quality of life research performed in children with bleeding disorders – A scoping review*. Haemophilia 2024;30(2):295-305

Hägele P, Staus P, Scheible, Ruhlmann A, Heeg M, Klemann C (..) ALPID study group (a.o. **Huisman EJ**): *Diagnostic evaluation of paediatric autoimmune lymphoproliferative immunodeficiencies (ALPID): a prospective cohort study*. Lancet Haematol 2024;11:e114-e126

Kruimer DM, Stavleu DC, Mulder RL, LCM Kremer, Tissing WJE, Loeffen EAH and guideline panel: (a.o **Huisman EJ**): *Prophylactic red blood cell transfusions in children and neonates with cancer: an evidence based clinical practice guideline*. Support Care Cancer 2024;4:32:766-770

Huisman EJ, Le Poole K, Dorresteijn E, Swaneveld F, Ootjers CS Vrielink H: *Aferese bij kinderen, aandachtspunten en valkuilen.* Ned Tijdschr Hematol 2024;2:80-88

Kreft C, **Huisman EJ** (shared 1st authors), Cnossen MH, Alphen va, FPJ, Zwaan van der, C, Leeuwen van, K, Spaendonk van R, Porcelijn, L, Veen CSB, Biggelaar van den, M, Haas de, M, A.B. Meijer, A.J. Hoogedijk. *Proteomic landscapes of inherited platelet disorders with different etiologies*. JTH 2023; 21(2):359-372

Henricks LM, **Huisman EJ,** Lopriore E, Lukken JS, De Haas M, Ootjer CS, Albersen A: *Acute hemolytic transfusion reaction after transfusion of fresh frozen plasma in a neonate – preventable by using solvent/detergent-treated pooled plasma?* Transfusion Med 2023;32:174-178

Bronkhorst-van der Helm MW, Weerkamp F, Huisman A, **Huisman EJ**, Russcher H: *Interference of bovine hemoglobin-based oxygen carrier-20 (Hemopure) on four hematology analyzers*. J Lab Hematol 2023;45:869-874.

Arora S, Goel R, Al-Riyami AZ, Al-Rawas AH, Al Hosni S, (…) **Huisman EJ** et al: *International Forum on Small-Volume Transfusions in Neonates and Paediatric Patients: Summary*. Vox Sang. 2023;118:223-229.

Arora S, Goel R, Al-Riyami AZ, Al-Rawas AH, Al Hosni S, (…) **Huisman EJ** et al: *International Forum on Small-Volume Transfusions in Neonates and Paediatric Patients: Responses.* Vox Sang. 2023;118:230-251.

Huisman EJ, Luijnenburg SL, Kamerbeek A, Raab MAE, Verzijl R, Van Wijk R: *De kunst van kijken: Uw diagnose?* Ned Tijdschr Hematol 2023;20:410-411

Hermans MAW, Pasmans SGMA, Arends NJT, van den Bosch TPP, van Daele PLA, van Doorn MBA, **Huisman EJ**, Mooyaart AL, Dammen J: *Histopathological characteristics are instrumental to distinguish monomorphic from polymorphic maculopapular cutaneous mastocytosis in children*. Clin Exp Dermatol 2022;47:1694-1702

Hermans MAW, Irvine W, Arends NJT, (…) **Huisman EJ** et al: *Diagnostiek en behandeling van mastocytose: toelichting op de richtlijn van 2022*. Ned Tijdschr Hematol 2022;19:311-320

Van Schaik J, Braakhuis MS, **Huisman EJ**, Heitink-Polle, K.: Verworven oorzaken van anemie: acute auto-immuun hemolytische anemie op de kinderleeftijd. Prakt Pediatrie 2022, edition of March

Atmar K, Ruivekamp CA, Hooimeijer L, Nibbeling EA, Eckhardt CL, **Huisman EJ**, Lankester AC, Bartels M, Santen G, Smiers FJ, Burg van der M, Mohseny AB: *Diagnostic Value of a Protocolized In-Depth Evaluation of Pediatric Bone Marrow Failure: A Multi-Center Prospective Cohort Study*. Front Immuno 2022;13:1-13

Huisman EJ, Brooimans AR, Mayer S, Joosten M, De Bont L, Dekker M, Rammeloo ELM, Smiers FJ, Van Hagen PM, Zwaan CM, De Haas M, Cnossen MC, Dalm VASH: *Patients with chromosome 11q deletions are characterized by inborn errors of immunity involving both B- and T-lymphocytes*. J Clin Immunol 2022;42:1521-1534

Zivkovic M, Kremer Hovinga ICL, Blaauwgeers MW, Kruip MW, Beckers EAM, Coppens M, Eikenboom J, Galen van KPM, Tamminga RYJ, Cnossen MH, **Huisman EJ**, Urbanus RT, Schutgens REG: *Inzichten in aangeboren trombocytenstoornissen verkregen uit de Trombocytopathie in Nederland (TiN)-studie*. Ned Tijdschr Hematol 2022;19:132-140

Veen CSB, **Huisman EJ**, Romano LGR, Schipaanboord CWA, Cnossen MH et al: *Outcome of surgical interventions and deliveries in patients with bleeding of unknown cause: an observational study*. Thromb Hemos 2021;121:1409-1416

Huisman EJ, Crighton GL: *Pediatric fibrinogen PART I - Pitfalls in fibrinogen evaluation and use of fibrinogen replacement products in children*. Front Pediatr 2021;9;617500e

Crighton GL, **Huisman EJ**: *Pediatric fibrinogen PART II - Overview of indications for fibrinogen use in critically ill children.* Front Pediatr 2021;9;647680e

A Machotta, **EJ Huisman**, IM Appel, SE Luijnenburg, M Lopez-Yurda, MH Cnossen, CH van Ommen: *Prophylactic fibrinogen concentrate administration in surgical correction of paediatric craniosynostosis*. Eur J Anesthesiology 2020

Veen CSB, **Huisman EJ**, Cnossen MH, Kom-Gortat R, Rijken DC, Leebeek FWG et al: *Evaluation of thromboelastometry thrombin generation and plasma clot lysis time in patients with bleeding of unknown cause: A prospective cohort study.* Haemophilia 2020;26:e106-e115

E Lopriore, **E Huisman,** S Fustolo-Gunnink, PM Snijder, IK Reis, S Stanworth: *Allogeneic cord blood transfusions for extremely preterm neonates: an extremely promising proof of concept*. Br J Haematol 2020;191:150-151

Fustolo-Gunnink SF, Fijnvandraat K, van Klaveren D, Stanworth S (…), **Huisman EJ** et al: *Preterm neonates benefit from low prophylactic platelet transfusion threshold despite varying risk of bleeding or death.* Blood 2019;134:2354-2360

Bruun MT, Yazer MH, Spinella PC, (…) **Huisman E** et all: *Vox Sanguinis International Forum on paediatric indications for blood component transfusions: Summary.* VoxSang 2019;114:523-530.

Curley A, Stanworth SJ, Willoughby K, (et all), **PlaNeT2 MATISSE Collaborators (a.o. Huisman EJ):** *Randomized Trial of Platelet-Transfusion Tresholds in Neonates*. N Eng J Med 2019;380:242-251

Fustolo-Gunnink SF, **Huisman EJ**, van der Bom JG, van Hout FMA, Makineli S, Lopriore E, Fijnvandraat K: *Are thrombocytopenia and platelet transfusions associated with major bleeding in preterm neonates? A systematic review*. Blood Rev 2019;36:1-9

Compendium kindergeneeskunde, *Transfusiegeneeskunde bij kinderen*, **EJ Huijssen-Huisman**, Y de Rijke, Revision Ch57, Febr 2018

Meinderts SM, Oldenborg PA, Beuger BM Klei TRL, Johanson J, Kuijpers TW, Matozaki T, **Huisman EJ**, De Haas M, Van den Berg TK, Van Bruggen R: *Human and murine splenic neutrophils are potent phagocytes of IgG-opsonized red blood cells*. Blood Adv 2017;1:875-886

Huijssen-Huisman EJ, Stigter RL, Westerhof H, Cnossen MH, Beishuizen A: *De kunst van kijken: Uw diagnose?* Ned Tijdschr Hematol 2017;14:93-94

Blumental S, **Huisman E** (shared 1st author), Cornet MC, Ferreiro C, De Schutter I (et al). *Pandemic A/H1N1v Influenza 2009 in Hospitalized Children: a Multicenter Belgian Survey.* Biomed central (BMC) Infect Dis. 2011;11:313

De Smet K, De Maeseneer M, **Huijssen-Huisman E**, Van Gorp V, Hachimi-Idrisi S, Ernst C: *A rare cause of dyspnea due to chylothorax*. Emergency Radiology 2010;11:503

Huijssen EJ, Sluis TR, Besten den H: *Decubitus bij dwarslaesiepatiënten, nog altijd een zwakke plek?* Ned. Tijdschrift voor Geneeskunde 2009;153:318

Van Mook WNKA, De Grave WS, **Huijssen-Huisman EJ**, de Witt-Luth M, Dolmans DH, Muijtjens AM, Schuwirth LW, van der Vleuten CP: Medical Education: *Factors inhibiting assessment of students' professional behaviour in the tutorial group during problem based learning. Medical Education 2007:56;849-856*

LIST OF ABBREVIATIONS

11q	long arm of chromosome 11
22q	long arm of chromosome 22
α_v/β_3	platelet vitronectin receptor
A or a	alpha granule
AA	arachidonic acid
ACMG	American College of Medical Genetics and Genomics
AD	autosomal dominant
ADP	adenosine diphosphate
ALL	acute lymphoblastic leukemia
ATE	adenotonsillectomy
ATP	adenosine triphosphate
APTT	activated partial thromboplastin time
AR	autosomal recessive
ATE	adenotonsillectomy
ATP	adenosine trisphosphate
BAT	bleeding assessment tool
BD	bleeding disorder(s)
BDMA	benzyldimethylamine
BDUC	bleeding disorder(s) of unknown cause
BSS	Bernard-Soulier syndrome
c	codon
C	Celsius
CarerQoL	Care related quality of life
CADD	Combined Annotation Dependent Depletion
CD	cluster of differentiation
CHIP-CE	Child health and illness profile - Child edition
CI	confidence interval
CHO-KLAT	Canadian hemophilia outcome-Kids life assessment tool
CHQ	Child health questionnaire
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CMV	cytomegalovirus
CNS	central nervous system
CNV	copy number variation
COX	cyclo-oxygenase
CPX	Carboxy-terminal domain protein kinase
CT	closure time
CTP	congenital thrombocytopenia

CV	coefficient of variation
D	domains
Da	Dalton
DDAVP	1-deamino-8-D-arginine vasopressin, (also: desmopressin)
Del	deletion
DDSA	dodecenylsuccinic anhydride
DHR	dihydrorhodamine
DNA	deoxyribonucleic acid
DOM	DoReMi study patient identifier
DPS	delta storage pool defect(s)
DTT	dithiotreitol
Dupl	duplication
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ENT	ear-nose-throat
EPF	electronic platelet files
EPI	epinephrin
EQ	Euro Quality of Life
Ery	erythrocyte
EV	empty vector
F(xx)	family (number)
F	female
FDR	false discovery rate
fL	femtoliter
FPD	functional platelet disorder(s)
Fs	frame shift
FVIII	factor VIII
FVIII:Act	factor VIII activity
FIX	factor IX
FXI	factor XI
FNAIT	fetal-neonatal alloimmune thrombocytopenia
FRET	fluorescence resonance energy transfer
FSC	forward scattered area
GAD	generalized anxiety disorder
GDP	guanosine diphosphate
GFP	green fluorescence protein
GI	gastro-intestinal
GMP	granulocyte-monocyte progenitor
GO	gene ontology

GP	glycoprotein (defect)
GSEA	Gene set enrichment analysis
GT	Glanzmann Thrombasthenia
GTP	guanosine triphosphate
GWAS	genome-wide association studies
h	hour(s)
HC	healthy control
Hemo-Sat	Health-related quality of life and satisfaction
HET	heterozygous
HLA	human leucocyte antigen
Haemo-QOL	Haemophilia quality of life
HC	healthy control
HCD	higher-energy collisional dissociation
HCl	hydrochloric acid
Hemi	hemizygous
HMB	heavy menstrual bleeding
HMP	hematopoietic malignancy predisposition
HPA	human platelet antigen
HPLC	high performance liquid chromatography
HRQoL	health-related quality of life
HSC	hematopoietic stem cell
HSP	Hermansky-Pudlak syndrome
HSV	herpes simplex virus
iBAQ	intensity based absolute quantitation
IFMS	International federation of medical specialists
Ig	Immunoglobulin(s)
IgRT	immunoglobulin replacement therapy
Im	intramuscular
INR	international normalized ratio
IP(F)D	inherited platelet (function) disorder(s)
IP	immunoprecipitation
IPF	immature platelet fraction
IQR	inter quartile range
IRT	item response theory
ISTH	International society of thrombosis and hemostasis
ITP	immune mediated thrombocytopenia
ITQOL	Infant and toddler quality of life
IVTsgRNA	In Vitro Transcribed single guide RNA
Karyo	karyogram
Kids ITP Tool	Kids immune mediated thrombocytopenia tool

KI	knockin
KO	knockout
L	liter
L	levels in HRQoL-research
LC-MS	liquid chromatographic mass-spectrometry
LFQ	label-free quantification
LTA	light transmission aggregometry
LRTI	lower respiratory tract infection
µg	microgram
µL	microliter
µM	micrometer
M	molar mass
M	mitochondrion
Mb	megabytes
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCID	minimally clinically important difference
MCV	mean corpuscular volume
MDS	multidimensional scaling
ME	mean error
MEC	medical ethic committee, medisch-ethische toetsingscommissie
(e-)MEP	(erythroid-biased) megakaryocyte-erythroid progenitor
MFI	melt flow index
MFI	mean fluorescence intensity
min	minutes
MK	megakaryocyte
MKi	megakaryocyte involved in immunity
MkP	megakaryocyte progenitor
MKl	lung megakaryocytes
MKn	niche-supporting megakaryocyte
mL	milliliter
mM	mini-molar mass
MMP	multipotent progenitors,
MNA	methylnadic anhydride
MPV	mean platelet volume
MRI	magnetic resonance imaging
mRNA	messenger RNA
ms	milliseconds
MS	mass-spectrometry
MYH-RD	MYH9-related disorders

m/z	mass/charge number of ions
µg	microgram
µM	micrometre
n	number
NA	not applicable
NaCl	natrium (sodium) chloride
nL	nanoliter
nos	not otherwise specified
NM	not mentioned
NSAID	non-steroid anti-inflammatory drug(s)
O	open canicular system
OCA	ocular albinism syndrome
OR	Odds ratio
p	protein
P	patient (number, also DOM-number)
PC	procoagulation function
PC(A)	principle component (analysis)
PCR	polymerase chain reaction
PD	platelet disorder(s)
PDUC	platelet disorder of unknown cause
PedsQL	Pediatric quality of life inventory
PFA	Platelet Function Analyzer
PFA-ADP	PFA with ADP cartridge
PFA-EPI	PFA with epinephrin cartridge
PGG2	prostaglandin G2
PGH2	prostaglandin H2
PHA	phytohemagglutinin
PHQ	patient health questionnaire
PID	primary immunodeficiency (also: inborn errors of immunity)
PLT-F	platelets visualized by immunofluorescence
PMA	phorbol 12-myristate 13-acetate
pMK	platelet producing megakaryocyte
PPM	parts per million
PRP	platelet rich plasma
PT	prothrombin time
Py	per year
QOL	quality of Life
QUAL-HEMO	Hemophilia age group-specific quality of life questionnaire
RBC	red blood cell
rFVIIa	recombinant activated FVII

Risto	ristocetin
RIVM	Dutch national institute for public health and environment (Also: Rijksinstituut voor volksgezondheid en milieu)
RNA	ribonuceleic acid
RSV	respiratory syncytial virus
S	seconds
SAPD	syndrome associated platelet disorders
scIg (RT)	subcutaneous immunoglobulin (replacement therapy)
SD	standard deviation
SE	standard error
SEB	staphylococcal enterotoxin B
Sec	seconds
SF	short form health survey
SFL	side fluorescence light
SNP	single nucleotide polymorphism
SPD	storage pool disease
SRM	super resolution microscopy
ST-HSC	short-term hematopoietic stem cell (or multipotent progenitor)
T	time point
TAPQOL	TNO-AZL preschool children quality of life
TAR	thrombocytopenia with absent radius
TEM	transmission electron microscopy
TLR	toll-like receptor
tNGS	targeted next generation sequencing
TPO	thrombopoietin
TRAP	thrombin receptor-activating peptide
TXA	tranexamic acid
TXA2	thromboxane
(I)U	(international) units
U or star	unidentified, crystalloid structure
Un	untransduced
URTI	upper respiratory tract infection
UTI	urinary tract infection
VAS	visual analogous scale
VMA T2	vesicular monoamine transporter 2
VUS	variant of unknown significance
VWF	von Willebrand factor
VWF:Act	von Willebrand factor activity
VWF:Ag	von Willebrand factor antigen
VWF:CB	von Willebrand factor collagen binding

VWD	von Willebrand disease
VZV	varicella zoster virus
WBC	white blood cell
WES	whole exome sequencing
WGA	whole genome array
WGCNA	weighted gene co-expression network analysis
WGS	whole genome sequencing
WHO	World health organization
WT	wild-type
XLD	x-linked dominant
XLR	x-linked recessive
XLT	x-linked thrombocytopenia
y	year(s)
Y	young

PHD PORTFOLIO

Name Elisabeth Janneke Huisman
Department Pediatric Hematology and Oncology
PhD period 12 2016 – 12 2025
Promotors Prof. dr. M.H. Cnossen, Prof. dr. M. de Haas

PhD Training	Year	Work load ECTS*
General courses		
Epidemiology and Research courses		
Biostatistical Methods I: Basic Principles	2016	5.7
CPO course	2016	0.3
EndNote course (Medical Library)	2016	0.2
Systematic Literature Retrieval in PubMed (Medical Library)	2016	0.4
Tulips Grant writing course	2017	1.5
BROK course (Basiscursis Regelgeving Klinisch Onderzoek)	2018	1.5
Boerhave Epidemiologie	2020	3.0
Biomedical English writing course	2020	2.5
SPSS Statistiek cursus HbO	2022	5.0
BROK course recertification	2022 & 2025	1.2
Scientific Integrity	2022	0.3
		21.6
Specific courses		
ISTH Advanced training Course on platelet defects, Oxford, UK	2016	1.5
Achieve trainings course blood disorders, Milaan	2016	1.0
Achieve exchange program, GOSH, London	2016	3.0
Teach the teacher III	2019	0.3
MolMed Excel workshop	2019	0.3
MolMed NGS in DNA Diagnostics course	2019	0.6
Leiderschapscursus artsen	2020	2.0
ISTH master class pediatric ITP	2022	0.3
		9.0
Presentation, conferences and seminars		
Oral plenary lecture		
International Society of Blood Transfusion (ISBT), Milaan	2025	1.0
Oral abstract presentations		
European Congress on thrombosis and haemostasis (ECTH)	2018	1.0
Sanquin nascholing "Genomics and proteomics in immunohematology"	2018	0.6
Nederlandse Vereniging van Bloedtransfusies (NVB)	2019, 2022	2.0

PhD Training	Year	Work load ECTS*
European 11q association meeting, Germany	2022	1.0
Poster abstract presentations		
International Society on Thrombosis and Hemostasis, Milaan (ISTH)	2020	0.3
International Society on Thrombosis and Hemostasis. London	2022	0.3
International Society on Thrombosis and Hemostasis, Toronto	2023	0.3
European Hematology Association	2024	0.3
International Society on Thrombosis and Hemostasis, Toronto	2025	0.3
		7.1
Conferences/symposia attended & organized		
Attended		
American Society of Hematology	2016, 2020, 2022	0.9
International Society on Thrombosis and Hemostasis	2020, 2022, 2023	0.9
European Association of Haemophilia and Allied Disorders	2020	0.3
Nederlandse Vereniging voor Bloedtransfusies	2016 - 2024	2.1
Nederlandse Vereniging voor Kindergeneeskunde	2016, 2022, 2023	0.9
Landelijk symposium Kind en Transfusie	2022 - 2025	1.5
European Hematology Association	2024	0.3
International Society of Blood Transfusion	2025	0.3
		7.2
Organized		
Nederlandse Vereniging voor Bloedtransfusies 2020 till -		6.0
Annual Symposia Kind en Transfusie 2021 till -		5.0
		11.0
Teaching activities & protocol development		
Teaching acitivities		
Sanquin UTG education days	2020	0.3
TRIP Meet the expert - annual	2016 - 2025	2.4
Nederlandse Vereniging voor Kindergeneeskunde, satelliet symposium	2016, 2022, 2023	0.9
Teaching the basics of clinical transfusion medicine to A(N)IOS	2019 - 2025	1.8
Developing Cyberpoli congenital and immune mediated thrombocytopenia (www.cyberpoli.nl)	2023	2.0
Developing website for the Netwerk Kind en Transfusie (www.kinden-transfusie.nl)	2023	1.5
		8.9
Protocol development		
Dutch Blood transfusion protocol Richtlijnendatabase	2018-2019	1.0
Beter Keten Zorgpad ITP bij kinderen (Chairman)	2020-2022	1.5
Dutch Mastocytosis protocol Richtlijnendatabase	2022	1.0
Dutch protocol on Treatment options in blood refusal (Vademecum Hematology)	2024-2025	1.0

PhD Training	Year	Work load ECTS*
Dutch AIHA protocol Richtlijnendatabase	2024 till -	1.0
European AIHA protocol EHA – transfusion WG (WG lead)	2024 till -	1.0
European AIHA protocol EHA – pediatrics WG	2024 till -	1.0
		7.5
Supervision of medical students or A(N)IOS		
Coaching 4 students during their master thesis (master)	2017 - 2020	4.0
Coaching 2 medical students throughout bachelor (minor)	2016, 2017	0.4
Teaching in the minor of medical students	2016, 2017	1.3
Teaching the basics of clinical transfusion medicine to A(N)IOS	2019 - 2024	1.5
Coaching PhD student	2025	1.0
		8.2
Membership and Others		
Weekly thrombosis and hemostasis authorization and discussion	2016 till 2022	6.0
Monthly DoReMi study group meeting	2017-2019	2.0
Member medical advisory board TRIP Dutch hemovigilance surveillance	2016 till -	1.0
Member medical advisory board ITP patients association	2021 till -	1.0
Member Board of Nederlandse Vereniging voor Bloedtransfusies	2019 till -	1.0
Member scientific committee of the Nederlandse Vereniging voor Bloedtransfusies	2021 till -	1.0
Chair 'Beraadsgroep weigering behandeling bloedproducten'	2022 till -	4.0
Co-lead ISBT international working group on Cord Blood transfusions	2025 till -	0.3
		16.3
Awards and grants		
Professor Heimburger Award	2016	1.0
		1.0
Total		**97.8**

Legend *ECTS are European Credit Transfer Accumulation System, One ECTS represents 28 hours. A minimum of 30 ECTS are required

ABOUT THE AUTHOR

Elisabeth (Elise) Janneke Huisman, was born on the 20th of November in Sliedrecht. After receiving her Gymnasium diploma in 1997 at the Guido de Brès College in Rotterdam, she moved to Eindhoven to study Medical Imaging and Radiotherapeutical Techniques at the Fontys Hogeschool (propedeuse). Hereafter, she started in 1998 at Maastricht University in Maastricht to study Medicine. During her bachelor, she followed a six-week elective course at the Department of Pediatric Oncology of the Erasmus MC Sophia Children's Hospital which was supervised by Prof. Dr. Karel Hählen. This motivated her to continue with her special interest in Pediatrics, with a subsequent elective in Starship Children's Hospital in Auckland, New-Zealand.

In 2004, she obtained her medical degree and started as a Pediatric house officer (ANIOS) at the Reinier de Graaf Gasthuis in Delft. Subsequently at the end of 2005, she worked on the pediatric intensive care unit (PICU) at Leiden University Medical Hospital. Afterwards, she broadened her medical experience by working at the Rijndam Rehabilitation Clinic in Rotterdam. It is there that she discovered that the search for a diagnosis is what motivated her most. She subsequently decided to definitely apply for an internship in Pediatrics. In 2007, at the University Hospital of Brussels (UZB), she started with her medical training to become a pediatrician. She finished her pediatric training in the Juliana Children's Hospital in The Hague in 2012 and then worked as a general pediatrician at the Sint Franciscus Gasthuis, currently Franciscus Vlietland. In 2013, she subsequently started with her fellowship Pediatric Hematology at both the Erasmus MC Sophia Children's Hospital and Transfusion Medicine at Sanquin.

After becoming a pediatric hematologist, she continued working in the Erasmus MC Sophia Children's Hospital and Sanquin. She started her PhD in 2016 under supervision of Prof. Dr Marjon H. Cnossen and Prof. Dr. Masja de Haas. Within the CRESCENDO study (PI Prof. Dr. Marieke Kruip) she initiated the DoReMi study after being confronted with two patients with a diagnosis of platelet dysfunction that could not be otherwise specified by current tests, but in time, turned out to be just the first symptom of a broader problem including immunological problems. She hypothesized that earlier and more specific diagnoses in this patient group are important to improve and individualize care. This was the start of this thesis and her specific research interests. During her PhD project, she followed many courses and gave several oral presentations. Besides patient related research, she launched the Cyberpoli website 'Bloedplaatjesstoornissen' for children and adolescents in close collaboration with the 'Stichting Artsen voor Kinderen' to further develop clinical care.

She now works as a pediatric hematologist in the Erasmus MC Sophia Children's Hospital mainly diagnosing and treating patients in the Specialized Hematology outpatient clinic

and is a consultant in the Blood Transfusion Department of the Erasmus MC. She is the chair of the Erasmus MC "Beraadsgroep Weigering Behandeling Bloedproducten". She also works as a consultant for the Unit of Transfusion Medicine with the special task of improving the Pediatric Transfusion care chain. This has resulted in the erection of the Dutch "Netwerk Kind en Transfusie" in 2022, connecting researchers, promoting studies in the fields of neonatal and pediatric transfusion medicine and improving the field of pediatric transfusion medicine by educational activities. She has developed several protocols within the field of pediatric hematology and transfusion medicine.

Find gratitude in little things and your well of gratitude will never run dry

(Unknown)

DANKWOORD

"On ne voit bien qu' avec le coeur", is één van mijn favoriete citaten uit het geweldige boek "Le petit prince" van Antoine de Saint-Exupéry. Ik denk dat dit ook geldt voor het voorrecht om deze PhD te mogen schrijven. Zonder **alle patiënten en hun ouders** was deze thesis nooit tot stand gekomen. En achter alle n-nummers, data, figuren, tabellen, zie ik de namen en gezichten van deze kinderen. Het is hartverwarmend en een voorrecht om als arts en onderzoeker een stukje mee op te mogen lopen met alle kinderen en hun ouders. Dank!

Maar '*mon coeur*' ziet daarnaast ook een schare aan geduldige, betrokken, deskundige en heel intelligente collega's. Zonder deze ondersteuning was mijn academische groei toch maar een vlakke helling geweest. Dank dus ook aan jullie allemaal met een aantal mensen in het bijzonder.

Lieve **Marjon, prof. dr. Cnossen**, jij had je eigen promotie tijdens mijn traject. Waar je begon als mijn co-promotor, ben je nu, in het afronden van mijn thesis betrokken als mijn promotor. Die plek verdien je volledig! En zo zie je maar, het is op meerdere fronten nuttig om lang over een thesis te doen… In de jaren samen heb je met niet alleen als (co) promotor, maar ook als collega geholpen. Soms was ik maar blij dat ik vooraf toch niet goed wist waar ik aan was begonnen. Maar jij wist het door al je ervaring heel goed en kon mij toch weer een stap verder brengen. Ik heb veel geleerd van je onverwoestbaar optimisme, en natuurlijk je 'rode pen' feedback techniek. Dank Marjon!

Lieve **Masja, prof. dr. De Haas**, je kwam precies op tijd als tweede promotor erbij! Mijn PhD-thesis was nooit gelukt zonder de hulp van heel veel mensen binnen Sanquin maar zeker ook niet zonder jou. Jij hebt me geholpen om alles te coördineren en iedereen te motiveren! Je inhoudelijke kennis van immunohematologie en transfusies was van grote meerwaarde, dank je dat ik daar van kon 'meesnoepen'. En in een korte periode dat Marjon er tijdelijk niet was, heb je mijn begeleiding geïntensiveerd. Ik heb de duidelijke, heldere feedback als heel prettig ervaren! Je talent om soms uit te zoomen en weer focus in een project te krijgen is echt van grote waarde. Dus ook aan jou, dank Masja!

Beste **Michel**, **prof. dr. Zwaan,** bedankt dat je de eerste twee jaar mijn promotor wilde zijn. Ondanks dat je als kinderoncoloog geen directe betrokkenheid had met mijn thesisonderwerp, wist je elke keer als we samen evalueerde de helikopter view te pakken, zodat de grote lijnen en praktische kanten van het PhD-traject niet zouden ondersneeuwen. Heel nuttig bij een dokter die gek is op details. Dus dank je Michel.

Beste **prof. dr. Leebeek, prof. dr. Schutgens** en **prof. dr. Reis,** oftewel, beste **Frank, Roger** en **Irwin,** veel, und natürlich vielen Dank, voor het plaatsnemen in de beoordelings-

commissie. En dank aan **prof. dr. Kruip**, **dr. Novotný** en **dr. Dalm** voor hun deelname aan de promotiecommissie. Zonder al jullie expertise, moeite en tijd om mijn proefschrift te lezen en te beoordelen, had ik hier niet gestaan.

Graag wil ik ook mijn andere **collega's van de kinderhematologie** bedanken. Lieve **Helee**n en **Saskia**, kamergenootjes en collega's, dank jullie wel voor het meedenken, meelezen, moed inspreken, klaagmuur zijn, op het juiste moment lief knikken en heel veel koffie zetten! En niet te vergeten, het feit dat jullie het laatste jaar een deel van de patiëntenzorg hebben overgenomen, zodat ik die laatste spurt kon maken! Heel bijzonder. En een speciale dank aan **Anke** en **Ineke**, mijn lichtende voorbeelden in geordend werken, PIF's opsturen en archiveren, inscannen, opplakken, inlijsten en als nodig was, ook nog een strik erom. Dank je dat mij zoveel hebt geholpen met de papieren tijger die automatisch meekomt als je een PhD-traject doorloopt! En natuurlijk dank aan **Eva, Sasja, Caroline, Simone, Elyse** en **Ruth** voor alle morele support en interesse in mijn voortgang, maar ook de ultieme kwaliteit om strategisch van onderwerp te veranderen als het even tegenzat. Ik gun elk Sophia-team zulke fijne collega's!

Lieve **Caroline**, **doctor van Veen** en expert op 'unexplained bleeding disorders'. Voor jou een speciaal dankwoord. We wisten toch niet waar we aan begonnen samen… Het ging met hobbels en bobbels, maar we kwamen er. Honderd vijftig patiënten konden we includeren in de gehele Crescendo. Jij vloog me al snel voorbij in kennis en kunde. Mooi om te zien dat jouw PhD-weg de naam van jouw studie eer aan deed! Wat heb je hard gewerkt om alle data te verzamelen en te analyseren. En wat heerlijk dat het ook weer een keer klaar is. Ik wens jou en je gezinnetje het allerbeste. Daarnaast, **Marieke**, een bijzonder dank aan jou, want als PI van Caroline kreeg je mij er gewoon bij. Dank voor je adviezen en kennis over begrepen en onbegrepen stollingsstoornissen, maar ook voor het lef en de visie die je toonde dat je mijn kleine niche wilde 'adopteren' in jouw grotere muziekstuk. Ik heb veel geleerd van de heldere en inhoudelijke vragen die jij kan stellen zodat ik in een korte tijd een beeld had van wat nog nodig is.

Beste **Virgil**, het was echt een plezier samen te werken. Het uitstapje over de grenzen van bloedplaatje en rode bloedcellen naar witte bloedcellen, heeft mij en mijn werk verrijkt. Ik was al overtuigd dat een kruisbestuiving tussen verschillende deelspecialisaties waardevol is, maar nu weet ik het zeker! Dank je voor de heel fijne samenwerking en de introductie in de kwetsbare maar ook prachtige wereld van 11q. Voeg ik meteen jou, **Cindy** eraan toe. Dank voor jouw prachtige voorbeeld van ouderschap en onvoorwaardelijke liefde, voor je warme betrokkenheid en bevlogenheid.

Dear **Guannan**, I miss you dear friend, and I miss the coffee times, Chinese noodles and good discussions about all important things in life! Thank you for being a colleague, but

so much more, a friend. **Prof. dr. Raat, Hein**, dank dat je mij introduceerde in de wereld van kwaliteit. Jouw passie voor dit onderwerp is besmettelijk.

Naast de Erasmus MC en Sophia kinderziekenhuis collega's wil ik een warm dankwoord richten aan mijn Sanquin-collega's. Lieve **Věra**, medisch directeur van medische transfusie en donorzaken, maar nimmer te beroerd om tijd voor mij vrij te maken. 'Děkuji', oftewel dank je voor je immer luisterende oor en vooral je warme support op het moment dat ik het hardst nodig had. Het vertrouwen wat je in mijn stelt is hartverwarmend. Dank!

Uiteraard een dank aan al mijn andere **collega's van de UTG** van Sanquin die betrokken zijn of zijn geweest wil ik ook bedanken. In het bijzonder een dank aan het kinder-power team met nieuwe veelbelovende en enthousiaste collega's om het vak van pediatrische transfusiegeneeskunde een plek te geven. **Pauline, Laura, Cornelis en Corine**, op een lange samenwerking samen! En dan een speciale ereplek voor **Leo van de Watering**. Met jouw engelengeduld heb ik mijn 'vrees voor SPSS' overwonnen! En jouw hulp bij het uitleggen van de analyses, de keuzes die je moet maken, de parameters die meegewogen moeten worden, zijn voor mij van groot belang geweest. Dank, dank Leo!

Naast de UTG wil ik ook het team van de **Sanquin research** bedanken. **Sander, Arjan, Maartje, Iris, Floris** en **Carmen**, zonder jullie inzet, energie en tijd lagen de samples nog steeds ingevroren te wachten. Maar jullie expertise is heel uniek en niet zomaar te evenaren! Een 'special thanks to **Huan** and **Emile**, for our iPSC adventure that has brought such great new insights'. En natuurlijk **Marieke, Ellen, Leendert, Karin en Resi**. Dank voor het brainstormen, voor het op weg helpen van het project, het uitwerken van allerlei verschillende onderdelen en vragen. En dan zeker, naast **Elly, Suzanne** en **Roxanne,** een dank aan alle analisten op het trombocyten- en leukocyten serologie laboratorium van Sanquin.

Mijn dank gaat ook naar alle PhD's op de 'puppykamer' die ondanks dat ze hun eigen PhD-traject doorliepen, toch tijd maakten voor mij. Dank aan **Jossi** en **Iris** en in het bijzonder **Tine**, voor alle keren dat ik zelfs in de avond even gauw jou belde en om advies vroeg; en **Ferdows** voor je bijzondere kennis van statistiek, stolling en je bescheiden, vriendelijke support. We gaan vast nog meer van jou horen.

Alexander, ook jou wil ik bedanken. Wat een expertise heb jij! Zonder jouw ICT-skills waren de figuren in het desmopressine artikel nog altijd een spaghetti-brei geweest of bij lange na niet zo illustratief geworden. Dank je voor de fijne en constructieve samenwerking.

Ook iedereen van het **hemostase laboratorium** wil ik bedanken voor alle keren dat er weer een LTA moest gebeuren, de ene keer keurig op tijd afgestemd, de andere keer ad

hoc of op korte termijn zodat een afname gecombineerd kon worden met bijvoorbeeld een operatie of andere bloedafname. Altijd hebben jullie het kind centraal gezet. Dank voor het mooie voorbeeld wat jullie hierin zijn!

In het bijzonder nog een dankwoord voor **Riekje, Rolinda, Debby** en **Heidi**, trouwe partners in het oplossen van logistieke knelpunten of onverwachte resultaten. En **Moniek**, dank dat je voor mij tijd vrij hebt willen maken wat zeker in de beginfase van het project zo kostbaar was. En ik je kennis mocht lenen, zodat ik eindelijk echt eens begreep hoe LTA's werkten (en hoe niet).

Mijn dank ook voor de vier studenten die ik mocht begeleiden tijdens hun masteronderzoek. **Pit, Jaleesha, Samone** en in het bijzonder **Caroline,** jullie hebben meer dan een steentje bijgedragen.

Verder, **collega kinderartsen** die patiënten verwezen voor de studie**,** dank Elisabeth, Frans, Idske, Louis, Marjo en Marielle. **Gerlant van Berlaer, Anne Malfroot** en **Iris de Schutter**, jullie hebben, denk ik zonder het te weten, mijn interesse in onderzoek gewekt. **Dick Mul**, aan jou ook een dankwoord, want jij adviseerde mij om een fellowship te zoeken omdat je geloofde dat dit bij mij zou passen. Jij attendeerde mij op de vacature voor fellow kinderhematologie en bloedtransfusie. Nou, the rest is history! **Inge Appel** en **Auke Beishuizen**, dank dat ik jullie als collega's mocht hebben toen ik bij de kinderhematologie in het Sophia kinderziekenhuis begon. Kostbaar! En vooruitkijkend, wat een mooi cord blood consortium hebben we met **Jip, Pauline, Irwin, Enrico, Thomas, Emile** en **teams**!

En dan niet te vergeten, lieve **prime-time vriendinnen**! Dank jullie voor de lieve appjes, support, geïnteresseerde vragen en bloemen die ik van jullie kreeg tijdens mijn PhD-project. Je zou maar zulke vriendinnen hebben!! Ik kom gauw weer op vrijdag koffiedrinken.

Lieve **pa, ma en familie**, dank voor jullie excellente talent om gewoon lekker verder te gaan met het leven. Werk is werk, en als ik er wat over kwijt wil, dan mag dat.

En als laatste, dank aan mijn gezin. Lieve **Koos**, dank voor je nimmer aflatende steun en geloof in mij. Voor al je uren extra oppassen, koken, of naar de voetbal sjezen omdat ik nog zat te blokken op een hoofdstuk dat 'bijna-af' was, althans dat dacht ik soms, vaak… En natuurlijk, lieve **Florenz, Noor en Reyald**, jullie maken ons leven zo veel mooier! Het is een voorrecht om jullie te zien opgroeien.

