# CONGENITAL PLATELET DEFECTS

## CLINICAL FEATURES AND DIAGNOSTIC TESTS

**Maaike Blaauwgeers**

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# CONGENITAL PLATELET DEFECTS

## CLINICAL FEATURES AND DIAGNOSTIC TESTS

**AANGEBOREN BLOEDPLAATJESSTOORNISSEN**

KLINISCHE KENMERKEN EN DIAGNOSTISCHE TESTEN

(met een samenvatting in het Nederlands)

Proefschrift

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door

Maaike Willemijn Blaauwgeers
geboren 16 december 1988
te Woerden

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# 1

# GENERAL INTRODUCTION

# PLATELETS

Platelets are anucleate cell fragments derived from bone marrow megakaryocytes. Unactivated platelets have a biconvex discoid shape, a diameter of 1-4 micrometer and a mean platelet volume of 7-11 fl. The normal platelet count is 150-450 x $10^9$/l and their lifespan is 7-10 days[1]. The main physiological role of platelets is to support hemostasis. Upon vascular injury, platelets undergo a series of functional responses, which enables the rapid formation of a platelet plug at the site of injury, thereby preventing excessive blood loss. First, platelets adhere to the exposed extracellular matrix, primarily mediated by two platelet adhesion receptors: glycoprotein (GP) Ib-IX-V that binds to von Willebrand factor (VWF) and integrin α2β1 that binds to collagen. Adhesion triggers rapid signal transduction, mediated by tyrosine kinases and G-protein coupled receptors, leading to platelet activation. Activation results in shape change, release of content of storage granules into the surrounding environment, thromboxane formation, exposure of procoagulant surface and inside-out activation of integrins. The ultimate step in the activation cascade is the formation of platelet aggregates. This is mediated by a conformational change in integrin αIIbβ3, which exposes the binding side for fibrinogen and allows fibrinogen to bind with high affinity. As the fibrinogen molecule has bilateral symmetry, it can bind to the αIIbβ3 receptor on two adjacent platelets resulting in platelet aggregation[2-6]. Increased platelet reactivity can lead to a thrombotic phenotype, while decreased platelet function can result in a bleeding phenotype.

## PLATELET DEFECTS

Platelet defects can be classified as defects in platelet number, platelet function or a combination of both. They can be acquired or congenital. Acquired platelet defects are caused by a variety of factors, including certain drugs, uremia and liver disease[7]. Congenital platelet defects (CPDs) can be due to defects in megakaryopoiesis and proplatelet formation or due to defects in the expression and function of surface membrane receptors, the formation and secretion of platelet granules, transcription factors or proteins involved in the signaling pathways[8,9]. For at least 42 types of CPDs, the underlying genetic defect has been identified[10] and over 300 candidate genes associated with platelet function and thrombopoiesis have been reported[11-14]. In this thesis, the term CPD refers to congenital platelet defects resulting in a bleeding phenotype.

## PREVALENCE OF CPDS

CPDs are considered rare bleeding disorders. The World Federation of Hemophilia reports approximately 3000 patients with CPDs worldwide[15]. The true prevalence is unknown and likely underestimated due to misdiagnosis or failure to recognize the presence of a platelet defect. Studies have suggested that CPDs may even be more prevalent than von Willebrand disease (VWD)[16,17].

## BLEEDING PHENOTYPE AND BURDEN OF DISEASE

Typical bleeding manifestations of CPDs are unexplained or extensive bruising, bleeding from mucous membranes such as epistaxis, oral cavity bleeds and menorrhagia, and bleeding following a challenge to the hemostasis system such as dental extraction, surgery or childbirth[1]. Bleeding phenotype varies greatly among patients and even within patients over time. In contrast to hemophilia[18] and VWD[19,20], the most frequently reported congenital bleeding disorders, bleeding phenotype of patients with CPDs and impact of CPDs on health status-related quality of life (HR-QoL) are poorly described. Phenotypic characterization of CPDs is necessary, since this could help physicians recognize CPD subtypes and inform new patients on prognostic implications regarding their bleeding phenotype.

## DIAGNOSTIC TESTS FOR CONGENITAL PLATELET DEFECTS

Diagnostic tests for CPDs include assessment of patient history with a standardized questionnaire (ISTH Bleeding Asssessment Tool (ISTH-BAT)), platelet count and morphology, platelet function assays and other tests evaluating platelet surface markers and platelet granule content[21,22]. Diagnosing CPDs is challenging due to the complexity of platelet function and consequently the large diversity of potential platelet defects. In addition, many tests are not validated or sufficiently standardized for use in a clinical setting and there is a lack of universal criteria for the diagnosis of a platelet defect that underlies a bleeding tendency, especially for mild defects. However, proper identification of CPDs is required for a clear understanding of their prevalence, adequate counseling of the patients and their relatives and improvement in their management. Therefore, there is an unmet need for improved platelet function diagnostics.

## AIM AND OUTLINE OF THE THESIS

The aim of this thesis is to evaluate the clinical characteristics and diagnostic approach of CPDs in the Netherlands. Therefore, we have initiated a nationwide cross-sectional study on CPDs, the Thrombocytopathy in the Netherlands (TiN) study. In collaboration with the 6 haemophilia treatment centers, adult patients suspected for or diagnosed with a CPD were included. The objective of the TiN study was to evaluate the diagnostic yield of advanced diagnostic tests. Another objective was to obtain insight into the bleeding phenotype of patients with CPDs and the influence of CPDs on HR-QoL.

**Chapter 2** provides an overview of the currently available diagnostic tests, describes their advantages and limitations and discusses alternatives for more effective platelet function assays. **Chapter 3** gives insight into the prevalence and severity of bleeding symptoms of CPD patients, assesses differences in bleeding phenotype between different types of CPDs and between men and women and provides diagnostic characteristics of several types of CPDs. The ISTH-BAT is used for evaluation of the patients' bleeding symptoms and is designed as a physician-administered questionnaire. Completing this questionnaire is time-consuming and requires expertise. Therefore, **Chapter 4** evaluates whether the use of an self-administered BAT (self-

BAT) is a reliable and feasible alternative to save valuable time during outpatient clinic visits. Currently, many diagnostic laboratories do not assess platelet nucleotide content, resulting in an under diagnosis of storage pool disease. Most tests to evaluate platelet nucleotide content are time-consuming and cannot be performed in patients with thrombocytopenia. Mepacrine staining of platelet dense granules has been suggested as a diagnostic tool for platelet storage pool disease. In **Chapter 5**, the diagnostic accuracy of flow cytometric mepacrine fluorescence for diagnosing storage pool disease is prospectively evaluated in patients suspected for a CPD. DNA-based analysis has become increasingly important for diagnosing CPDs and recent studies have suggested moving genetic analysis 'upward' in the diagnostic approach. However, the best timing of genetic analysis remains unclear. Therefore, **Chapter 6** investigates the diagnostic yield of genetic analysis performed as a first-line investigation alongside initial functional analysis of platelet function in unselected patients in whom a CPD is suspected. Repeated bleeds can have a significant impact on HR-QoL, since it can hinder activities of daily living and social functioning. Even though HR-QoL has become more important in patient-centered care, studies investigating HR-QoL in patients with CPDs are limited. **Chapter 7** studies HR-QoL in a large cohort of patients with suspected or confirmed CPDs, compares the results to the general Dutch population and assesses the association between HR-QoL and bleeding severity. Finally, the results of this thesis are placed in a broader perspective in **Chapter 8.**

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# CONGENITAL PLATELET FUNCTION DISORDERS: CURRENT DIAGNOSTIC STRATEGY AND FUTURE PERSPECTIVES

Maaike Blaauwgeers, Ivar van Asten, Albert Huisman,
Rolf Urbanus and Roger Schutgens

*Ned Tijdschr Hematol 2016;13:332-40*

## SUMMARY

Congenital platelet function disorders (PFDs) are a relatively rare cause of symptomatic bleeding and are characterized by mucocutaneous bleeds and prolonged bleeding after surgery and childbirth. The prevalence is probably underestimated due to under-diagnosis. Severe PFDs are readily identified using routine diagnostic tests. For the diagnosis of mild platelet function disorders, the current diagnostic tests are often insufficient. In this review, we will discuss the advantages and disadvantages of the currently available diagnostic tests and we provide alternatives for more effective platelet function assays.

## INTRODUCTION

Platelet function disorders (PFDs) are defects of primary hemostasis and can be classified according to platelet function into adhesion, activation, secretion and aggregation defects (Figure 1). Most commonly, PFDs are caused by defects in platelet membrane receptors, but defects in signal transduction, transcription factors and the cytoskeleton also occur. The most well-known disorders are Bernard Soulier syndrome (BSS), Glanzmann thrombasthenia and storage pool disease (SPD)(Figure 2). BSS is caused by the absence or decreased surface expression of the GPIb/IX/V complex, resulting in failure of the platelets to bind VWF, leading to deficient adhesion. In Glanzmann thrombasthenia, platelets fail to aggregate due to a functional defect or deficiency of the membrane integrin αIIbβ3. SPD is characterized by a decreased number of alpha or dense granules or a defective granule content release mechanism. Examples are gray platelet syndrome (GPS) and Hermansky-Pudlak syndrome (HPS). GPS is associated with an absence of α-granules and their contents, resulting in a typical gray appearance of platelets on peripheral smears. In HPS, dense granules are lacking. Characteristic features of this disorder include a low platelet ADP content and the presence of albinism[1].

Similar to von Willebrand disease (VWD), typical bleeding manifestations of PFDs are mucocutaneous bleeds and bleeding following invasive procedures or childbirth. PFDs are rare, although some studies suggests that the prevalence of PFDs is similar to that of VWD. The prevalence of PFDs might be underestimated due to misdiagnosis[2]. Severe platelet disorders are usually identified in childhood due to bleeding problems at a young age. In contrast, mild platelet disorders are more likely to become evident in adulthood, as the exposure to events associated with bleeding risks increases with age, and bleeds may only present after an appropriate challenge like dental extraction or surgery[3].

The current diagnostic work-up of PFDs starts with a patient history, followed by screening tests and platelet function assays. With this strategy, mainly severe PFDs (like Glanzmann thrombasthenia) are diagnosed. Mild platelet dysfunctions are more difficult to diagnose and the currently available assays are often insufficient[2]. However, an accurate diagnosis is important for clinical management. Life-threatening or disabling bleeds can be prevented by educating patients on when to seek medical attention and early treatment. For instance, prophylactic treatment before surgery can decrease the risk of severe blood loss. Furthermore, misdiagnosis can lead to unnecessary and potential harmful treatments.

In this review we will discuss the currently available diagnostic tests and their limitations and provide alternatives for more effective platelet function assays.

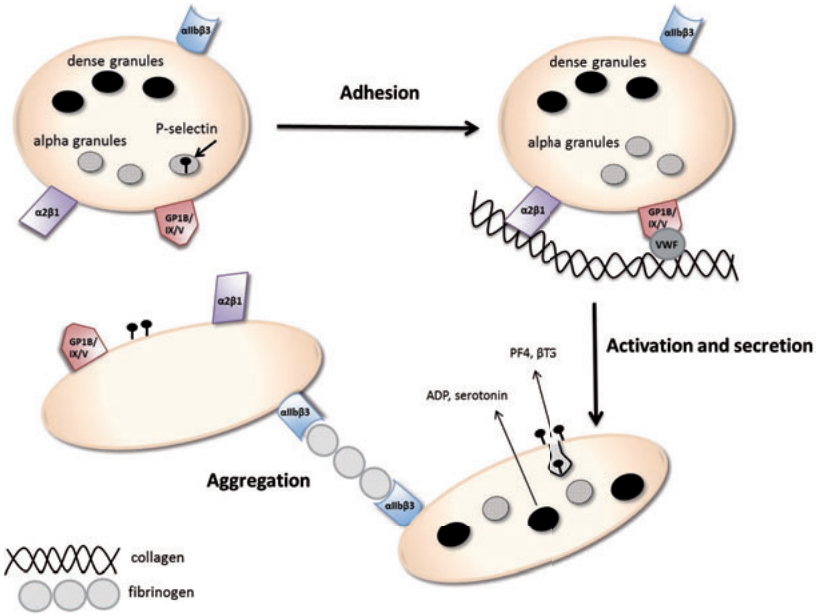


**Figure 1.** Schematic overview of platelet function. Upon vascular injury, platelets adhere to von Willebrand factor (VWF) via the glycoprotein Ib/IX/V-receptor and to collagen via the α2β1-receptor. Adhesion triggers signal transduction leading to platelet activation, shape change and degranulation of the alpha and dense granules. Release of the granule content, such as ADP and serotonin, leads to secondary activation through paracrine and autocrine activation. Binding of fibrinogen to the glycoprotein αIIbβ3-receptor leads to platelet aggregation. Examples of platelet agonists are collagen, ADP and thrombin among others. ADP, adenosine diphosphate; bTG, beta-thromboglobulin; GP, glycoprotein; PF4, platelet factor 4; VWF, von Willebrand factor.

## CURRENT DIAGNOSTIC STRATEGIES

### Patient history: the bleeding assessment tool

To assess bleeding symptoms, it is advised to use a standardized questionnaire, a Bleeding Assessment Tool (BAT). The BAT systematically evaluates the frequency and severity of different bleeding symptoms and the total of all items results in a bleeding score. A frequently used BAT is the Tosetto score[4]. A normal Tosetto bleeding score (≤3) has a very high negative predictive value (99,2%) in the diagnosis of VWD. A high score requires further laboratory testing[5]. However, this score is not validated to use in PFD patients. Therefore, the International Society for Thrombosis and Haemostasis developed the ISTH-BAT, a BAT that can be used in both VWD and PFD patients[6]. The cut-off values for a normal ISTH-BAT bleeding score are ≤3 for men and ≤5 for women[7]. Currently, the BAT is only used as a screening tool to assess in which patients further laboratory tests are required.

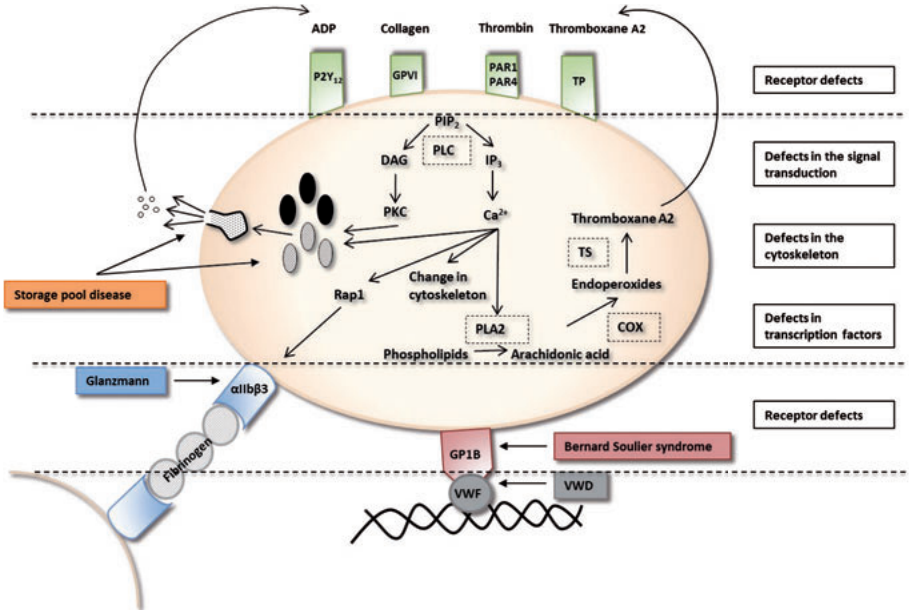


**Figure 2.** Schematic view of different types of platelet function disorders. COX, cyclooxygenase; DAG, diacylglycerol; GP, glycoprotein; $IP_3$, inositol trisphosphate; PAR, protease-activated receptor; $PIP_2$, phosphatidylinositol bisphosphate; PLA2, phospholipase A2; PLC, phospholipase C; PKC, protein kinase C; TS, thromboxane synthase; TP, thromboxane receptor; VWD, von Willebrand disease; VWF, von Willebrand factor.

## Screening tests for platelet function

A bleeding problem due to thrombocytopenia, VWD or a disorder of secondary hemostasis needs to be ruled out before proceeding to more specific platelet function testing. A complete blood count provides information about the number of platelets and mean platelet volume (MPV). In thrombocytopenic patients, the fraction of reticulated platelets (immature platelets containing some residual RNA) can be used to distinguish between defective platelet production and peripheral loss (consumption or clearance) of platelets[8]. Other blood count parameters can point towards underlying bone marrow pathology as a cause for thrombocytopenia. Thrombocytopenia can occur in combination with a functional platelet defect, like BSS and GPS[1]. The diagnostic work-up for thrombocytopenia should thus also include tests to assess platelet function, although the results from the most frequently used platelet function assays should be interpreted with caution when the platelet number is below 100x109/L. Measurement of vWF antigen (vWF:Ag), vWF activity (vWF:Rco or vWF:Act[9]) and FVIII activity should be performed to rule out VWD[10]. PT and APTT are used to evaluate and exclude coagulation disorders. When the bleeding score is high and VWF, PT and APTT measurements are normal, a platelet function disorder as a cause for the bleeding tendency is more likely[11]. Morphology of platelets can be studied using a peripheral blood smear (Figure 3). MYH9-related disorders, GPS and pseudothrombocytopenia due to clumping of platelets can be diagnosed with this technique.

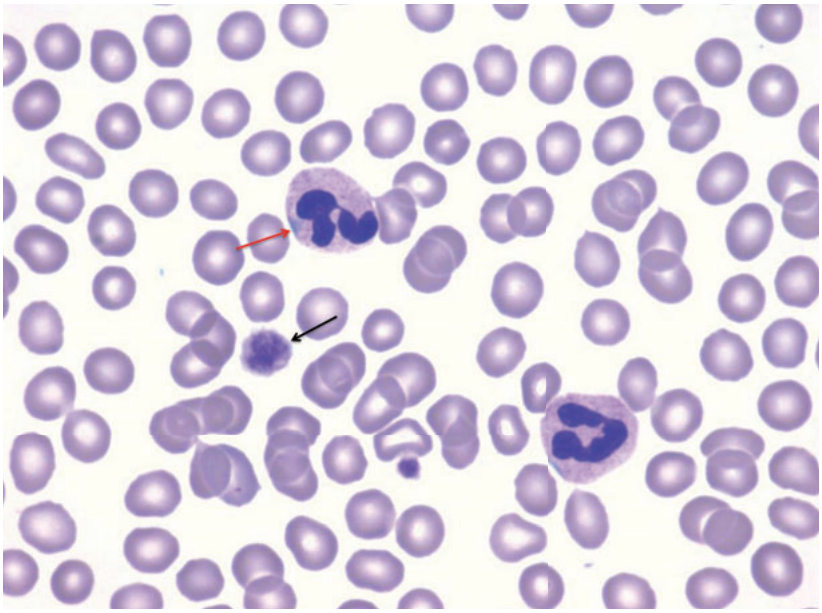

**Figure 3.** Peripheral blood smear of MYH9-related disease with giant platelets (black arrow) and Döhle body inclusions (red arrow).

Other screening tests for disorders of primary hemostasis are the bleeding time and the Platelet Function Analyzer (PFA-100). The bleeding time is the time until cessation of bleeding after a standardized incision in the arm[12]. The PFA-100 is a system that mimics a damaged vessel wall. It utilizes a membrane coated with either collagen and epinephrine or collagen and ADP to stimulate platelet activation. Blood is forced through an aperture in this membrane, inducing the formation of a platelet plug, which leads to closure of the aperture. The time until aperture closure, the closure time, is indicative for platelet function[13]. Both bleeding time and PFA-100 have a low sensitivity and specificity for PFDs. Therefore, the clinical value of these tests is debatable: a prolonged bleeding time does not lead to a specific diagnosis and a normal bleeding time does not rule out the presence of a PFD[14]. For this reason, we strongly advise to use a standardized BAT as the only screening tool for the presence of a primary hemostasis disorder, since a high bleeding score is sufficient to proceed to specific platelet function testing, regardless of the outcome of the other tests.

## Light transmission and lumiaggregometry

After excluding VWD and coagulation disorders as possible causes for a bleeding tendency, the next step in the diagnostic approach is the assessment of platelet function with specific tests. The most frequently used assay is light transmission aggregometry (LTA). LTA measures the ability of platelets to aggregate after stimulation with several agonists in various concentrations. It measures the permeability of light through a platelet rich plasma suspension that is continuously

stirred. Addition of an agonist will induce platelet activation and consequently shape change, leading to a slight decrease in light permeability. Next, platelets will aggregate and form platelet 'thrombi', resulting in an increase in light transmission through the sample. Changes in light transmission are monitored in real time during the test and are displayed as an aggregation trace (Figure 4). Defects in activation or aggregation result in an abnormal curve.

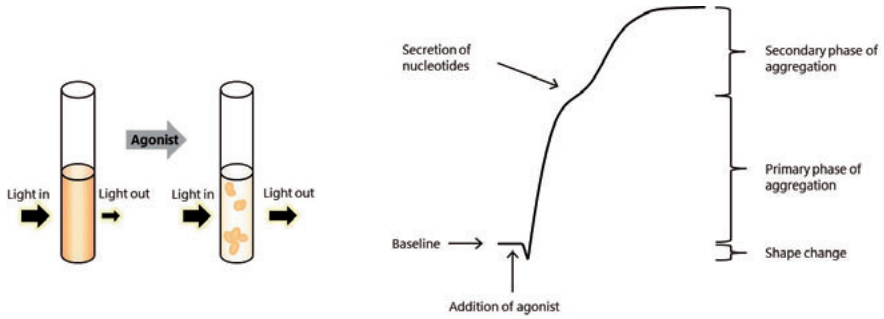


**Figure 4.** Light transmission aggregometry. Addition of the agonist leads to activation and shape change. Spreading of the platelets will decrease the light transmission (shape change). Next, platelets will aggregate and the light transmission will increase (primary phase of aggregation). Release of granule contents will lead to a secondary phase of activation and aggregation (secondary phase of aggregation). The different phases of activation and aggregation are displayed as a curve.

The most frequently used agonists are ADP, collagen, arachidonic acid and epinephrine. The response to several agonists should be assessed to distinguish between different types of PFDs. In addition, VWF-dependent platelet agglutination should be measured with ristocetin, to aid in the diagnosis of BSS and VWD type 2B. The main advantage of LTA is that several platelet activation pathways can be studied. However, LTA has a low sensitivity (49%) resulting in under-diagnosis of mild platelet function disorders[15]. Furthermore, LTA is operator dependent, time consuming and requires large volumes of blood and a minimal platelet count of 100x109/L, making it an unsuitable test for thrombocytopenic patients and small children[15,16].

Patients with SPD can have aberrant LTA results, such as an absent secondary phase of aggregation after stimulation with ADP, but normal LTA results do not exclude the presence of SPD. SPD is characterized by a decreased platelet ADP content[17]. Platelet nucleotide content can be determined in platelet lysates. The ATP concentration is measured in platelet lysates using a luciferase assay. ADP will then be converted to ATP by a pyruvate kinase reaction and the ATP concentration is re-measured. The difference between both measurements is the platelet ADP concentration. Measuring ATP and ADP concentration in platelet lysates only gives information on the theoretical secretion capacity of the platelets.

Another diagnostic test for SPD is lumiaggregometry, where ATP release from activated platelets is measured by adding luminol and luciferase to the plasma. Using this approach, it is

impossible to distinguish between storage and release defects. Lumiaggregometry has a high specificity (99%) and a moderate sensitivity (78%) for SPD[18].

The above mentioned functional assays are often insufficient to diagnose mild PFDs. Studies have shown that up to 60% of patients with a high suspicion for PFDs have normal LTA results[2]. In these cases, it is necessary to perform additional laboratory tests.

## ADDITIONAL LABORATORY TESTS

Additional diagnostic tests for PFDs include a variety of standardized and experimental tests. These are discussed below.

### Flow cytometry

Flow cytometry is a laser based approach for cell sorting and counting. Blood is perfused through a narrow chamber, enabling the passage of single cells through a laser. The light scattering is determined by the type, size and granule content of cells, allowing the sorting of cells based on their morphology. The expression of several platelet membrane receptors can be quantified with fluorescent antibodies[19]. The main advantage of flow cytometry is the requirement of only a small sample of blood. Disadvantages are the expenses for fluorescent antibodies and the limited availability of flow cytometers.

### Platelet Activation Test (PACT)

In addition to the currently used platelet function assays, the UMC Utrecht uses an extra diagnostic test: the Platelet ACtivation Test (PACT). The PACT is based on platelet activation induced by addition of a specific agonist to whole blood. Activation state of platelets is measured on a flow cytometer by quantifying P-selectin expression on the outer membrane of platelets and fibrinogen binding to the membrane glycoprotein αIIbβ3 (Figure 5). The degree of P-selectin expression gives an indication of the platelet activation and secretion function, while the fibrinogen binding the platelet activation and aggregation capacity measures. The most important activation pathways include the thrombin activation pathway via PAR1 and PAR4, the collagen activation pathway via GPVI, the ADP activation pathway via P2Y12 and the thromboxane activation pathway via TP. All activation pathways can be investigated separately, in a single experiment[20].

A huge benefit of the PACT is the ability to measure platelet function in thrombocytopenic patients. Platelet function tests in thrombocytopenia are essential, since thrombocytopenia itself usually does not results in a bleeding phenotype. LTA requires a platelet count of at least 100x109/L to give reliable results[21], while the PACT reliably measures platelet function with platelet counts as low as 5x109/L. Furthermore, the PACT only requires maximum 200 µL of blood, while the LTA needs at least 15 ml, making the PACT suitable to use in small children. Currently, the PACT is being validated to use as a diagnostic tests for PFDs[22].

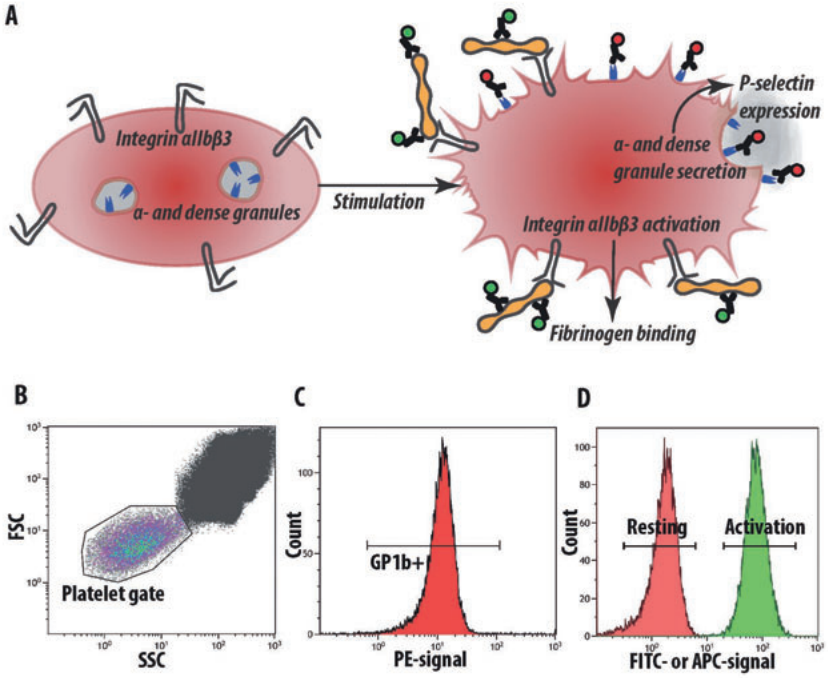


**Figure 5.** Platelet Activation Test (PACT). Platelets in whole blood are stimulated and activation will lead to binding of fibrinogen and expression of P-selectin. With APC- and FITC-labelled antibodies the degree of P-selectin expression and fibrinogen binding is quantified (A). Platelets are identified with forward and sideward scatter (B). Then, GP1b positive events (C) are selected to quantify the degree of activation. Activation of platelets results in a shift to the right of both the FITC and APC signal (D). APC, allophycocyanin; FITC, fluorescein isothiocyanate; FSC, forward scatter; GP, glycoprotein; PE, phycoerythrin; SSC, side scatter.

## Electron microscopy

Electron microscopy is used to visualize the platelet size, morphology and presence of alpha and dense granules. Platelet disorders that can be diagnosed with electron microscopy include GPS and other forms of α- and δ-storage pool disease. The agreement between experts is high[23], but the use of electron microscopy is very expensive its availability is limited.

## Flow chamber-based experiments

Platelet adhesion and aggregation under flow can be measured in vitro with flow chamber-based methods. These methods are based on the perfusion of whole blood over a platelet-activating surface, such as collagen, at physiological shear rates. Rate of adhesion and aggregation, surface coverage with platelet thrombi and thrombus volumes can be measured[24]. Adhesion defects, such as a defect in the collagen receptor α2β1, can be ascertained with such assays[25]. Flow chamber-based assays are currently only used in experimental settings.

### Proteomics and mass spectrometry

The proteome is the entire set of proteins of an organism or a cell. Proteomics is the large-scale study of visualization, quantitation and identification of these proteins. Proteins can be separated with several techniques such as electrophoresis and chromatographic methods. Mass spectrometry is used to identify and quantify the proteins in conjunction with protein sequence databases. Proteomics can be used in platelet science for deciphering defects in the platelet signaling cascades[26]. Routinely use of this technique for the diagnoses of PFDs is not yet validated.

### Whole-exome sequencing

If the aforementioned tests give inconclusive results, attempts can be made to identify the underlying genetic defect. PFDs can be caused by a mutation in one or more of the genes involved in megakaryocyte development, platelet formation or platelet function. Whole-exome sequencing (WES) permits the simultaneous analysis of the whole exome. All protein-coding genes are selectively captured from the DNA and the nucleotide sequence of the nucleotides is determined. Then, so called sequence reads are produced: small sequences of nucleotides approximately 100 base pairs in length. These sequence reads are aligned and compared to reads of a reference genome to identify variations.

One of the major assets of WES is that a large amount of (candidate)genes can be examined in a single experiment. Underlying genetic defects for HPS[27] and GPS[28] were identified with this technique. The biggest challenge is to identify the mutation that affects the clinical phenotype. In ~60% of the patients a functional defect can be identified by combining functional assays with genetic analysis[29]. WES is routinely performed in the UMC Utrecht using a selected gene platform.

## CONCLUSION

A variety of tests can be used to evaluate patients with suspected platelet function disorders. The diagnosis of severe PFDs is relatively straightforward. Mild PFDs are frequently misdiagnosed and the currently available tests are often insufficient. A considerable amount of patients with a suspected primary hemostasis disorder will not receive a definitive diagnosis, even after extensive laboratory testing. On the one hand this can be explained by the low sensitivity of LTA and the poor standardization and reproducibility of the majority of the routinely used diagnostic tests. On the other hand we must realize that the etiology of mild PFDs is often multifactorial and since LTA measures all activation pathways separately, mild defects might be missed. Whole-exome sequencing is hopeful, but the major challenge remains to identify the disease causing mutation and platelet function testing in combination with genetic analysis is essential. The use of the PACT as additional test seems promising, there are indications for a higher sensitivity and

specificity as compared to LTA. Moreover, the PACT can be used in thrombocytopenic patients. The PACT is currently being validated to use as a diagnostic test for platelet function disorders.

## 'THROMBOCYTOPATHY IN THE NETHERLANDS' - STUDY



Although research on PFDs has increased in recent years and innovations take place in the field of diagnostic tests, Dutch patients with PFDs are not well studied. We set up the 'Trombocytopathy in the Netherlands' (TiN) study to gain more insight into clinical course and quality of life of these patients. In addition, we will investigate if diagnostic approaches of PFDs can be improved with new and innovative laboratory tests such as those described above, and we aim to validate a few of these new diagnostic tests. The TiN study is the first study in the Netherlands to combine clinical characteristics with functional assays, mass spectrometry and whole-exome sequencing and generates a unique platform to better understand PFDs. The results of this study will help to improve the clinical management of patients with PFDs in the Netherlands.

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# 3

# BLEEDING PHENOTYPE AND DIAGNOSTIC CHARACTERISTICS OF PATIENTS WITH CONGENITAL PLATELET DEFECTS

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## SUMMARY

We report the analyses of the bleeding phenotype and diagnostic characteristics of a large cohort of adult patients with a confirmed congenital platelet defect (CPD). A total of 96 patients were analyzed and they were classified as Glanzmann thrombasthenia, Bernard-Soulier syndrome, dense granule deficiency, defects in the ADP or thromboxane A2 (TxA2) pathway, isolated thrombocytopenia or complex abnormalities. The median ISTH-BAT bleeding score was 9 (IQR 5-13). Heavy menstrual bleeding (HMB) (80%), post-partum hemorrhage (74%), post-operative bleeds (64%) and post-dental extraction bleeds (57%) occurred most frequently. Rare bleeding symptoms were bleeds from the urinary tract (4%) and central nervous system (CNS) bleeds (2%). Domains with a large proportion of severe bleeds were CNS bleeding, HMB and post-dental extraction bleeding. Glanzmann thrombasthenia and female sex were associated with a more severe bleeding phenotype.

## INTRODUCTION

Congenital platelet disorders (CPDs) are rare bleeding disorders caused by congenital defects in platelet production or platelet function. Patients typically present with a mucocutaneous bleeding tendency. Common symptoms include epistaxis, unexplained or extensive bruising, oral cavity bleeds, heavy menstrual bleeding (HMB) and bleeding following a hemostatic challenge such as surgery, dental extraction and childbirth[1]. CPDs are clinically heterogeneous; the frequency and severity of symptoms vary greatly among different types of CPDs, among patients with the same disorder and within patients over time[2].

The bleeding phenotype can be evaluated with the ISTH Bleeding Assessment Tool (ISTH-BAT). The ISTH-BAT was designed to underscore the importance of repetitive minor bleeding in addition to more severe bleeds and can be used in all hemorrhagic disorders[3]. The ISTH-BAT is primarily designed as a screening tool. Since the ISTH-BAT documents large variety of bleeding symptoms, it is also used for the phenotyping of patients[4,5].

Very few studies have reported the bleeding phenotype in adult patients with CPDs and most studies focused on a few specific types of CPD[6,7] or a specific mutation[8,9]. Phenotypic characterization of the whole spectrum of CPDs is necessary, since this could help physicians recognize CPD subtypes and inform new patients on prognostic implications regarding their bleeding phenotype. In this study, we aimed to evaluate the bleeding phenotype of a large cohort of adult patients with CPDs and to search for correlations between the bleeding score and different laboratory phenotypes of CPDs.

## METHODS

### Participant selection

Data were derived from patients included in the 'Thrombocytopathy in the Netherlands' (TiN) study; a nationwide cross-sectional study to collect data on clinical features, functional assays and genetics in a population of patients with or suspected for a CPD. Patients were included in the TiN study when von Willebrand disease or a coagulation factor deficiency were excluded and when (1) they were previously diagnosed with a CPD, or (2) they had previously abnormal platelet count or function test results, or (3) they exhibited a predominantly mucocutaneous bleeding tendency compatible with a platelet function disorder. Within the TiN study, a CPD was confirmed when abnormal platelet count or function was found on at least two occasions, of which one was in our diagnostic laboratory. For the current evaluation, we included only TiN patients in whom a CPD diagnosis was confirmed.

### Bleeding phenotype

The ISTH-BAT was used for evaluation of the patients' bleeding symptoms and was administered by an experienced physician prior to platelet function testing. It contains questions on 14

domains: epistaxis, cutaneous bleeding, bleeding from minor wounds, urinary tract bleeding, gastrointestinal bleeding, oral cavity bleeding, post-dental extraction bleeding, post-operative bleeding, heavy menstrual bleeding (HMB), post-partum hemorrhage (PPH), muscle hematomas, hemarthrosis, central nervous system (CNS) bleeding and one final domain on other bleeding symptoms. Each domain was scored on a scale ranging from 0 to 4 points. We classified a bleeding symptom as severe when the domain score was 3 or higher, since this indicates that the bleeding symptom required medical treatment. The total of all domains resulted in a bleeding score ranging from 0-56. The cut-off values for an abnormal bleeding score are >3 for men and >5 for women[10].

## Laboratory assessment

Laboratory tests were performed for platelet count, aggregation in response to 4 agonists (ADP, arachidonic acid, collagen, ristocetin), platelet ADP and ATP content, surface receptor expression with flow cytometry and whole-exome sequencing (WES) with a selected 76 gene panel. Platelet morphology, grey platelets and leukocyte inclusion bodies were assessed in a peripheral blood smear. The cut-off value for abnormal platelet aggregation was determined for every agonist and was based on the 2.5th percentile plus the coefficient of variation of 52 healthy donors (Table S1). The cut-off value for abnormal platelet receptor expression was determined based on the 2.5th percentile of 49 healthy donors (Table S2). Dense granule deficiency was diagnosed when the ADP content was lower than 1.4 µmoles/ $10^{11}$ platelets, based on the 2.5$^{th}$ percentile of 49 healthy donors. In line with the American College of Medical Genetics guidelines, a genetic variant was stated to be causal when a (likely) pathogenic variant (class 4 or 5, respectively)[11] was identified in one or more of the selected genes that corresponded to the platelet phenotype.

## Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 25, GraphPad Prism software version 6 and RStudio version 0.99. Descriptive results for continuous variables were presented as medians (IQR) and categorical variables were presented as frequencies (percentages). The difference in bleeding score between types of CPDs and between men and women was evaluated with linear regression analysis. Correlations between bleeding score and number of abnormal agonists in light transmission aggregometry (LTA) and between bleeding score and ADP content were calculated with non-parametric Spearman's rank correlation. Correlation coefficients (ρ) of 0.20-0.39 were considered weak, 0.40-0.59 moderate, 0.60-0.79 strong and >0.8 very strong[12]. Only moderate or stronger correlations were considered relevant.

## RESULTS

A CPD diagnosis was confirmed in 96 TiN patients. The majority of patients were women (61/96, 64%) (Table 1). The median age was 38 years (IQR 28-53) for women and 40 years (IQR 26-57) for men. The median bleeding score was 9 (IQR 7-15) for women and 6 (IQR 3-12) for men.

**Table 1.** Patient characteristics.

| | | N=96 |
|---|---|---|
| Sex | Women, n (%) | 61 (64) |
| Age | Women, median (IQR) | 38 (28-53) |
| | Men, median (IQR) | 40 (28-57) |
| Bleeding score | Women, median (IQR) | 9 (7-15) |
| | Men, median (IQR) | 6 (3-12) |
| Type of CPD | ADP pathway defect, n (%) | 22 (23) |
| | Bernard-Soulier syndrome, n (%) | 4 (4) |
| | Complex abnormality*, n (%) | 6 (6) |
| | Dense granule deficiency, n (%) | 13 (14) |
| | Glanzmann thrombasthenia, n (%) | 14 (14) |
| | Isolated thrombocytopenia, n (%) | 22 (23) |
| | TxA2 pathway defect, n (%) | 15 (16) |

CPD, congenital platelet defect; IQR, interquartile range; TxA2, thromboxane A2. * Complex abnormality was diagnosed when the patterns of platelet function defects did not support the diagnosis of a particular type of CPD.

### Classification of patients based on diagnostic characteristics

#### *Glanzmann thrombasthenia*

Glanzmann thrombasthenia (GT) was diagnosed in 14 patients based on decreased or absent aggregation in response to ADP, arachidonic acid and collagen and decreased αIIbβ3 expression. The median bleeding score was 21 (IQR 16-22) (Figure 1). The median platelet count was $183x10^9$/L (IQR 133-226) and in 5/14 patients the platelet count was below the normal range. The median αIIbβ3 expression was 1.9% (IQR 1.4-3.3). Genetic mutations were identified in all 14 patients.

#### *Bernard-Soulier syndrome*

Bernard-Soulier syndrome (BSS) was diagnosed in four patients based on a decreased aggregation in response to ristocetin and decreased GP1b-V-IX expression. The median bleeding score was 9 (IQR 1-15). All patients had macrothrombocytopenia with a median platelet count of $53x10^9$/L (IQR 16-80) and median MPV of 16.3 fL (IQR 15.9-16.4). The median GP1b-V-IX expression was 24.2% (IQR 16.5-26.9) and genetic analysis confirmed BSS in all patients.

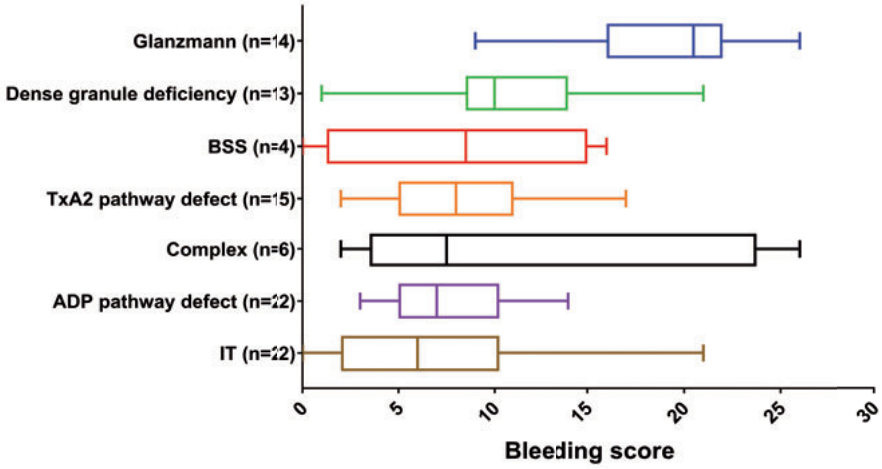


**Figure 1.** Bleeding score per type of congenital platelet defect. Boxes represent median and interquartile range, whiskers represent minimum and maximum. BSS, Bernard-Soulier syndrome; IT, isolated thrombocytopenia; TxA2, thromboxane A2.

*Dense granule deficiency*

Dense granule deficiency was diagnosed in 13 patients based on a platelet ADP content below 1.4 µmoles/$10^{11}$ platelets. The median bleeding score was 10 (IQR 9-14). The median platelet count was 165x$10^{9}$/L (IQR 69-303) and in 6/13 patients the platelet count was below the normal range. The median platelet ADP content was 0.9 µmoles/$10^{11}$ platelets (IQR 0.5-1.2). LTA results were abnormal in 6/13 patients, but did not reveal a specific pattern. Genetic mutations were identified in five patients.

*ADP pathway defect*

An isolated ADP pathway defect was diagnosed in 22 patients based on decreased aggregation in response to ADP. A normal aggregation in response to arachidonic acid differentiates them from patients with a thromboxane A2 (TxA2) pathway defect (Figure S1A). The median bleeding score was 7 (IQR 5-10). The median aggregation in response to ADP was 54% (IQR 47-61). Platelet ADP content was normal in all patients. Genetic mutations were identified in three patients.

*Thromboxane A2 pathway defect*

A TxA2 pathway defect was diagnosed in 15 patients based on decreased aggregation in response to arachidonic acid (AA), with or without defective aggregation in response to other agonists (Figure S1B). The median bleeding score was 8 (IQR 5-11). The median aggregation in response to AA was 10% (IQR 4-14). Platelet ADP content was normal in all patients. Genetic mutations were identified in two patients.

*Isolated thrombocytopenia*

An isolated thrombocytopenia (IT) was diagnosed in 22 patients based on a low platelet count, normal platelet function and normal platelet ADP content. The median bleeding score was 6 (IQR 2-10). The median platelet count was $80x10^9$/L (IQR 49-126) and median MPV 9.3 fL (IQR 8.5-12.2). Genetic mutations were identified in two patients.

*Complex abnormalities*

A complex abnormality was diagnosed in 6 patients. A complex abnormality was diagnosed when the patterns of platelet function defects did not support the diagnosis of a particular type of CPD as described above. The median bleeding score was 8 (IQR 4-24). No genetic mutations were identified in this subgroup.



## Bleeding symptoms in patients with CPDs

Most frequently occurring bleeding symptoms were HMB (49/61, 81% of women), PPH (20/27, 74% of women who gave birth) and post-operative bleeds (47/73, 64% of patients who underwent surgery) (Figure 2). Rare bleeding symptoms were bleeds from the urinary tract (4/96, 4%) and CNS bleeds (2/96, 2%).

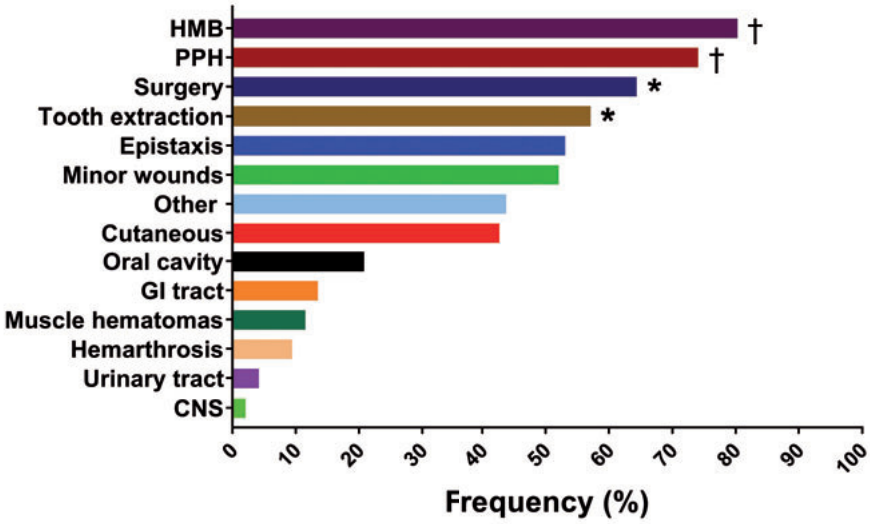


**Figure 2.** Prevalence of bleeding symptoms in patients with congenital platelet defects (CPDs). Proportion of CPD patients who experienced the bleeding symptom (ISTH-BAT domain score ≥1). CNS, central nervous system; GI, gastrointestinal; HMB, heavy menstrual bleeding; PPH, postpartum hemorrhage. * Frequencies are based on patients who underwent tooth extraction (n=63) or surgery (n=73). † Frequencies are based on women who have been menstruating (n=61) or gave birth (n=27).

We classified a bleeding symptom as severe when the domain score was 3 or higher. Both CNS bleeds were classified as severe (Figure 3A). Other domains of the ISTH-BAT with a large proportion of severe bleeds were HMB (47/49, 96% of women who have experienced HMB), post-dental extraction bleeding (31/36, 86% of patients who experienced bleeding after tooth extraction) and post-operative bleeding (35/47, 75% of patients who experienced bleeding after surgery). Bleeds from minor wounds were relatively common (50/96, 52%), as were bleeds in the 'other' category (42/96, 44%), but these bleeds were rarely severe: 9 of 50 patients (18%) had severe bleeds from minor wounds, none of 42 patients reported severe 'other' bleeds.

By combining the frequency and severity of the bleeding symptoms, the contribution of each domain to the sum of all bleeding scores can be calculated (Figure 3B). Bleeding symptoms with the largest contribution to the bleeding score were HMB (19%), post-operative bleeding (17%), post-dental extraction bleeding (13%) and epistaxis (12%).

The bleeding score was significantly higher in women than in men (β 3.5, 95% CI 0.9 to 6.1). When the bleeding score was calculated without the domains HMB and PPH, there was no significant difference (β 0.1, 95% CI -2.1 to 2.4).

## Bleeding symptoms according to type of CPD

Adjusted for age and sex, patients with GT had a significantly higher bleeding score than patients with other types of CPDs. As compared to other types of CPDs, GT patients more frequently ($p<0.01$) experienced epistaxis (100% vs 45%), urinary tract bleeds (21% vs 1%), gastrointestinal bleeds (50% vs 7%), oral cavity bleeds (71% vs 12%) and hemarthrosis (36% vs 5%)(Figure 3C). For the other types of CPDs, the bleeding phenotype was not distinctive. The bleeding phenotypes of CPDs other than GT were similar.

## Association between bleeding score and laboratory measurements

The bleeding score showed a moderate correlation with the number of abnormal agonists in LTA (ρ 0.45, 95% CI 0.23 to 0.63). When GT patients were not taken into account, the bleeding score did not correlate with the number of abnormal agonists in LTA (ρ 0.16, 95% CI -0.10 to 0.42). Also, the bleeding score did not correlate with a lower platelet ADP content (ρ 0.03, 95% CI -0.19 to 0.25), nor with a lower platelet count (ρ 0.17, 95% CI -0.03 to 0.36).

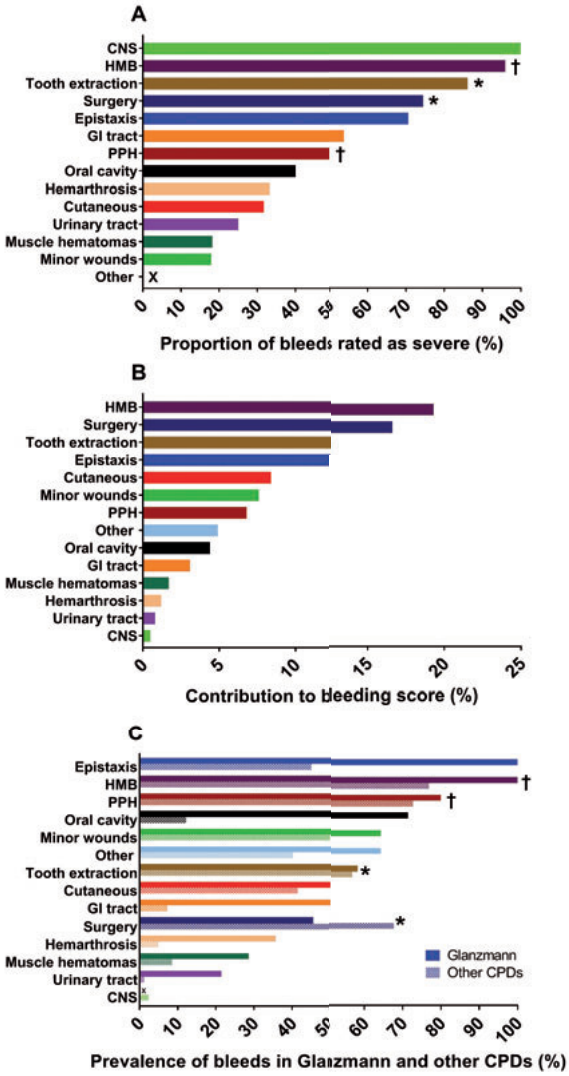


**Figure 3.** Prevalence of (severe) bleeding symptoms and contribution of bleeding symptoms to the bleeding score. (A) Prevalence of severe bleeding in patients with CPDs. Proportion of CPD patients who experienced the bleeding symptom and in whom the bleeding symptom was severe (ISTH-BAT domain score ≥3). (B) Contribution of the bleeding symptoms to the bleeding score. The frequency and severity of the bleeding symptoms are combined to calculate the contribution of each domain to the sum of all bleeding scores. (C) Prevalence of bleeding symptoms in patients with Glanzmann and patients with other types of CPDs. Proportion of Glanzmann patients (solid bars) and patients with other types of CPDs (striped bars) who experienced the bleeding symptom (ISTH-BAT domain score ≥1). CNS, central nervous system; CPD, congenital platelet defect; GI, gastrointestinal; HMB, heavy menstrual bleeding; PPH, post-partum hemorrhage; X, Not applicable. * Frequencies are based on patients who underwent tooth extraction or surgery. † Frequencies are based on women who have been menstruating or gave birth.

## DISCUSSION

This is the first study to report the bleeding phenotype and diagnostic characteristics of a large cohort of adult patients with CPDs. Patients were classified as Glanzmann thrombasthenia, Bernard-Soulier syndrome, dense granule deficiency, defects in the ADP or thromboxane A2 (TxA2) pathway, isolated thrombocytopenia or complex abnormalities. Most frequently occurring bleeding symptoms were HMB, PPH and post-operative bleeds. Glanzmann thrombasthenia and female sex were associated with a more severe bleeding phenotype. The bleeding score was not correlated with the number of abnormal agonists in LTA, nor with a lower platelet ADP content or lower platelet count.

Eighty percent of women reported HMB and in 96% of cases it was classified as severe. Moreover, HMB accounted for almost 20% of the sum of all bleeding scores. And, when the bleeding score in women was calculated without the domains HMB and postpartum hemorrhage, there was no difference between men and women. Taken together, this indicates that HMB is a considerable health problem in women with CPDs. Serious bleeding complications like CNS bleeds, gastrointestinal bleeds and hemarthrosis were rare, although more common in patients with GT as compared to other types of CPDs.

Studies on bleeding phenotype in patients with CPD mostly focus on a specific subtype or a specific mutation. One previous study reported bleeding scores in a cohort of patients with CPDs[13],but they did not report on the frequency of bleeding symptoms. Their study population consisted of mostly children and the Pediatric Bleeding Questionnaire was used. Another study reported the bleeding phenotype of patients with Von Willebrand disease[4], a primary hemostasis defect with similar symptoms. They used the Tosetto bleeding score[14] to assess bleeding symptoms and their patients most frequently reported HMB, cutaneous bleeds and prolonged bleeding from minor wounds.

It is debatable whether the ISTH-BAT is the most suitable tool for assessing the severity of bleeding symptoms, since domains of the ISTH-BAT saturate easily. Patients who have experienced a single bleed after surgery that required DDAVP treatment score the same as patients with multiple bleeds after surgery that required several transfusions. Also, CNS bleeds are always classified as severe, since the score is either 0, 3 or 4 for that domain. Despite its limitations, the ISTH-BAT is at the moment the best tool to assess bleeding symptoms, because it takes both the frequency and the severity of the whole spectrum of bleeding symptoms into account.

In our study, there was a risk for selection bias. Patients with a clinical suspicion for a CPD were referred for inclusion in the TiN study and it is likely that the referral rate was higher for patients with clinically important bleeds, such as HMB or bleeding after a hemostatic challenge, than for patients with clinically less important bleeds, such as cutaneous bleeds. This might explain the high prevalence of these bleeding symptoms in our population. In addition, for some patients it was not reported whether they had undergone tooth extraction. When patients

reported no bleeding after tooth extraction, in 6% of cases it was not reported whether they had undergone tooth extraction at all. These patients were not included in the analysis for that domain, which could have led to a slight overestimation of the prevalence. Also, the ISTH-BAT was not completed at the time of diagnosis for some patients, but at the time of inclusion in the study. These patients possibly had more hemostatic challenges, since they had more time to develop bleeding symptoms, resulting in a higher bleeding score. Some of these bleeds might have required treatment, which would also have led to an increase in the bleeding score.

The strength of our study is the inclusion of a large number of CPD patients. To the best of our knowledge, this is to first study to evaluate the bleeding phenotype and diagnostic characteristics in a large cohort of adult patients with various subtypes of CPDs. Insight into the bleeding phenotype of these patients can lead to better counseling of patients regarding the prognostic implications of their bleeding phenotype. It will create more awareness on the clinical picture of CPDs amongst doctors and in society. We incorporated explicit diagnostic criteria for CPDs based on expert opinion. Standardized diagnostic criteria will reduce under- and misdiagnosis in patients. Moreover, it will allow cluster analysis in patients with similar phenotypes and further unravelling of the pathophysiology of platelet defects.

In conclusion, in CPD patients most frequently occuring symptoms are HMB and bleeding after a hemostatic challenge. Glanzmann thrombasthenia and female sex are associated with a more severe bleeding phenotype.

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## SUPPLEMENTARY TABLES

**Table S1.** Cut-off values for light transmission aggregometry (LTA).

| LTA | ADP 2.5 | ADP 5.0 | Arachidonic acid | Collagen 1.0 | Collagen 4.0 | Ristocetin |
|---|---|---|---|---|---|---|
| 2.5th percentile + CV | 36 | 73 | 60 | 67 | 78 | 75 |

CV, coefficient of variance



**Table S2.** Cut-off values for platelet receptor expression with flow cytometry.

| Receptor expression | α2bβ3 | α2β1 | GP1b-V-IX | GP6 |
|---|---|---|---|---|
| 2.5th percentile | 32 | 57 | 60 | 33 |

## SUPPLEMENTARY FIGURES

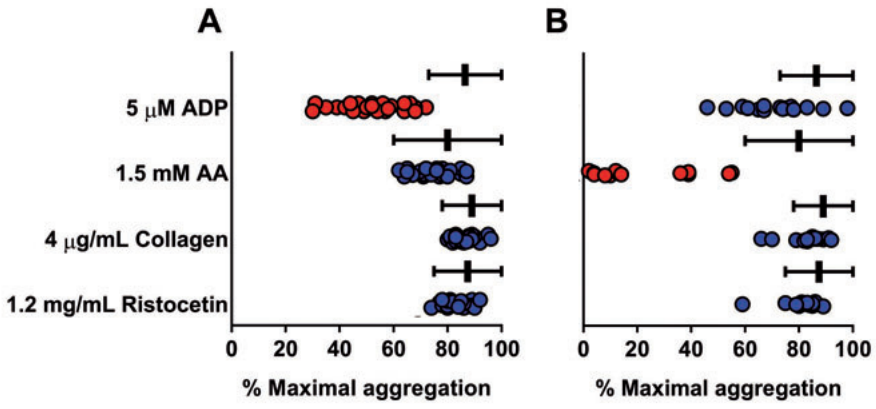


**Figure S1**. Aggregation in response to ADP, arachidonic acid (AA), collagen and ristocetin in patients with (A) an ADP pathway defect and (B) a TxA2 pathway defect. Bars represent reference values (range) for healthy controls.

4

# RELIABILITY AND FEASIBILITY OF THE SELF-ADMINISTERED ISTH BLEEDING ASSESSMENT TOOL

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*TH Open. 2019;3(4):e350–e355*

## SUMMARY

**Background**

Standardized bleeding assessment tools (BATs), such as the International Society for Thrombosis and Hemostasis (ISTH)-BAT, are screening instruments used during the diagnostic work-up of suspected bleeding disorders. A self-administered ISTH-BAT (self-BAT) would enhance screening and save time during an outpatient clinic visit.

**Aim**

This study was aimed to investigate the reliability and feasibility of the self-BAT.

**Methods**

The electronic self-BAT was created from the ISTH-BAT and paper-version of self-BAT and optimized by patients and physicians. Patients with a (suspected) congenital platelet defect (CPD), who had previously undergone physician-administered ISTH-BAT assessment, were invited to complete the self-BAT. Optimal self-BAT cut-off values to detect a bleeding tendency as defined by the ISTH-BAT, were evaluated by ROC curve analysis to reach a sensitivity ≥95%. Reliability was tested by assessing sensitivity, specificity and intraclass correlation (ICC). Feasibility was evaluated on comprehension and length of self-BAT.

**Results**

Both versions of the BAT were completed by 156 patients. Optimal cut-off values for self-BAT to define a bleeding tendency were found to be identical to those of the ISTH-BAT. Normal/abnormal scores of the ISTH-BAT and self-BAT were agreed in 88.5% (138/156, 95% CI 0.83-0.93) of patients. The sensitivity and specificity of the self-BAT to detect a bleeding tendency were 96.9% and 48.1%, respectively. The ICC was 0.73. Self-BAT questions were graded by 96.8% (151/156) as 'very easy' 'easy' and 'satisfactory' and questionnaire length as 'exactly right' by 91% (142/156) of patients.

**Conclusion**

In patients with a (suspected) CPD, the self-BAT is sufficiently reliable and feasible to detect a bleeding tendency, which supports its use as a screening tool.

## INTRODUCTION

Bleeding disorders include von Willebrand Disease (VWD), platelet disorders, hemophilia and other clotting factor deficiencies[1]. Clinical symptoms range from continued bleeding after injury to severe spontaneous bleeding. Bleeding disorders can be difficult to diagnose due to inconclusive and expensive laboratory tests. Over the past few years, several standardized bleeding assessment tools (BATs) have been created in order to aid in distinguishing normal from abnormal bleeding during the diagnostic work-up of a suspected bleeding disorder and to grade the bleeding severity[2].

In 2010, the International Society for Thrombosis and Hemostasis (ISTH) has endorsed a new BAT (ISTH-BAT), which is currently implemented in clinical practice widely to detect mild bleeding disorders and to assess the severity of the bleeding symptoms[3]. By completing this 14-domain questionnaire, a bleeding score is determined ranging from 0 to 56 points. The ISTH-BAT can be used for all types of congenital bleeding disorders and can be used to select patients in whom further laboratory investigations are necessary[4,5]. The ISTH-BAT has a high sensitivity and negative predictive value (NPV) for bleeding disorders that makes it suitable as a screening tool[2,6].

The ISTH-BAT is designed as a physician-administered questionnaire, yet completing the questionnaire is time consuming and requires expertise[2]. Recently, a self-administered ISTH-BAT (self-BAT) was generated in Canada[7]. Their aim was to generate, optimize, and validate a self-BAT as a screening tool for patients referred for suspected VWD. In the preliminary analysis, reliability of the self-BAT was considered excellent with an intraclass correlation coefficient (ICC) of 0.87 compared with the ISTH-BAT. In the final analysis, sensitivity and specificity of the self-BAT outcomes were found comparable to the ISTH-BAT, using laboratory-defined diagnosis of VWD as a reference standard.

Patients suspected of a congenital platelet disorder (CPD) comprise a large proportion of the patients seen at the outpatient clinic of hemophilia treatment centers (HTC). A (Dutch) self-BAT for this population is not yet available. HTC physicians conduct the ISTH-BAT as part of the standard procedure for bleeding assessment in the diagnostic workup of this population. A self-BAT completed at home would save valuable time during an outpatient clinic visit and enhance screening before referral to the HTC for a suspected CPD. Therefore, our aim was to assess the reliability and feasibility of the Dutch online self-BAT in patients referred for assessment of CPDs.

## METHODS

### Study participants

This study included patients who participated in the cross-sectional “Thrombocytopathy in the Netherlands” (TiN) study between February 2016 and December 2017 (Supplement 1)[8]. The TiN study investigated 201 patients with a bleeding tendency who were suspected of or had

been diagnosed with a CPD. Assessment included the ISTH-BAT score (physician-administered), platelet characteristics and function, and DNA analysis. To validate the self-BAT in this cohort, all TiN patients were reinvited to complete the self-BAT questionnaire. Only TiN participants who had previously declined participation in future studies and deceased participants were excluded (n = 3 and n = 1, respectively). Nonrespondents were contacted via telephone and e-mail. The ethics board of the University Medical Center Utrecht (UMCU) confirmed that the Medical Research Involving Human Subjects Act does not apply (reference number: 18-329).

## ISTH Bleeding Assessment Tool

The 14 ISTH-BAT domains cover epistaxis, cutaneous bleeding, minor cutaneous wounds, hematuria, gastrointestinal bleeding, oral cavity bleeding, tooth extraction, surgical bleeding/major trauma, menorrhagia, postpartum bleeding, muscle hematomas, hemarthrosis, central nervous system bleeding and one final domain on other bleeding symptoms. Each domain scores from 0 (absence of bleeding symptoms) to 4 (symptoms requiring extensive medical intervention), and the overall bleeding score is determined by summing the scores for all domains[3]. An abnormal bleeding score has been determined for men >3 and for women >5[9].

## Self-administered ISTH Bleeding Assessment Tool

At the beginning of 2016, the Dutch ISTH-BAT had been incorporated into the electronic patient records of UMCU and a Dutch self-BAT designed for children was back and forward translated by another institute, during which the diagnostic accuracy of this preliminary version of the self-BAT was evaluated by receiver operator characteristic (ROC) curves, using laboratory assessment as per the reference standard[10]. This preliminary self-BAT formed the basis of the current electronic self-BAT. The self-BAT was then tested by one pediatric and five adult hemophilia treating physicians of the Van Creveldkliniek and by two male and two female members of the Dutch Hemophilia Patient Organization. In addition, seven naive patients suspected of a bleeding disorder completed the self-BAT prior to their first visit to the clinic. Unclear self-BAT questions were adjusted till easy understanding was achieved. Medical terms were replaced by common words (such as epistaxis to nosebleeds) and at various points examples were given (muscle hematoma was explained by a description of the clinical symptoms; Self-Bat Dutch version can be provided on request). Once no noteworthy alterations were required, the self-BAT was sent to the TiN patients who had approved participating in this side study via a secured link by e-mail during July and August 2018. Each patient completed the self-BAT at home, without assistance. A reminder was circulated after 1 month in case of missing or incomplete responses. Incomplete questionnaires were excluded after study closure.

## Statistical analysis

ISTH-BAT questionnaires from the previous TIN study were compared with the current self-BAT questionnaires. Incomplete self-BAT questionnaires were excluded from the analyses. To assess

optimal cut-off values for men and women for the self-BAT, ROC curves were constructed, and a sensitivity of at least 95% to detect bleeding tendency defined by the ISTH-BAT was sought to avoid false negative bleeding assessments which are undesirable for a screening instrument. We hypothesized that the self-BAT score would be higher than the ISTH-BAT score, possibly warranting a different cut-off value for the self-BAT to define a bleeding tendency in accordance to the ISHT-BAT definitions. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were assessed using the optimal cut-off scores for the self-BAT. Also the absolute agreement was calculated; we hypothesized that in less than 10% of cases, the outcomes of the surveys (normal or abnormal bleeding) would be discrepant.

For intersurvey reliability, we considered an ICC of >0.75 and Limits of Agreement (LoA) ±3 (that is, 10% of the ISTH-BAT range) between the two survey types as good[11]. A Bland– Altman plot was constructed to visualize the LoA. A sensitivity analysis was performed to detect any relevant differences between the TiN patients who did and who did not agree to participate in this self-BAT study. The influence of age, sex, and time between self-BAT and ISTH-BAT assessment on the difference between self-BAT and ISTH-BAT scores were visualized with scatter plots and analyzed using univariable and multivariable linear regression analysis. For comparison of the medians, the Wilcoxon signed rank test was used, considering a p-value of <0.05 as significant.

Feasibility was assessed from the patients' perspective on the time necessary to complete the questionnaire and the difficulty of questions (3- and 5-point Likert's scales). In addition, patients were asked to provide feedback on improvement and positive aspects of the questionnaire (free text). Data were analyzed using IBM SPSS Statistics Version 25 (IBM Corp., Armonk, New York, United States).

## RESULTS

During the inclusion period, 197 patients were contacted from the TiN study. Those patients not willing to complete the self-BAT (9%, 17/197) and those patients who did not respond to our invitation (8%, 16/197), failed to submit (5%, 9/197) or complete (1%, 1/197) the questionnaire were excluded. The final cohort consisted of 156 patients, 78% (156/201) from the original TiN study. Baseline characteristics are depicted in Table 1. The included patients were 77% women, had a median age of 43 years, median ISTH-BAT total score of 10 (interquartile range (IQR) 7-14) with a final CPD diagnosis in 48% of patients. In comparison, TiN patients who did not participate in the self-BAT were 89% (40/45, p=0.005) women, with a median age of 31 years (p=0.000), but comparable median ISTH-BAT total score of 9 (IQR 7-11, p=0.132) and final CPD diagnosis in 42% (19/45, p=0.272).

**Table 1.** Baseline characteristics and BAT scores of included patients.

| | | N=156 | |
|---|---|---|---|
| Sex | Women, n (%) | 120 (77) | |
| Age | Median (range) | 43 (18-76) | |
| Classification* | Congenital platelet defect, n (%) | 77 (48) | |
| | Glanzmann Thrombasthenia | | 13 (17) |
| | Bernard-Soulier Syndrome | | 4 (5) |
| | Dense granule deficiency | | 8 (10) |
| | ADP pathway defect | | 15 (20) |
| | TxA2 pathway defect | | 11 (14) |
| | Isolated thrombocytopenia | | 20 (26) |
| | Complex abnormalities# | | 6 (8) |
| | Possible congenital platelet defect, n (%) | 16 (10) | |
| | Acquired platelet defect, n (%) | 3 (2) | |
| | Von Willebrand Disease, n (%) | 1 (2) | |
| | Unexplained bleeding tendency, n (%) | 59 (38) | |
| ISTH-BAT score | Median (range) | 10 (0-26) | |
| | Score 0 (floor score), n (%) | 5 (3) | |
| | Score >5 for women, n (%) | 104 (87) | |
| | Score >3 for men, n (%) | 25 (69) | |
| Self-BAT score | Median (range) | 12 (0-33) | |
| | Score 0 (floor score), n (%) | 2 (1) | |
| | Score >5 for women, n (%) | 110 (92) | |
| | Score >3 for men, n (%) | 29 (81) | |

ADP, Adenosine diphosphate; TxA2, Thromboxane A2. * Final diagnosis at the end of the 'Thrombocytopathy in the Netherlands' study. # The patterns of platelet function defects did not support the diagnosis of one particular congenital platelet defect.

## Cut-off values for the self-BAT

The optimal cut-off values for the self-BAT were determined to be >3 for men and >5 for women, the same as for the original ISTH-BAT (Supplement 2)[9]. The self-BAT score was significantly higher than the ISTH-BAT score (median difference = 2.00, range = 16, Table 1 and Figure 1).

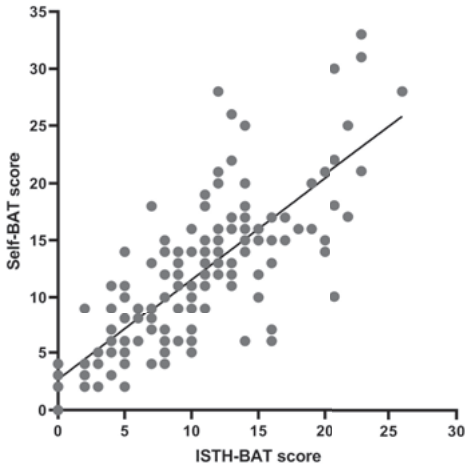


**Figure 1.** Scatter plot of self-BAT and ISTH-BAT scores. Linear model: self-BAT score= 2.62 +0.89*ISTH-BAT score.

## Reliability

The sensitivity, specificity, PPV and NPV of the self-BAT to detect a bleeding tendency were 96.9%, 48.2%, 89.9% and 76.5%, respectively (Table 2). The normal/abnormal outcome classification of the ISTH-BAT and self-BAT were agreed in 138/156 (88.5%, 95% CI: 0.83-0.93) patients, slightly lower than our hypothesized agreement of 90%. The exact agreement percentages and agreement ±1 point per domain are shown in Table 3 and Supplement 3. Both over- and underreporting of symptoms in the self-BAT compared with the ISTH-BAT was seen on the individual domains.

**Table 2.** Test performance of the self-BAT.

| | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| Overall | 96.9 (92.3-99.2) | 48.2 (28.7-68.1) | 89.9 (86.1-92.8) | 76.5 (53.4-90.2) |
| Women | 97.1 (91.8-99.4) | 43.8 (19.8-70.1) | 91.8 (87.9-94.5) | 70.0 (40.1-89.0) |
| Men | 96.0 (79.7-99.9) | 54.6 (23.4-83.3) | 82.8 (71.4-90.2) | 85.7 (44.9-97.8) |

Data is presented as percentages (95% CI). Cut-off value used was >5 for women and >3 for men. NPV, negative predictive value; PPV, positive predictive value.

The Bland-Altman plot of the differences between ISTH-BAT and self-BAT score versus the average of the two scores is presented in Figure 2, with a LoA of -9.65 to 6.67, compared with our hypothesis of ±3. The ICC was 0.73 (95% CI: 0.64-0.79).

With increasing age, the self-BAT score showed an on average 0.059 (range: 0.029 to 0.088) per year increase based on the regression coefficient of multivariable linear regression analysis. Women had a somewhat larger absolute difference in scores (-0.001 [range: -1.092 to 1.091]).

The time elapsed between both BAT assessments did not affect the difference between both scores (-0.001 [range: -0.001 to 0.003]) (Supplement 4).

**Table 3.** Percentage agreement between self-BAT and ISTH-BAT (domain) scores.

| | ISTH-BAT | Self- BAT | % Exact Agreement* | % Agreement ±1# |
|---|---|---|---|---|
| **Outcome classification** | | | | |
| Normal bleeding | 27 | 17 | NA | NA |
| Abnormal bleeding | 129 | 139 | NA | NA |
| **Score per domain, median (range)** | | | | |
| 1 Epistaxis | 0 (0-4) | 1 (0-4) | 62.8 | 84.0 |
| 2 Cutaneous bleeding | 0 (0-3) | 0 (0-4) | 57.7& | 82.7 |
| 3 Minor cutaneous wound | 1 (0-3) | 1 (0-4) | 57.1& | 85.9 |
| 4 Hematuria | 0 (0-4) | 0 (0-3) | 89.7 | 94.2 |
| 5 Gastrointestinal bleeding | 0 (0-4) | 0 (0-4) | 87.8 | 91.7 |
| 6 Oral cavity bleeding | 0 (0-4) | 0 (0-4) | 74.4 | 87.2 |
| 7 Tooth extraction | 0 (0-4) | 2 (0-4) | 55.1& | 71.8& |
| 8 Surgical bleeding/Major trauma | 2 (0-4) | 2 (0-4) | 60.3 | 76.3& |
| 9 Menorrhagia | 3 (0-4) | 3 (0-4) | 74.4 | 92.9 |
| 10 Post-partum bleeding | 0 (0-4) | 0 (0-4) | 75.0 | 90.4 |
| 11 Muscle hematomas | 0 (0-4) | 0 (0-4) | 86.5 | 93.6 |
| 12 Hemarthrosis | 0 (0-3) | 0 (0-4) | 83.3 | 89.7 |
| 13 Central Nervous System bleeding | 0 (0-4) | 0 (0-4) | 98.1 | 98.7 |
| 14 Other bleeding symptoms^ | 0 (0-3) | 1 (0-4) | 54.5& | 89.7 |

NA, not applicable. * Percentage exact agreement is defined as exact same score. # Percentage agreement ± 1 is defined as same score or one point difference. ^ Excessive umbilical stump bleeding, cephalohematoma, suction bleeding, venipuncture bleeding and bleeding during intercourse. & Exact agreement% under the 60%, Agreement +/- 1 under 80%.

## Feasibility

The questions were graded as 'very easy', 'easy' and 'satisfactory' in 96.8% (151/156) of the patients (Table 4). The length of the questionnaire was experienced as 'exactly right' in 91% (142/156) of the patients (Table 5). Recurrent themes on suggestions for improvement where to add an 'I don't know/I don't remember' option and free text to allow for comments and elaboration. Patients felt that free text would aid in cases where standard answers would not seem applicable, for example when prophylaxes had been administered before surgery. Patients at an older age reported not being able to remember details of their bleeding history and women during/after menopause reported having difficulty in reporting their earlier menstrual cycle. Positive remarks focused on the clear and easy to understand nature of the questions.

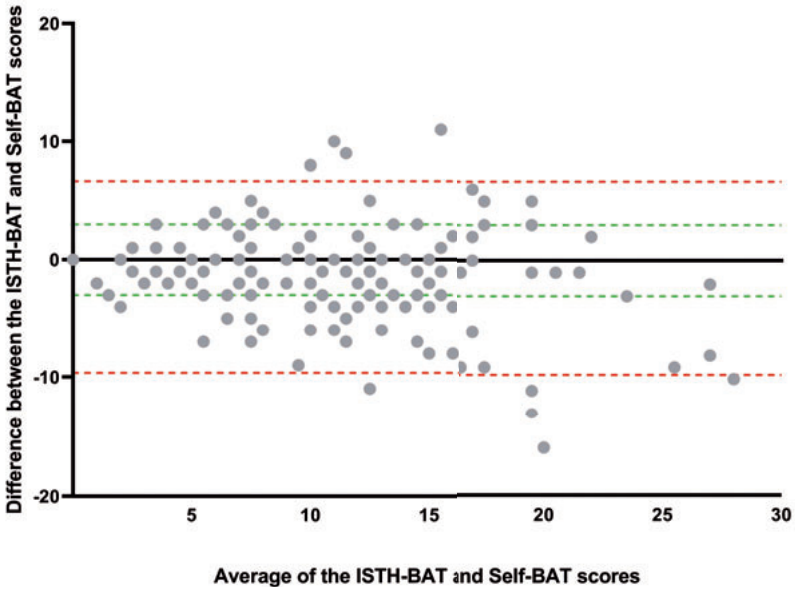


**Figure 2.** Bland-Altman plot of the differences between ISTH-BAT and self-BAT score versus the average of the two scores. ISTH-BAT, International Society for Thrombosis and Hemostasis bleeding assessment tool; self-BAT, self-administered ISTH-BAT. Red line, limits of agreement (LoA) from study: -9.65 to 6.67; Green line, beforehand determined LoA of ±3.

**Table 4.** Evaluation of the difficulty of questions of the self-BAT.

| | Number of patients | % of patients |
|---|---|---|
| Very difficult | 3 | 1.9 |
| Difficult | 2 | 1.3 |
| Satisfactory | 46 | 29.5 |
| Easy | 57 | 36.5 |
| Very easy | 48 | 30.8 |

**Table 5.** Evaluation of the length of the self-BAT.

| | Number of patients | % of patients |
|---|---|---|
| Too short | 8 | 5.1 |
| Exactly right | 142 | 91.0 |
| Too long | 6 | 3.9 |

## DISCUSSION

The present study assessed the reliability and feasibility of a self-BAT for screening of patients referred to a hemophilia treatment center with a suspected or known CPD. The optimal cut-off values for defining a bleeding tendency were found to be similar to those of the original ISTH-BAT. The self-BAT appears reliable with a sensitivity of 96.9% to detect a bleeding tendency. Patients valued the clear and easy to understand nature of the questions and the length of the questionnaire was experienced as appropriate.

This is the first study to evaluate reliability and feasibility of the self-BAT in patients referred for assessment of CPDs. A previous Canadian study evaluated the self-BAT in patients with VWD and showed that their self-BAT was a reliable screenings tool with an ICC of 0.87 and sensitivity of 78% to detect VWD[7]. The major difference between the Canadian self-BAT and the current self-BAT is the language (English versus Dutch), the wording used and hard copy versus digital questionnaires, whereas the scoring system is similar. We found a higher sensitivity; however, this refers to the ability in detecting a bleeding tendency comparable to the ISTH-BAT, instead of detecting a particular bleeding disorder. Their cut-off values for the self-BAT to define a bleeding tendency were comparable to our study. The high sensitivity of 97% indicates very few false negative bleeding assessments in a population with a suspected or diagnosed CPD, a desirable feature of screening instruments. Even though the ISTH-BAT and self-BAT scores are highly correlated, a discrepancy of 11.5% of normal/abnormal cases and larger than expected LoA were found. The discrepant cases are bordering the cut-off value for a bleeding tendency, in very few cases, leading to a 'false negative' score (2.5%, 4 of 156). The LoA discrepancy can be explained by the larger dispersion seen in higher self-BAT scores, far above the cut-off value for a bleeding tendency and thus of no implication for the diagnostic workup. On the contrary, a potential concern of the moderate specificity of 48% might be overdiagnosis and medicalization. The specificity could be increased if higher cut-off values were chosen, yet this would result in a loss of sensitivity (and thus the effectiveness as a screening tool for further diagnostic/laboratory workup). Lastly, relatively low NPV of the self-BAT in the current study is explained by the high prevalence of bleeding tendency in the investigated population. More research is needed to define evidence based criteria to safely exclude an inherited bleeding disorder with an ISTH-BAT score below the cut-off for an increased bleeding tendency[2,6].

Strengths of this study include the large cohort in which this self-BAT was evaluated and the extensive pretesting in health care workers and naive patients. A limitation of this study is the overrepresentation of female patients and the lack of an accurate gold-standard test. The participation bias (older patients and more females) could have influenced the results, although the median ISTH-BAT scores and proportion of patient with a bleeding tendency did not differ in the TiN patients who declined participating of this self-BAT study. We compared the data of our self-BAT to the ISTH-BAT, although the ISTH-BAT is recommended for clinical use, the test–retest reliability of both BATs has not yet been evaluated.

We recommend the use of the self-BAT in the clinic, but results should be discussed and interpreted with caution during consultation. Higher self-administered scores can occur when patients interpret normal bleeding events as abnormal. Discrepant cases (normal/abnormal bleeding) commonly differed by only a few points on the total score, but resulted in >10% of the cases in a discrepant outcome. The ICC of 0.73 is acceptable and can be explained by the dispersion seen in higher scores. Although a larger dispersion in differences between BATs was seen in the elderly, the dispersion was symmetric, and therefore it is unlikely that recall bias caused the discrepancies. By discussing the self-BAT results with a physician during the following outpatient clinic visit, scores can be adjusted accordingly. This would still be more time efficient than the current ISTH-BAT and coincides with its use as a screening tool and preliminary assessment of bleeding history.

Future studies on secondary and primary care centers are needed to assess potential use of the self-BAT in these settings and to assess the potential for its use as a screening instrument for clinically relevant bleeding disorders in general. In conclusion, the self-BAT has a high sensitivity to detect a bleeding tendency as defined by the current standard ISTH-BAT, which supports its use as a screening tool, and was valued by patients for its clear questions and appropriate length.

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# 5

# FLOW CYTOMETRIC MEPACRINE FLUORESCENCE CAN BE USED FOR EXCLUSION OF PLATELET DENSE GRANULE DEFICIENCY

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## SUMMARY

**Background**

δ-storage pool disease (δ-SPD) is a bleeding disorder characterized by a reduced number of platelet dense granules. The diagnosis of δ-SPD depends on the measurement of platelet ADP content, but this test is time consuming and requires a relatively large blood volume. Flow cytometric analysis of platelet mepacrine uptake is a potential alternative, but this approach lacks validation, which precludes its use in a diagnostic setting.

**Objectives**

To evaluate the performance of platelet mepacrine uptake as a diagnostic test for δ-SPD.

**Methods**

Mepacrine fluorescence was determined with flow cytometry before and after platelet activation in 156 patients with a suspected platelet function disorder, and compared with platelet ADP content as reference test. Performance was analyzed with a receiver operating characteristics (ROC) curve.

**Results**

17/156 patients had δ-SPD based on platelet ADP content. Mepacrine fluorescence was inferior to platelet ADP content in the identification of patients with δ-SPD, but both mepacrine uptake (area under the ROC curve (AUC) 0.87) and mepacrine release after platelet activation (AUC 0.80) had good discriminative ability. In our tertiary reference center, mepacrine uptake showed high negative predicitive value (97%) with low positive predictive value (35%). Combined with a negative likelihood ratio of 0.1, these data indicate that mepacrine uptake can be used to exclude δ-SPD in patients with a bleeding tendency.

**Conclusion**

Mepacrine fluorescence can be used as a screening tool to exclude δ-SPD in a large number of patients with a suspected platelet function disorder.

## INTRODUCTION

Platelets play an important role in haemostasis by forming a platelet plug upon vascular injury. When platelets are activated, they secrete the content of their storage organelles, alpha- and dense granules, to promote further platelet activation and coagulation[1,2]. One of the molecules secreted from dense granules is ADP, which promotes secondary platelet activation via the P2Y12 receptor and is essential for thrombus stability[3].

Defects in platelet dense granules can be classified into storage pool disease (δ-SPD) and secretion defects. δ-SPD results from either a decreased number or complete absence of dense granules or a decreased granule content, like the empty sack syndrome[4]. Secretion defects are associated with a defective release mechanism due to impaired signal transduction or granule trafficking[5].

Platelet secretion disorders, in particular dense granule disorders, are the most common inherited platelet function disorders and may be more prevalent than von Willebrand disease[6]. Nonetheless, there is no consensus on the best laboratory practice to detect these disorders and the methodology is poorly standardized[7,8]. The current approach to evaluate platelet dense granule secretion includes lumiaggregometry and the measurement of ADP and ATP in platelet lysate using bioluminescence[9,10]. Lumiaggregometry is currently the most often used method, but cannot distinguish between a decreased granule number or a secretion defect[9]. Measuring ADP and ATP content in platelet lysates will diagnose patients with storage pool deficiency[11], but is insensitive for secretion defects[12]. Interestingly, many diagnostic laboratories do not measure platelet nucleotide content, resulting in potential underdiagnosis of δ-SPD[13,14]. In addition, none of these tests can be performed in patients with thrombocytopenia. Another used method to diagnose δ-SPD is to count the total number of dense granules per platelet with whole mount transmission electron microscopy (TEM)[15,16]. However, this technique is challenging and not widely available. Therefore, there is an unmet need for an easy and rapid diagnostic tool to evaluate platelet dense granule secretion.

Flow cytometry has been recommended by the ISTH/SSC guidelines as a tool to diagnose patients with a platelet function disorder, and has been shown to have added value to LTA in diagnosing platelet function disorders[9,17]. Platelet granule markers, such as CD63 and P-selectin, have also been used in the screening of mild platelet function disorders on the flow cytometer, but require platelet stimulation before analysis. Mepacrine, a fluorescent acridine derivative which binds adenosine nucleotides[18], has been used to measure platelet dense granule content. Several studies showed decreased platelet mepacrine fluorescence in patients with δ-SPD[19-22], and implementation of mepacrine fluorescence in a diagnostic algorithm for platelet function disorders has been proposed[23]. However, although current data on mepacrine fluorescence are promising, the performance of mepacrine fluorescence has not yet been compared with routine diagnostic tests for δ-SPD in a real-life clinical setting [24,25].

In the present study, we validated a flow cytometric mepacrine fluorescence assay for dense granule content in patients with a suspected platelet function disorder.

## METHODS

### Participants

*Healthy volunteers:* Blood from healthy individuals was obtained via the Mini Donor Service, a blood donation facility for research purposes that is approved by the medical ethics committee of the University Medical Center (UMC) Utrecht. All donors provided written informed consent, in accordance with the declaration of Helsinki and self-reported to be free from antiplatelet drugs or non-steroid anti-inflammatory drugs for at least ten days prior to blood donation.

*Patients:* Two different patient cohorts were used in this study. Cohort 1 consisted of 7 patients with a previously diagnosed δ-SPD (ADP content lower than 1.7 µmol/$10^{11}$ platelets) and was used to provide proof of principle for diagnostic mepacrine fluorescence. Cohort 2 included patients from the Thrombocytopathy in the Netherlands (TiN) study and was used to validate the flow cytometric mepacrine uptake. The TiN study is a nationwide cross-sectional study to collect data on clinical characteristics, functional assays and genetics in a population of patients with a suspected platelet disorder. Patients were included when Von Willebrand disease or a coagulation factor deficiency was excluded and (1) they had previously abnormal platelet counts or platelet function test results, or (2) they exhibited a predominantly mucocutaneous bleeding tendency compatible with a platelet function disorder. After a single hospital visit, laboratory tests were performed for platelet count, aggregation in response to 4 agonists, nucleotide content, surface receptor expression via flow cytometry and genetic analysis with a selected primary hemostasis gene panel. Platelet mepacrine content and release were measured at the time of inclusion as well. In total, the TiN cohort included 173 patients with a bleeding tendency in whom a platelet function disorder was suspected and in 156 patients both mepacrine fluorescence and platelet ADP content was measured. All patients were aged ≥18 years and were referred to the Van Creveldkliniek for platelet function testing. Donors and patients declared to be free from any anti-platelet drugs. The medical ethics review board of the UMC Utrecht approved this study and patients provided written informed consent in accordance with the declaration of Helsinki.

### Blood collection and platelet preparation

Peripheral venous blood from patients and controls was collected with venipuncture into 3.2% sodium citrate Vacutainer® tubes (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometric assays were performed in whole blood, whereas the other tests required Platelet Rich Plasma (PRP). PRP was obtained by centrifugation of whole blood at 160g without brake for 15 minutes at 20°C. Platelet Poor Plasma (PPP) was obtained by centrifugation of whole blood at 2000g for

10 minutes and was used to adjust PRP concentration to $250x10^9$ platelets/L. All experiments were performed within 1-6 hours after blood collection.

## Flow cytometric determination of dense granule content

Five µL whole blood was diluted 1:10 (v:v) in HEPES buffered saline (HBS; 10mM HEPES, 150mM NaCl, 1mM $MgSO_4$, 5mM KCl, pH 7.4), which contained 100µM mepacrine (Sigma Aldrich, Zwijndrecht, The Netherlands) and 15µg/mL in-house developed PE-conjugated anti-GP1b nanobodies (clone 17), with or without 25µM protease activating receptor (PAR)-1 activating peptide SFLLRN (PAR1-AP; Bachem, Weil am Rhein, Germany). Whole blood was incubated for 10 minutes at 37°C, after which samples were fixed with 0.148% formaldehyde, 137mM NaCl, 2.7mM KCl, 1.12mM $NaH_2PO_4$, 10.2mM $Na_2HPO_4$, 1.15mM $KH_2PO_4$, 4mM EDTA, pH 6.8 for 20 minutes at room temperature and analyzed on a BD FACSCanto II (BD Biosciences). The flow cytometer was calibrated every week to maintain stable fluorescent intensity. Platelets were identified based on forward and sideward scatter, as well as GPIbα-expression. Mepacrine fluorescence was normalized on the median fluorescence of the healthy control population and was expressed as normalized Median Fluorescent Intensity (nMFI). The coefficient of variation for mepacrine uptake was 2.3%. Flow cytometric analysis was reproducible within 6 hours after blood collection (data not shown).



## Platelet ADP concentration

One mL PRP with a platelet count between 100 and $250x10^9$/L was diluted 1:3 (v:v) in ice cold 86.4% ethanol, 10mM EDTA, pH 7.4. Platelets were lysed by vortex and 1 freeze/thaw cycle and samples were stored at -80°C until further processing. Platelet lysates were split into two fractions. The first fraction was incubated with 95µM phosphoenolpyruvate and 25µg/mL pyruvate kinase in 0.2M Tris-Maleate, 10mM KCl, 15mM $MgSO_4$, pH 7.4 at 37°C for 15 minutes to convert all ADP to ATP. Reactions were stopped by heating the samples for 10 minutes at 80°C. The second fraction was used without prior treatment. ATP levels in both fractions were determined with the ATPLite 1 step kit (Perkin Elmer, Waltham, MA, USA) on a Spectramax L luminometer (Molecular Devices, Sunnydale, CA, USA) according to the protocol of the manufacturer. ATP levels were derived from an ATP calibration curve. ADP concentrations were calculated by subtracting the ATP concentration of the second fraction from the first. ADP levels were expressed in µmol/$10^{11}$ platelets.

## Quantification of dense granules with TEM

Platelet dense granule numbers were counted using TEM images made with the Jeol1010 microscope (Jeol, Peabody, MA, USA). Formvar-coated grids were stabilized with carbon (Edwards Auto306) and coated with 100 µg/mL fibrinogen for 20 minutes at room temperature. Coated grids were blocked with 1% BSA in HBS. Platelets were allowed to adhere to the grids for 1 minute, after which the grids were rinsed with demi water and air dried. Images of 10 platelets at

12.000x magnification were taken for every subject. Six independent individuals were instructed to quantify the dense granule number in all images according to the guidelines for dense granule identification[26]. Observers were blinded to the case or control status of the sample.

### Data analysis

Statistical analysis was performed with GraphPad Prism software version 6 (San Diego, CA, USA) and IBM SPSS Statistics 21 (Armonk, NY, USA). Variables were analyzed for a normal distribution with the Shapiro-Wilk test. Non-normally distributed variables were transformed with a Box-Cox power transformation, after which normality was checked again. Cut-off values for mepacrine fluorescence and mepacrine release were determined in normally distributed data using the 2.5th percentile from 89 healthy controls. The lower cut-off value for platelet ADP content (<1.4 µmol/$10^{11}$ platelets) was based on the 2.5$^{th}$ percentile of 49 healthy controls. Inter-test agreement between the different tests was expressed as Cohen's kappa coefficient. The $R^2$ Pearson's correlation coefficient was calculated for the correlation between platelet size or platelet count and mepacrine uptake. The discriminative ability of mepacrine fluorescence and mepacrine release was determined with the area under a receiver operator characteristic curve (AUC) in patients with a bleeding tendency without prior diagnosis in whom a platelet function disorder was suspected. Researchers were blinded for the δ-SPD diagnosis during analysis.

## RESULTS

### Mepacrine fluorescence and mepacrine release show good agreement in patients with previously diagnosed δ-SPD.

Seven patients with a previously diagnosed δ-SPD were enrolled in this study and were compared with healthy controls. This population included 4 female and 3 male patients with an ADP concentration ranging from 0.3 to 1.21 µmol/$10^{11}$ platelets and an average dense granule number per platelet ranging from 0.3 to 1.15 (Table 1).

**Table 1.** Baseline characteristics of δ-storage pool disease patients

| | SPD patients n=7 | Reference value |
|---|---|---|
| **Sex, men** | 3 | NA |
| **Age (years)** | 48 (23.5-63) | NA |
| **Platelet count ($10^9$/L)** | 232 (183-306) | 150-450 |
| **MPV (fL)** | 7.2 (6.8-7.5) | 7.0-9.5 |
| **Platelet ADP content (µmol/ $10^{11}$ platelets)** | 1 (0.5-1.43) | 1.7-3.8 |
| **Number of dense granules** | 0.85 (0.5-1.15) | 4-6 |

Data are presented in median (IQR), with the exception of sex (n). IQR, interquartile range; MPV, mean platelet volume; NA, not applicable

Platelet ADP content (Figure 1A) was decreased in all patients with δ-SPD, whereas the platelet ADP/ATP ratio (Figure 1B) was increased in all patients with δ-SPD. In these 7 patients, platelet dense granule content was also determined with flow cytometry by measuring mepacrine uptake in resting platelets, or mepacrine release after PAR1-AP stimulation. The lower limit of normal (2.5$^{th}$ percentile) was 75.1%. Patients with δ-SPD had reduced mepacrine uptake compared with healthy controls (P<0.05) (Figure 1C). Platelet activation with 25µM PAR1-AP resulted in decreased mepacrine release in patients with δ-SPD (Figure 1D).

Mepacrine uptake did not correlate with platelet size ($R^2$=0.02; P-value 0.43) or platelet count ($R^2$=0.003: P-value 0.78). Mepacrine fluorescence was in perfect agreement (k=1) with platelet ADP content (Figure 2A) and mepacrine release after PAR1-AP activation was in good agreement (k=0.93) with platelet ADP content (Figure 2B). Mepacrine fluorescence was also in good agreement (k=0.93) with PAR1-AP induced mepacrine release (Figure 2C).

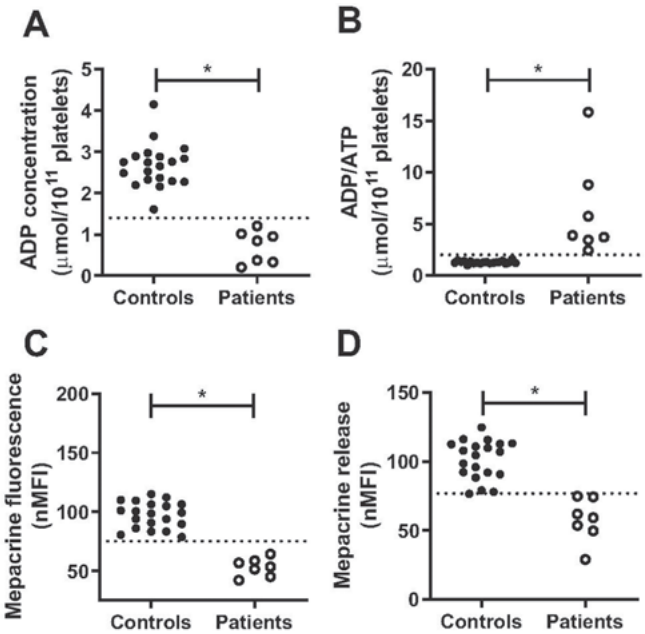


**Figure 1.** High discriminative ability of flow cytometric measurement of platelet dense granule content in patients with previously diagnosed δ-SPD. (A) Platelet ADP content, expressed as µmol/$10^{11}$ platelets measured with luminescence, (B) platelet ADP/ATP ratio, (C) normalized mepacrine fluorescence and (D) mepacrine release in 20 healthy controls (closed symbols) and 7 δ-SPD patients measured with flow cytometry. The dotted line represents the 2.5th percentile of the healthy control population. ADP, adenosine diphosphate; ATP, adenosine triphosphate; nMFI, normalized median fluorescent intensity; SPD, storage pool disease. * Indicates a p-value < 0.05.

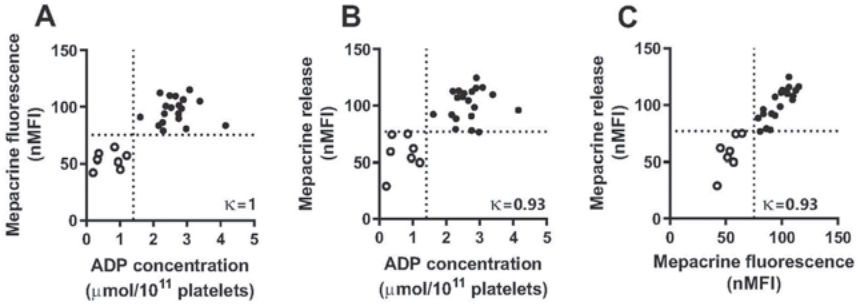


**Figure 2.** Statistically relevant agreement between mepacrine fluorescence and platelet ADP content in diagnostic testing of δ-SPD. Cohen's kappa was calculated to determine the agreement between (A) mepacrine fluorescence and platelet ADP content, (B) mepacrine release and platelet ADP content, and (C) mepacrine release and mepacrine fluorescence in 20 healthy controls (closed symbols) and 7 δ-SPD patients. Platelet ADP content is expressed in μmol/$10^{11}$ platelets. Mepacrine fluorescence and release are normalized on the median fluorescence of the healthy control group. The dotted line represents the 2.5$^{th}$ percentile of the healthy control population. ADP, adenosine diphosphate; nMFI, normalized median fluorescent intensity; SPD, storage pool disease.

## Validation of mepacrine fluorescence in patients with suspected platelet function disorders

To prospectively validate mepacrine fluorescence and mepacrine release in a relevant patient population, we compared these parameters with platelet ADP content as gold standard in the TiN cohort. This cohort included 173 patients with a bleeding tendency, in whom a platelet function disorder was suspected. Mepacrine fluorescence data were not performed in 17 patients. Consequently, 156 patients were used in the current analyses. Based on the 2.5$^{th}$ percentile of 49 healthy controls, the cut-off for normal platelet ADP content was set at 1.4 μmol/$10^{11}$ platelets. In total, 17 out of 156 patients had a platelet ADP content below 1.4 μmol/$10^{11}$ platelets and were diagnosed with δ-SPD (Table 2 and Figure 3A). Both mepacrine fluorescence (Figure 3B) and mepacrine release (Figure 3C) were decreased in these patients with δ-SPD. Mepacrine fluorescence (Figure 3D; $R^2$=0.18) and mepacrine release (Figure 3E; $R^2$=0.10) correlated with platelet ADP content. The discriminative ability of mepacrine fluorescence and mepacrine release were determined with the area under a receiver-operator curve (AUC) (Figure 3F).

**Table 2.** Baseline characteristics of patients with a suspected platelet function disorder

| | Non-SPD (n=139) | SPD (n=17) | Reference value |
|---|---|---|---|
| **Sex, men** | 22 (16) | 7 (41%) | NA |
| **Age (years)** | 38 (29-52) | 41 (31-56) | NA |
| **Bleedingscore** | 9 (7-12) | 10 (8-14) | Men >3, Women >5* |
| **Platelet count ($10^9$/L)** | 235 (190-282) | 200 (69-272) | 150-450 |
| **MPV (fL)** | 8 (7.2-8.7) | 7.4 (6.5-8.2) | 7.0-9.5 |
| **Platelet ADP content (µmol/ $10^{11}$ platelets)** | 2.5 (2.1-2.8) | 0.9 (0.5-1.2) | 1.4-3.8 |

Data are presented in median (IQR), with the exception of sex (n, %). IQR, interquartile range; MPV, mean platelet volume; NA, not applicable; SPD, storage pool disease. * See reference 29

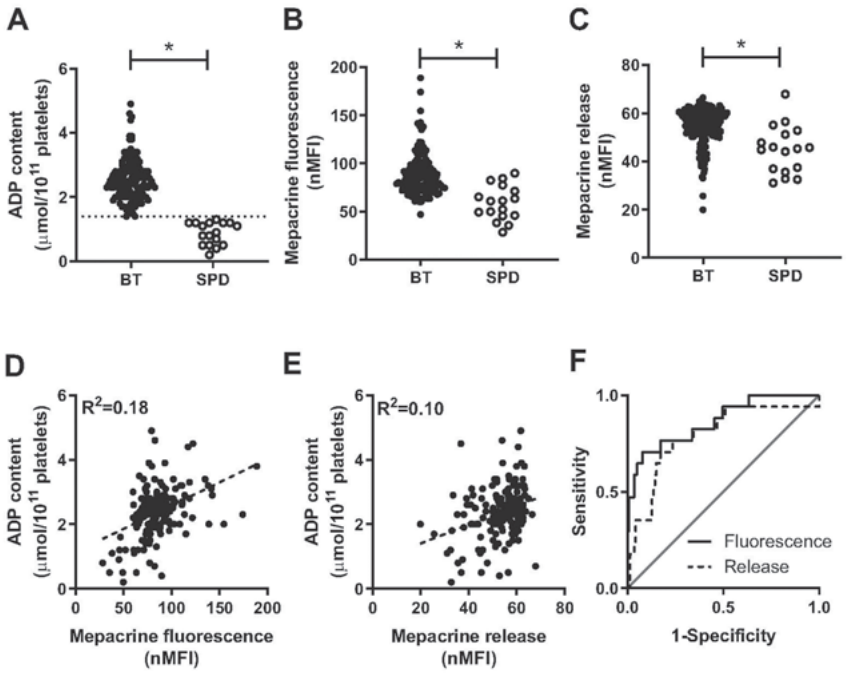


**Figure 3.** Diagnostic accuracy of flow cytometric mepacrine uptake and mepacrine release in patients with suspected platelet function disorders. (A) Platelet ADP content expressed as µmol/$10^{11}$ platelets, (B) mepacrine fluorescence expressed as normalized MFI, and (C) mepacrine release expressed as normalized MFI for all patients included in the validation cohort. Patients were classified as bleeding tendency without δ-SPD (BT; n = 139), or patients with a bleeding tendency and δ-SPD (SPD; n = 17). The cut-off value for ADP content was 1.4 µmol ADP/$10^{11}$ platelets. The correlation of mepacrine fluorescence (D) and mepacrine release (E) with platelet ADP content in all patients included in the validation cohort (n=156). (F) The discriminative ability of mepacrine fluorescence (AUC 0.87) and mepacrine release (AUC 0.79) with platelet ADP as reference test plotted in a ROC-curve. ADP, adenosine diphosphate; AUC, area under the curve; BT, bleeding tendency; nMFI, normalized median fluorescent intensity; ROC, receiver operator characteristic; SPD, storage pool disease. * Indicates a p-value <0.05.

Mepacrine fluorescence (AUC 0.87; 95% confidence interval [CI] 0.76-0.96) and mepacrine release (AUC 0.79; 95% CI 0.67-0.91) showed good discriminative ability for diagnosing δ-SPD. When a more stringent definition of δ-SPD, based on both platelet ADP content <1.4 µmol/$10^{11}$ platelets and an ATP/ADP ratio >2, was used, 15 patients met the diagnostic criteria for δ-SPD. This did not affect the AUC for mepacrine fluorescence (AUC 0.90, CI: 0.81-0.996, P=0.42). Based on the area under the ROC curve, the optimal diagnostic cut-off for mepacrine fluorescence was 71.2% of normal and 51.3% of normal for mepacrine release. Based on these cut-off values, the diagnostic accuracy for both mepacrine fluorescence and mepacrine release was determined (Table 3). With a sensitivity of 76.5% (CI: 50.1-93.2) and a specificity of 82.7% (CI: 75.4-86.6), mepacrine fluorescence showed moderate diagnostic accuracy. The diagnostic accuracy for mepacrine release was similar. The 2.5$^{th}$ percentile of healthy controls, a commonly used cut-off value in clinical laboratories, showed a similar diagnostic accuracy for mepacrine fluorescence, but the sensitivity of mepacrine release decreased to 35% (P=0.02). To evaluate the potential use of mepacrine fluorescence as a screening test for δ-SPD, diagnostic accuracy was determined at several cut-off values. At a cut-off value below 84.3% of normal, the sensitivity of mepacrine fluorescence was 94.1% and specificity was 50.4%. The positive likelihood ratio (LR+) was 1.9, indicating mepacrine fluorescence is a poor predictor of δ-SPD, but the negative likelihood ratio (LR-) was 0.1, indicating that mepacrine fluorescence can be used to exclude δ-SPD.

**Table 3.** Diagnostic accuracy of mepacrine fluorescence and mepacrine release at different cut-off values compared with platelet ADP content as gold standard.

**Mepacrine fluorescence**

| Cut-off (nMFI) | Sensitivity | Specificity | PPV | NPV | Cohen's κ | LR+ | LR- |
|---|---|---|---|---|---|---|---|
| 67.8# | 70.6 (44.0-89.7) | 88.5 (82.0-93.3) | 42.9 (30.1-56.6) | 96.1 (92.2-98.1) | 0.46 | 6.1 (3.5-10.7) | 0.3 (0.2-0.7) |
| 71.2* | 76.5 (50.1-93.2) | 82.7 (75.4-86.6) | 35.1 (25.7-45.9) | 96.6 (92.4-98.60) | 0.39 | 4.4 (2.8-6.9) | 0.3 (0.1-0.7) |
| 84.3^ | 94.1 (71.3-99.9) | 50.4 (41.8-59.0) | 18.8 (15.9-22.2) | 98.6 (91.2-99.8) | 0.16 | 1.9 (1.5-2.3) | 0.1 (0.02-0.8) |

**Mepacrine release**

| Cut-off (nMFI) | Sensitivity | Specificity | PPV | NPV | Cohen's κ | LR+ | LR- |
|---|---|---|---|---|---|---|---|
| 43.5# | 35.3 (14.2-61.7) | 87.8 (81.1-92.7) | 26.1 (13.9-43.6) | 91.7 (88.6-94.1) | 0.20 | 2.9 (1.3-6.3) | 0.7 (0.5-1.1) |
| 51.3* | 76.5 (50.1-93.2) | 76.3 (68.3-83.1) | 28.3 (20.9-37.0) | 96.4 (91.8-98.4) | 0.30 | 3.2 (2.2-4.8) | 0.3 (0.1-0.7) |

LR, likelihood ratio; nMFI, normalized median fluorescent intensity; NPV, negative predictive value; PPV, positive predictive value. * indicates the optimal cut-off value derived from the ROC curve. # indicates the cut-off value derived from the 2.5th percentile of mepacrine fluorescence or release in healthy controls. ^ indicates the optimal cut-off value as screening test for δ-SPD

## DISCUSSION

In the present study, we show that patients with δ-SPD have both decreased mepacrine uptake and decreased mepacrine release after platelet stimulation compared with healthy controls. Because of the high negative predictive value (NPV), but low positive predictive value (PPV), mepacrine fluorescence can be used for exclusion of δ-SPD in patients with a suspected platelet function disorder.

Flow cytometry has been recommended by the ISTH/SSC guidelines in the diagnostic work-up of patients with platelet function disorders[9]. It has been shown that flow cytometry has added value to the current diagnostic work-up of patients with suspected platelet function disorders, but its value in diagnosing δ-SPD in particular has not been validated[19]. We are the first to prospectively evaluate the diagnostic accuracy of flow cytometric mepacrine fluorescence in patients with a suspected platelet function disorder. Previous studies already showed that mepacrine uptake is decreased in patients with δ-SPD[22,24,25]. One of these studies compared flow cytometric mepacrine assays with routine diagnostic tests in patients with δ-SPD [22]. Similar to these studies, we found that mepacrine fluorescence allows perfect discrimination between patients with confirmed δ-SPD and healthy controls. In contrast to these findings, the performance of mepacrine fluorescence was inferior to platelet ADP measurements in the prospective evaluation of δ-SPD in unselected patients with a bleeding tendency in whom a platelet function disorder was suspected.

We evaluated both mepacrine fluorescence in resting platelets and mepacrine release after platelet stimulation. Of these two parameters, mepacrine fluorescence seems more specific, since it provides a direct measure of platelet dense granule content without the necessity of platelet stimulation. Mepacrine release requires platelet stimulation and therefore cannot discriminate between impaired platelet activation and a secretion defect, because both result in decreased mepacrine release. This is reflected by the superior diagnostic accuracy of mepacrine fluorescence compared with mepacrine release in unselected patients with a bleeding tendency, in whom other platelet function disorders are common.

The strength of this study is that a selected cohort of patients with a suspected platelet function disorder was used for validation of mepacrine fluorescence. All tests were performed simultaneously and researchers were blinded for the diagnosis. Therefore, there was no selection bias in this cohort. A potential weakness of our study is that we used platelet ADP content as a reference test. As a consequence, we could have missed δ-SPD caused by a secretion defect. Moreover, we could have falsely diagnosed δ-SPD in patients with a decreased metabolic adenosine nucleotide concentration, which is characterized by a low ATP/ADP ratio. This is not likely to have influenced the outcome of our study, as application of a more stringent definition of δ-SPD that includes an ATP/ADP ratio > 2 resulted in a similar performance of mepacrine fluorescence. Another limitation of this study is that the diagnosis of δ-SPD was not confirmed

in a second visit to our diagnostic center. This may have led to an overestimation of the number of patients with SPD in our study.

We report a high NPV for mepacrine fluorescence, which suggests mepacrine fluorescence can be used to exclude δ-SPD. Our validation cohort consisted of undiagnosed patients with a bleeding tendency in whom a platelet function disorder was suspected and therefore reflects the real-life patient population seen at a tertiary referral center. As a result, the prevalence of δ-SPD in our study population was relatively high (11%) compared with the expected prevalence of δ-SPD in the general population. This might have caused an overestimation of the NPV of mepacrine fluorescence. However, we also found a low negative likelihood of mepacrine fluorescence for δ-SPD (LR- 0.1), which is independent of the prevalence and supports the ability of mepacrine fluorescence to exclude δ-SPD.

The current diagnostic approach for δ-SPD does not include a rapid screening test and could benefit from an additional test like flow cytometry. Unlike the currently available diagnostic tools, flow cytometry is applicable in thrombocytopenic samples and requires only a small sample volume, allowing rapid exclusion of δ-SPD in children[27,28]. Our data indicate that flow cytometric analysis is very reproducible, even immediately after blood collection.

Taken together, these data indicate that flow cytometric measurement of dense granule parameters is a potential tool for the screening of δ-SPD. The presented method requires a minimal amount of whole blood and can be used to for the exclusion of δ-SPD and for the selection of patients that require further extensive testing.

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# 6

# THE LIMITATION OF GENETIC TESTING IN DIAGNOSING PATIENTS SUSPECTED FOR CONGENITAL PLATELET DEFECTS

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## SUMMARY

Identification of congenital platelet defects (CPDs) is challenging and usually requires highly specialized tests and multiple hospital visits. DNA-based analysis has become increasingly important for diagnosing CPDs and recent studies have suggested moving genetic analysis 'upward' in the diagnostic approach. This study reports on the diagnostic yield of genetic analysis performed as a first-line investigation in a prospective cohort of patients suspected for a CPD. Only 5% (8/156) of patients received a molecular diagnosis. Therefore, genetic testing with a selected gene panel should only be performed in patients in whom a platelet number or function defect is confirmed.

# INTRODUCTION

Congenital platelet defects (CPDs) are rare disorders of primary hemostasis caused by congenital defects in platelet production or function. Identification of CPDs is challenging due to the lack of awareness resulting in late or missing referrals, the lack of diagnostic criteria, absence or limitations of laboratory tests and poor standardization of the available tests[1]. However, an accurate diagnosis is important for proper counseling and management of patients and to avoid ineffective and potentially harmful treatments due to misdiagnosis, like idiopathic thrombocytopenic purpura (ITP).

DNA-based analysis has become increasingly important for diagnosing CPDs[2]. Genetic analysis can be useful to confirm a suspected phenotypic diagnosis and to identify patients with an increased risk for associated pathologies, such as myelofibrosis (*NBEAL2*), renal insufficiency (*MYH9*) and hematological malignancies (*RUNX1*). The International Society for Thrombosis and Haemostasis (ISTH) currently recommends to perform genetic analysis as a third-line investigation, that is, after extensive phenotyping and functional analyses have confirmed the presence of a platelet disorder[3]. Recent studies on the efficacy of genetic testing in selected patients with platelet disorders have suggested that genetic analysis could be moved 'upward' in the diagnostic approach in order to simplify and hasten the diagnosis of CPDs[4-6]. However, it remains unclear whether genetic analysis should be performed as a first-line investigation, alongside initial functional analysis of platelet function in unselected patients in whom a congenital platelet disorder is suspected.

In the Thrombocytopathy in the Netherlands (TiN) study, we assessed the diagnostic value of genetic analysis performed in parallel with routine laboratory tests in a prospective cohort of patients suspected of having a CPD.



# METHODS

## Participant selection

Three categories of patients were included in the study:

1. Patients suspected of having a CPD based on previous abnormal platelet counts, light transmission aggregometry (LTA) results or platelet ADP content without a molecular diagnosis
2. Patients suspected of having a CPD based on a predominantly mucocutaneous bleeding tendency compatible with a CPD, in whom other known causes of bleeding were excluded and in whom previous LTA results were normal
3. Patients suspected of having a CPD based on a predominantly mucocutaneous bleeding tendency compatible with a CPD, in whom other known causes of bleeding were excluded, newly referred for platelet function testing

### Laboratory assessment

Laboratory tests were performed for platelet count, aggregation response to four agonists (ADP, arachidonic acid, collagen, ristocetin), platelet ADP and ATP content, mepacrine staining, surface receptor expression with flow cytometry and whole-exome sequencing (WES) with a selected 76 gene panel (Table 1). Platelet morphology, grey platelets and leukocyte inclusion bodies were assessed in a peripheral blood smear.

### Definitions

A CPD was diagnosed when an abnormal platelet count or function was found on at least two separate occasions, of which one was in our diagnostic laboratory. A possible CPD was diagnosed when an abnormal platelet function was found once in our diagnostic laboratory or when abnormal platelet function test results were inconsistent with previous findings. In line with the American College of Medical Genetics guidelines, a genetic variant was stated to be causal when a (likely) pathogenic variant (class 4 or 5, respectively)[7] was identified in one or more of the selected genes that corresponded to the platelet phenotype.

## RESULTS

In total, 156 patients were included for analysis. In patients with previously abnormal laboratory results (n=96), a CPD was confirmed in 61 of 96 (64%) patients and a possible CPD was diagnosed in four of 96 (4%) patients. Eight of 96 (8%) patients received a molecular diagnosis and in 11 of 96 (11%) patients a variant of unknown significance was identified (Table 2 and Table 3). In patients with previously normal LTA results (n=39) and in newly referred patients (n=21), a possible CPD was diagnosed in 10 of 39 (26%) and 6 of 21 (29%) patients, respectively. No causal genetic variants were identified in these patients.

**Table 1.** Genes included in the WES gene panel for molecular screening of primary hemostatic disorders

| Target protein | Gene | Description | Gene | Description |
|---|---|---|---|---|
| Platelet agonist receptors | *ADRA2A* | G-protein coupled receptors | *GP9* | Bernard Soulier syndrome |
| | *ADRA2B* | G-protein coupled receptors | *ITGA2* | Bleeding disorder, platelet type 9 |
| | *CD36* | Bleeding disorder, platelet type 10 | *ITGA2B* | Glanzmann thrombasthenia |
| | *F2R* | G-protein coupled receptors | *ITGB1* | Bleeding disorder, platelet type 9 |
| | *F2R13* | G-protein coupled receptors | *ITGB3* | Glanzmann thrombasthenia |
| | *GP1BA* | Bernard Soulier syndrome | *P2RY12* | Bleeding disorder, platelet type 8 |
| | *GP1BB* | Bernard Soulier syndrome | *TBXA2R* | Bleeding disorder, platelet type 13 |
| | *GP6* | Bleeding disorder, platelet type 11 | | |

**Table 1.** (Continued)

| Target protein | Gene | Description | Gene | Description |
|---|---|---|---|---|
| Platelet granules | *AP3B1* | Hermansky-Pudlak syndrome 2 | *LYST* | Chediak-Higashi syndrome |
| | *BLOC1S3* | Hermansky-Pudlak syndrome 8 | *MLPH* | Griscelli syndrome |
| | *BLOC1S6* | Hermansky-Pudlak syndrome 9 | *MYO5A* | Griscelli syndrome |
| | *DTNBP1* | Hermansky-Pudlak syndrome 7 | *NBEAL2* | Gray platelet syndrome |
| | *HPS1* | Hermansky-Pudlak syndrome 1 | *PLAU* | Quebec platelet disorder |
| | *HPS3* | Hermansky-Pudlak syndrome 3 | *RAB27A* | Griscelli syndrome |
| | *HPS4* | Hermansky-Pudlak syndrome 4 | *VPS33B* | ARC syndrome |
| | *HPS5* | Hermansky-Pudlak syndrome 5 | *VIPAS39A* | ARC syndrome |
| | *HPS6* | Hermansky-Pudlak syndrome 6 | | |
| Signal transduction | *PLA2G4A* | Phospholipase A2 deficiency | *RGS2* | G-protein signaling |
| | *PTGS1* | Bleeding disorder, platelet type 12 | *TBXAS1* | Bleeding disorder, platelet type 14 |
| | *RASGRP2* | Bleeding disorder, platelet type 18 | | |
| Transcription factors | *CYCS* | Thrombocytopenia 4 | *HOXA11* | CTRUS syndrome |
| | *ETV6* | Thrombocytopenia 5 | *MECOM* | CTRUS syndrome |
| | *FLI1* | Bleeding disorder, platelet type 21 | *RBM8A* | TAR syndrome |
| | *GATA1* | GATA1-related disorder | *RUNX1* | FPD/AML |
| | *GFI1B* | Bleeding disorder, platelet type 17 | *STIM1* | Stormorken syndrome |
| Cytoskeletal and structural proteins | *ABCG5* | Sitosterolemia | *FYB* | CARST syndrome |
| | *ABCG8* | Sitosterolemia | *MASTL* | Thrombocytopenia 2 |
| | *ACTN1* | Bleeding disorder, platelet type 15 | *MYH9* | MYH9-related disorders |
| | *ANKRD26* | Thrombocytopenia 2 | *PRKACG* | Bleeding disorder, platelet type 19 |
| | *CDC42* | Takenouchi-Kosaki syndrome | *TUBB1* | TUBB1-related macrothrombocytopenia |
| | *FERMT3* | Leukocyte adhesion deficiency III | *WAS* | Wiskott-Aldrich syndrome |
| | *FLNA* | Filaminopathy | | |
| Collagen disorders | *COL1A1* | Ehlers-Danlos syndrome | *COL5A1* | Ehlers-Danlos syndrome |
| | *COL3A1* | Ehlers-Danlos syndrome | *COL5A2* | Ehlers-Danlos syndrome |
| Procoagulant disorders | *ANO6* | Scott syndrome | | |
| Blood vessel abnormalities | *ACVRL1* | Hereditary telangiectasia | *ENG* | Hereditary telangiectasia |
| Fibrinogen disorders | *FGA* | Dys/hypo/afibrinogenemia | *FGG* | Dys/hypo/afibrinogenemia |
| | *FGB* | Dys/hypo/afibrinogenemia | | |
| Other | *GBA* | Gaucher disease | *SLFN14* | Bleeding disorder, platelet type 20 |
| | *GNE* | GNE myopathy | *THPO* | Thrombocytemia 1 |
| | *MPL* | CAMT syndrome | *VWF* | von Willebrand disease |

ARC, arthrogryposis, renal dysfunction and cholestasis; CAMT, congenital amegakaryocytic thrombocytopenia; CARST, congenital autosomal recessive small-platelet thrombocytopenia; CTRUS, congenital thrombocytopenia with radioulnar synostosis; FPD/AML, familial platelet disorder with propensity to acute myelogenous leukemia; TAR, thrombocytopenia and absent radius; WES, whole exome sequencing.

**Table 2**. Results of laboratory and genetic testing per patient category

| Patient category | N | CPD | Possible CPD | Molecular diagnosis | VUS |
|---|---|---|---|---|---|
| Previously abnormal laboratory tests* | 96 | 61 (64) | 4 (4) | 8 (8) | 11 (11) |
| Previously normal LTA results# | 39 | 0 (0) | 10 (26) | 0 (0) | 1 (3) |
| Newly referred^ | 21 | 0 (0) | 6 (29) | 0(0) | 1 (5) |

Data are presented in number of patients (%). CPD, congenital platelet defect; LTA, light transmission aggregometry; VUS, variant of uncertain significance. * Patients suspected for a CPD based on previous abnormal platelet counts, LTA results or platelet ADP content without a molecular diagnosis. # Patients suspected for a CPD based on a predominantly mucocutaneous bleeding tendency compatible with a CPD, in whom other known causes of bleeding were excluded and in whom previous LTA results were normal. ^ Patients suspected for a CPD based on a predominantly mucocutaneous bleeding tendency compatible with a CPD, in whom other known causes of bleeding were excluded, newly referred for platelet function testing.

**Table 3.** Identified genetic variants in patients who received a molecular diagnosis

| Gene | Cases | Zygosity | Variant# | Protein change | Variant assessment^ | Platelet phenotype |
|---|---|---|---|---|---|---|
| *GATA1 (NM_002049)* | 1 | Hemi | c.647G>A[8] | p.Arg216Gln | Pathogenic | Thrombocytopenia, LTA not performed, ↓ADP content |
| *GP9 (NM_000174)* | 1 | Homo | c.182A>G[9] | p.Asn61Ser | Likely pathogenic | Macrothrombocytopenia, LTA not performed, ↓GP1b-V-IX expression |
| | 1 | Compound het | c.182A>G[9]; c.70T>C[10] | p.Asn61Ser; p.Cys24Arg | Likely pathogenic (both) | Macrothrombocytopenia, LTA not performed, ↓GP1b-V-IX expression |
| *P2RY12 (NM_176876)* | 1 | Het | c.772C>A[11] | p.Pro258Thr | Pathogenic | ↓ADP aggregation |
| | 1 | Het | c.293_294del[12] | p.Gln98fs | Likely pathogenic | ↓ADP aggregation |
| *RUNX1 (NM_001754)* | 1 | Het | c.610C>T[13] | p.Arg204* | Pathogenic | Microthrombocytopenia, ↓ADP content |
| | 1 | Het | c.602G>A[13] | p.Arg201Gln | Pathogenic | Thrombocytopenia, ↓ADP, AA, collagen aggregation, ↓ADP content |
| *SLFN14 (NM_001129820)* | 1 | Het | c.657A>C[14] | p.Lys219Asn | Pathogenic | Macrothrombocytopenia |

AA, arachidonic acid; ADP, adenosine diphosphate; Hemi, hemizygous; Het, heterozygous; Homo, homozygous; LTA, light transmission aggregometry; ↓, decreased. # References for previously reported genetic variants are depicted with numbers in square brackets. ^ Pathogenicity was assessed following the guidelines of the American College of Medical Genetics and Genomics[7].

## DISCUSSION

Genetic analysis can be useful to confirm a phenotypic diagnosis, to identify patients with increased risk for other medical conditions, such as hematological malignancies associated with *RUNX1* variants, and for genetic counseling of patients and family members. However, the timing of genetic analysis remains uncertain. In this study, we included several subgroups of patients suspected of having a CPD to properly assess when genetic analysis should be performed in the diagnostic procedure. Our study shows that the diagnostic yield of genetic analysis is limited in patients suspected for a CPD, since only 5% (8/156) of patients received a molecular diagnosis.

This is in contrast to the diagnostic rate of 47.8% for platelet count defects and 26.1% for platelet function defects reported in a recent study. There, 2396 patients with bleeding, thrombotic, and platelet disorders (BTPD) were screened with a panel of 96 BTPD-associated genes, in which the number of platelet associated genes was similar to our gene panel[15]. However, their diagnostic rate included variants of unknown significance, resulting in an overestimation. Leaving out variants of unknown significance strongly reduced the diagnostic rate. The differences between their and our study are also related to patient-selection. Our study reflects the real-life population of patients suspected for a CPD referred to outpatient clinics of hemophilia treatment centers. Their study included patients with a previously ascertained pathogenic variant, or patients with phenotypes strongly indicative of a particular disorder on the basis of laboratory abnormalities, with a high likelihood of having an inherited BTPD. In patients with either normal laboratory assays or assays not diagnostic of an established disorder, they reported a diagnostic rate of only 3.2%. Studies performed in the Iberian Peninsula, with a gene panel similar to ours, reported the identification of a molecular defect in 40%[16] and 68%[5] of patients with (suspected) CPDs. Their studies included large numbers of patients with Glanzmann thrombasthenia, Bernard-Soulier syndrome and MYH9-related disorders. Therefore, their study population does not reflect clinical practice and is not comparable to ours. A study performed in a pediatric population reported a positive molecular diagnosis in 23.8%[17]. Their cohort included a relatively large number of patients with thrombocytopenia (67% vs 22% in our cohort) and genetic testing was not performed as a first-line investigation.

We identified 16 possibly damaging variants of unknown significance in 9 different genes. However, these variants cannot be used in clinical practice in terms of patient management and treatment and although a causal relation between the mutations and the bleeding phenotype was plausible, family studies and functional or structural studies should be performed to determine whether these variants actually affect platelet function and contribute to the bleeding phenotype of these patients.

It is possible that limitations of WES have led to an underestimation of the number of patients with an identified genetic variant. First, large insertions and deletions might be missed. Second, regulatory and non-coding regions of the genome were not examined, and these regions might harbor variants essential for controlling transcriptional regulation or splicing. Third, by using

a selected gene panel we might have missed pathogenic variants in genes not included in the panel. Finally, we cannot exclude that an additive effect of multiple genetic variants that have escaped our selection, might underlie the CPD in individual patients.

In conclusion, genetic testing with a selected gene panel has limited diagnostic yield in patients suspected for a CPD and should only be performed in patients in whom a platelet number or function defect is confirmed.

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7

# CONGENITAL PLATELET DISORDERS AND HEALTH STATUS-RELATED QUALITY OF LIFE

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## SUMMARY

**Background**

Patients with congenital blood platelet disorders (CPD) demonstrate a predominantly mucocutaneous bleeding tendency. Repeated bleeds throughout life can have a significant impact on health status-related quality of life (HR-QoL), but few studies have investigated HR-QoL in patients with CPDs.

**Objectives**

To determine HR-QoL in patients with suspected or confirmed CPDs as compared with the general Dutch population and to assess the association between bleeding phenotype and HR-QoL.

**Methods**

Data were derived from the 'Thrombocytopathy in the Netherlands' (TiN) study, a cross-sectional study of individuals suspected for a congenital platelet defect. TiN patients with an increased ISTH Bleeding Assessment Tool (ISTH-BAT) score (>3 in adult men and >5 in adult women) were included for analysis. HR-QoL was assessed with the Short Form (SF)-36 survey. Bleeding symptoms were evaluated with the ISTH-BAT, resulting in a bleeding score.

**Results**

One hundred and fifty-six patients were analyzed of whom 126 (81%) were women. Sixty-two patients (40%) had a confirmed CPD. Compared to the general Dutch population, patients with a suspected or confirmed CPD reported decreased physical functioning, limitations in daily activities due to physical health problems, limitations in social activities, decreased energy levels and fatigue, pain and lower general health status. HR-QoL was not correlated with the ISTH-BAT score and was similar in patients with a confirmed CPD and those in whom a CPD could not be diagnosed.

**Conclusion**

A bleeding tendency in patients with a suspected or confirmed CPD significantly impacts HR-QoL, independent of a confirmed explanatory diagnosis.

# INTRODUCTION

Congenital blood platelet disorders (CPD) are disorders of primary hemostasis and can be due to defects in the adhesion, activation, secretion or aggregation of platelets[1]. Patients typically present with mucocutaneous bleeds or persistent bleeding following a hemostatic challenge such as dental extraction, invasive procedures or childbirth[2]. Although a study on the overall prevalence of CPDs has never been undertaken, it is suggested that it is similar to that of von Willebrand disease (VWD)[3].

Repeated bleeds throughout life can have a significant impact on quality of life, since it can hinder activities of daily living, social functioning and educational achievements. Health status-related quality of life (HR-QoL) is a multidimensional concept for evaluating the physical, mental, emotional and social health of an individual[4]. Assessment of HR-QoL has become increasingly important in patient-centered care, since it provides valuable information on the impact of the disease on daily activities and can guide clinical decision-making[5]. In contrast to hemophilia and VWD[6-9], information on HR-QoL in patients with CPDs is lacking.

The aim of the present study was to assess HR-QoL in adult patients with suspected or confirmed CPDs as compared with the general Dutch population and to study the association between HR-QoL and bleeding phenotype. To our knowledge, this is the first study on HR-QoL in a large cohort of patients with suspected or confirmed CPDs.

# METHODS



## 'Thrombocytopathy in the Netherlands' study

Data were derived from the 'Thrombocytopathy in the Netherlands' (TiN) study. The TiN study is a nationwide cross-sectional study to collect data on clinical characteristics, functional assays and genetics in a real-life population of patients with suspected or confirmed congenital platelet disorders. Inclusion and exclusion criteria of the TiN study can be found in the supplementary material. In all included patients, laboratory tests were performed for platelet count, aggregation to 4 agonists (ADP, arachidonic acid, collagen, ristocetin), platelet ADP and ATP content, mepacrine staining, surface receptor expression with flow cytometry and genetic analysis with a selected primary hemostasis gene panel. Platelet morphology, grey platelets and leukocyte inclusion bodies were assessed in a peripheral blood smear.

## Participant selection

For the current study, we evaluated data from TiN patients with a bleeding tendency defined as an increased ISTH bleeding assessment tool (ISTH-BAT) score (>3 in adult men and >5 in adult women [10]). We included new patients suspected for a CPD, as well as patients with a previously confirmed CPD. These patients are referred to as 'study group patients'. We excluded patients diagnosed with an acquired platelet defect.

## ISTH Bleeding Assessment Tool

The bleeding phenotype was assessed with the ISTH-BAT, administered by experienced physicians. The ISTH-BAT systematically evaluates 14 different bleeding symptoms, scored on a scale ranging from 0 to 4 points, and results in an ISTH-BAT bleeding score[11]. Higher scores indicate a more severe bleeding phenotype.

## Short Form-36

HR-QoL was assessed with the Dutch version of the Short Form (SF)-36 survey. The SF-36 is a generic measure assessing 8 physical and mental health domains: physical and social functioning, role limitations due to physical or emotional problems, general health, mental health, bodily pain and vitality (Table S1)[12, 13]. For each domain, scores are converted to a 0-100 scale, with higher values reflecting a better quality of life. Control data from the general Dutch population were obtained from a nationwide health status survey[14].

## Brief Illness Perception Questionnaire

The Dutch language version of the Brief Illness Perception Questionnaire (B-IPQ) was used to assess the cognitive and emotional representations of illness. The nine dimensions of the B-IPQ include consequences, timeline, personal control, treatment control, identity, concern, understanding, emotional response and causal factors (Table S2). All of the dimensions except causal factors are rated on a linear scale from 0 to 10. Higher scores reflect a more negative (unfavorable) illness perception , except for the dimensions personal control, treatment control and understanding, where a higher score indicates a more positive (favorable) illness perception[15]. Control data from the general Dutch population were not available.

## Definitions

The term 'study group patients' refers to all patients included in the current study, i.e. new patients suspected for a CPD where a diagnosis could not be confirmed, as well as patients with a confirmed CPD. In the TiN study, a confirmed CPD was diagnosed when abnormal platelet function was found on at least 2 occasions, of which one was in our diagnostic laboratory. These patients are referred to as 'confirmed CPD patients'. A bleeding tendency was defined as an increased ISTH bleeding assessment tool (ISTH-BAT) score (>3 in adult men and >5 in adult women [10]). Comorbidity was defined as the presence of one or more conditions with a duration of at least 6 months in the last year[16] and was self-reported .

## Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics 25 and RStudio version 0.99. Descriptive results were presented as medians (IQR) for continuous data and frequencies (percentages) for categorical data. Differences in SF-36 scores between study group patients and the general Dutch population were evaluated with linear regression analyses adjusted for

age, sex and comorbidity as other determinants of HR-QoL. For each SF-36 domain, a linear regression was performed, with the SF-36 domain as outcome and group (general population or study group patient) as determinant. A significance level of P≤0.01 was used to correct for multiple comparisons. Correlations between SF-36 domains and the ISTH-BAT score (as a proxy for bleeding phenotype), between B-IPQ dimensions and the ISTH-BAT score and between B-IPQ dimensions and the SF-36 domain general health perception were calculated with non-parametric Spearman's rank correlation. Correlation coefficients (ρ) of 0.20-0.39 were considered weak, 0.40-0.59 moderate, 0.60-0.79 strong and >0.8 very strong[17]. Only moderate or stronger correlations were considered relevant.

## RESULTS

### Patient characteristics

A total of 200 patients were included in the TiN cohort, of whom 31 did not have an objective bleeding tendency and 6 were diagnosed with an acquired platelet defect. Thus, 163 patients with a suspected or confirmed CPD and an objective bleeding tendency were included for the current analysis, of whom 156 patients completed the questionnaire and were included in the study group. Patient characteristics are shown in Table 1. The majority of patients were women (81%). The median age was 44 years for women (IQR 31-55) and 45 years for men (IQR 31-61). The median ISTH-BAT score was 11 for women (IQR 9-14) and 10 for men (IQR 7-13). A CPD was confirmed in 62/156 patients (40%). The most commonly observed comorbidities were hypertension (17%), type 2 diabetes mellitus (6%) and endometriosis (4%).



**Table 1.** Patient characteristics

| | | General Dutch population* *n* = 1742 | Study group patients# *n* = 156 | Confirmed CPD patients^ *n* = 62 |
|---|---|---|---|---|
| Sex | Women, n (%) | 761 (44) | 126 (81) | 41 (66) |
| Age | Men, median (IQR) | 49 (36-63) | 45 (31-61) | 53 (32-63) |
| | Women, median (IQR) | 41 (30-60) | 44 (31-55) | 45 (32-57) |
| Bleeding score | Men, median (IQR) | NA | 10 (7-13) | 10 (6-14) |
| | Women, median (IQR) | NA | 11 (9-14) | 11 (9-17) |
| Comorbidity | Yes, n (%) | 856 (49) | 92 (59) | 34 (55) |

CPD: congenital blood platelet disorder; IQR: interquartile range; NA: not available. * See reference 14. # Patients included in the study with a suspected or confirmed CPD. ^ Study group patients in whom a CPD was confirmed.

## Health-related quality of life

Compared to the general Dutch population, study group patients reported decreased physical functioning and limitations in the type or amount of regular daily activities due to physical health problems. They also reported limitations in social activities, decreased energy levels and fatigue, pain and lower general health status (Figure 1 and Table S3). HR-QoL was similar in patients with a confirmed CPD and those in whom a CPD could not be diagnosed (Table S4). Women reported significantly more pain than men. The other domain scores were not significantly different between men and women.

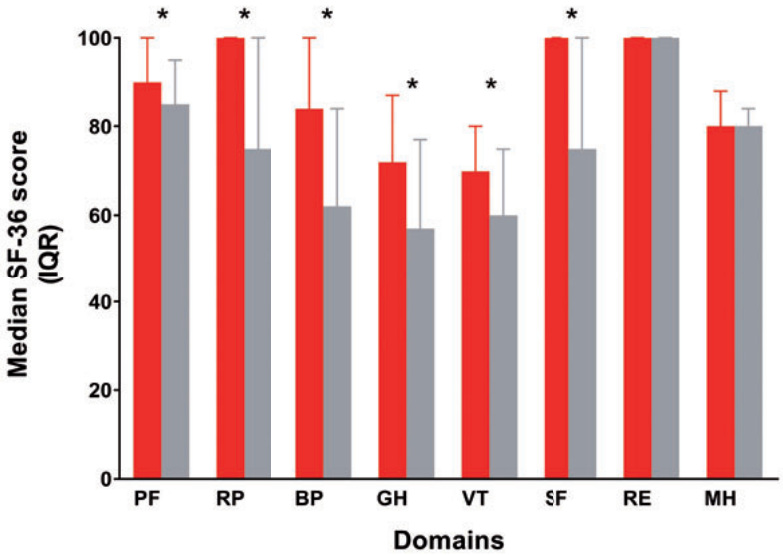


**Figure 1.** Health status-related quality of life. Differences in SF-36 domain scores between study group patients (grey) and the general Dutch population (red) were evaluated with linear regression analysis adjusted for age, sex and comorbidity. BP, bodily pain; GH, general health status; IQR, interquartile range; MH, mental health; PF, physical functioning; RE, role limitations due to emotional problems; RP: role limitations due to physical health problems; SF, social functioning; SF-36, Short Form-36; VT, vitality. Error bars represent the interquartile range. * P-value ≤0.01

## Association between HR-QoL and bleeding phenotype

The ISTH-BAT score, as a proxy for bleeding phenotype, was not correlated with any of the SF-36 domains (Table S5) nor with any of the B-IPQ dimensions (Table S6).

## Brief Illness Perception Questionnaire

Perceiving more consequences of bleeds, perceiving more physical complaints due to bleeds, perceiving more concerns about bleeds and perceiving a more extreme emotional response to bleeds were associated with lower general health perception (Table 2).

**Table 2.** Correlation between B-IPQ dimensions and SF-36 domain general health perception

| B-IPQ dimension | Spearman's ρ* |
|---|---|
| Consequences | -0.52 |
| Timeline | 0.12 |
| Personal control | 0.08 |
| Treatment control | 0.07 |
| Identity | -0.43 |
| Concern | -0.54 |
| Understanding | 0.14 |
| Emotional response | -0.46 |

Interpretation: Perceiving more consequences of bleeds is associated with lower general health perception. B-IPQ, Brief Illness Perception Questionnaire; SF-36, Short Form-36. * Correlation coefficients of ≥0.40 and ≤-0.40 were considered relevant.

## DISCUSSION

This is the first study on HR-QoL in a large cohort of patients with suspected or confirmed CPDs. Our patients reported decreased physical functioning, limitations in the type or amount of regular daily activities due to physical health problems, limitations in social activities, decreased energy levels and fatigue, pain and lower general health status as compared with the general Dutch population. HR-QoL was similar in patients with a confirmed CPD and those in whom a CPD could not be diagnosed and was not associated with the ISTH-BAT score. More negative illness perceptions were related to lower general health perceptions.

The domain general health reflects the perception of general health status. Patients with a suspected or confirmed CPD may believe their health is poor and likely to get worse due to recurrent bleeds and hospital visits. This might especially be true for patients without a definitive diagnosis, due to insecurity about their current and future health status. Also, patients perceiving more consequences of bleeds, more physical complaints due to bleeds, more concerns about bleeds or a more extreme emotional response to bleeds experienced lower general health status. The domain vitality measures energy levels and fatigue. Especially women with CPDs might experience fatigue due the development of iron-deficient anemia as a consequence of menorrhagia. The decrease in energy levels could also account for lower levels of social activity. The lower scores in the domains physical functioning, role physical and bodily pain are more difficult to explain, as in general, mucocutaneous bleeds do not induce physical impairment or pain. It could not be explained by the bleeding phenotype, since there was no correlation between the SF-36 domain scores and the ISTH-BAT score. Possibly, HR-QoL scores were influenced by illness perception. It is generally understood that HR-QoL and illness perception are related[18-20] and we found negative correlations between the SF-36 domain general health perception and the B-IPQ domains consequences, identity, concern and emotional

response. Possibly, patients were anxious to exercise because they feared bleeds and therefore felt physically impaired.

Previous studies on HR-QoL in patients with bleeding disorders mostly focused on patients with von Willebrand disease and hemophilia or on women with menstrual disorders[21-24]. Only a few studies included a limited number of patients with CPDs[25, 26]. These studies used other questionnaires than the SF-36 to assess HR-QoL and are therefore not comparable. Patients with VWD, another primary hemostasis defect with similar clinical characteristics, reported a reduced HR-QoL for the domains general health and vitality[9]. HR-QoL in patients with VWD was mainly affected by acute bleeds. This is in contrast to hemophilia where HR-QoL depends on long-term effects, such as orthopedic status, comorbidities and hepatitis C infection[27, 28] and where the domains physical functioning and general health were most affected[7].

There was a risk for selection bias. There is no database or registry for congenital platelet disorders in the Netherlands. Patients included in the study were mainly patients who recently visited a hemophilia treatment center. Besides that, patients with a more severe bleeding phenotype were perhaps more willing to participate in the study. Therefore, milder types of CPDs are likely underrepresented in our study population. This could explain the lower HR-QoL in our patients as compared to the general population. However, in our analysis HR-QoL was not correlated with the bleeding phenotype.

We reported no correlation between the ISTH-BAT score and domains of the SF-36. A possible explanation for this finding is that the ISTH-BAT score and HR-QoL capture different time frames: the ISTH-BAT score reflects symptoms throughout one's entire life, regardless of the symptoms still being present, while the SF-36 measures someone's health status in the preceding weeks to months. A patient with a high ISTH-BAT score due to severe bleeding problems in the past can have a relatively good quality of life due to the absence of bleedings in the past year. Another possible explanation is that only patients with an increased ISTH-BAT score were included and, as mentioned before, milder types of CPDs with lower ISTH-BAT scores were likely underrepresented in our study population. As a result, the calculated correlation coefficient might not reflect the true correlation between ISTH-BAT score and HR-QoL, since certain data are missing.

A major challenge of HR-QoL evaluation is the interpretation of the observed differences. A difference of 3-5 points has been considered clinically relevant [29]. However, although scores for the SF-36 domains range from 0-100, for some domains the score range is limited due to dichotomous answers and therefore it does not seem defensible to use this 3-5 point differences for all domains.

Evaluating HR-QoL has become increasingly important in patient-centered care, because it will help to comprehend the patient's perspective on the disease. Our patients showed an impaired HR-QoL independent of a confirmed CPD, indicating that a bleeding tendency is a considerable health problem on its own. Even after extensive laboratory testing, as advised by the ISTH[30], many patients remain without a definitive diagnosis due to the complexity of

platelet function testing. Accurately informing patients about their condition and treatment options and creating awareness among other physicians might improve the HR-QoL of patients with a bleeding disorder, even those without a definitive diagnosis. In addition, proper counseling of patients may modify the patient's perception of illness, which could improve their HR-QoL.

For future studies, it could be interesting to assess changes in HR-QoL from time of initial diagnosis, to explore whether education on the disease will benefit these patients. Since different populations may vary in their perception of health and associated comorbidities, it will be useful to conduct similar studies in other populations.

In conclusion, our study showed that a bleeding tendency in patients with a suspected or confirmed CPD significantly impacts HR-QoL, independent of a confirmed explanatory diagnosis and that this patient group is one to care for.

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## SUPPLEMENTARY TABLES

**Table S1.** Domains of the SF-36

| Domain | Explanation |
|---|---|
| *Physical domains* | |
| Physical functioning | Limitations in daily activities |
| Role physical | Limitations in the type or amount of regular daily activities due to physical health problems |
| Bodily pain | Presence of pain and its interference with normal activities |
| General health | Perception of general health status |
| *Mental domains* | |
| Vitality | Energy levels and fatigue |
| Social functioning | Limitations in social activities |
| Role emotional | Limitations in the type or amount of regular daily activities due to emotional problems |
| Mental health | Psychological distress |

**Table S2.** Dimensions of the Brief IPQ

| Item | Question |
|---|---|
| *Cognitive illness perception* | |
| Consequences | How much does your illness affect your life? |
| Timeline | How long do you think your illness will continue? |
| Personal control | How much control do you feel you have over your illness? |
| Treatment control | How much do you think your treatment can help your illness? |
| Identity | How much do you experience symptoms from your illness? |
| *Emotional illness perception* | |
| Concern | How concerned are you about your illness? |
| Emotional response | How much does your illness affect you emotionally? (e.g. does it make you angry, scared, upset or depressed?) |
| *Illness comprehensibility* | |
| Understanding | How well do you feel you understand your illness? |
| *Causal representation* | |
| Causal factors | List in rank-order the three most important factors that you believe caused your illness |

**Table S3.** Health status-related quality of life: linear regression analyses with the SF-36 domains as outcome and suspected or confirmed CPD (compared to general population) as determinant

| | General population (n=1742) | Study group patients (n=156) | Adjusted difference B [95% CI]* | P value# |
|---|---|---|---|---|
| Physical functioning | 90 (75 to 100) | 85 (61 to 95) | -5 [-8 to -2] | <0.01 |
| Role limitations due to physical functioning | 100 (50 to 100) | 75 (13 to 100) | -14 [-20 to -8] | <0.01 |
| Bodily pain | 84 (62 to 100) | 62 (42 to 84) | -8 [-11 to -4] | <0.01 |
| General health perception | 72 (60 to 87) | 57 (35 to 77) | -15 [-18 to -12] | <0.01 |
| Vitality | 70 (60 to 80) | 60 (40 to 75) | -8 [-12 to -5] | <0.01 |
| Social functioning | 100 (75 to 100) | 75 (63 to 100) | -10 [-14 to -7] | <0.01 |
| Role limitations due to emotional problems | 100 (67 to 100) | 100 (67 to 100) | 1 [-5 to 6] | n.s. |
| Mental health | 80 (68 to 88) | 80 (68 to 84) | 0 [-3 to 3] | n.s. |

Health status-related quality of life is reported in medians (IQR). CPD, congenital blood platelet disorder; N.s., not significant. Interpretation: the score for physical functioning is 5 points lower in study group patients as compared to the general Dutch population. * Adjusted for age, sex and comorbidity. # A significance level of P≤0.01 was used to correct for multiple comparisons

**Table S4.** Health status-related quality of life: linear regression analyses with the SF-36 domains as outcome and confirmed CPD (compared to no CPD) as determinant

| | Confirmed CPD (n=62) | No CPD (n=94) | Adjusted difference B* [95% CI] | P value# |
|---|---|---|---|---|
| Physical functioning | 85 (60 to 100) | 85 (65 to 95) | -3 [-10 to 4] | n.s. |
| Role limitations due to physical functioning | 75 (19 to 100) | 75 (0 to 100) | 0 [-13 to 14] | n.s. |
| Bodily pain | 62 (51 to 100) | 62 (41 to 84) | 3 [-5 to 11] | n.s. |
| General health perception | 52 (27 to 73) | 57 (40 to 77) | -8 [-15 to 0] | n.s. |
| Vitality | 58 (40 to 75) | 60 (42 to 75) | -6 [-12 to 1] | n.s. |
| Social functioning | 69 (59 to 88) | 75 (63 to 100) | -10 [-18 to -2] | n.s. |
| Role limitations due to emotional problems | 100 (67 to 100) | 100 (100 to 100) | -9 [-21 to 3] | n.s. |
| Mental health | 76 (63 to 84) | 80 (68 to 84) | -4 [-9 to 2] | n.s. |

Health status-related quality of life is reported in medians (IQR). CPD, congenital blood platelet disorder; N.s., not significant. Interpretation: the score for physical functioning is similar in patients with and without a confirmed CPD. * Adjusted for age, sex and comorbidity. # A significance level of P≤0.01 was used to correct for multiple comparisons

**Table S5.** Correlation between SF-36 domains and ISTH-BAT score.

| SF-36 domain | Spearman's ρ* |
|---|---|
| Physical functioning | -0.37 |
| Role physical | -0.30 |
| Bodily pain | -0.25 |
| General health perception | -0.25 |
| Vitality | -0.10 |
| Social functioning | -0.17 |
| Role emotional | -0.02 |
| Mental health | -0.04 |

* Correlation coefficients of ≥0.40 and ≤-0.40 were considered relevant.

**Table S6.** Correlation between B-IPQ dimensions and general health perception.

| B-IPQ dimension | Spearman's ρ* |
|---|---|
| Consequences | -0.52 |
| Timeline | 0.12 |
| Personal control | 0.08 |
| Treatment control | 0.07 |
| Identity | -0.43 |
| Concern | -0.54 |
| Understanding | 0.14 |
| Emotional response | -0.46 |

* Correlation coefficients of ≥0.40 and ≤-0.40 were considered relevant. Interpretation: Perceiving more consequences of bleeding is associated with lower general health perception

# 8

# SUMMARY AND DISCUSSION

# SUMMARY

Congenital platelet defects (CPDs) are rare disorders of primary hemostasis. Clinical characteristics and burden of disease are poorly described for CPD patients. Next to that, the current diagnostic tools lack sensitivity and specificity for CPDs. Hence, we are in need of improved platelet function diagnostics. This thesis aimed to gain insight into the bleeding phenotype and health-status related quality of life (HR-QoL) of patients with CPDs and to evaluate the diagnostic yield of advanced diagnostic tests. The study population consisted of patients who had been recruited for the 'Thrombocytopathy in the Netherlands' (TiN) study, the first large nationwide study on CPDs.

**Chapter 2** reviewed the currently available diagnostic tests for platelet function and their pitfalls. The laboratory diagnostics entail many different techniques and these are often insufficient to diagnose mild CPDs. We described flow cytometry and whole-exome sequencing (WES) as promising additional tests for diagnosing CPDs. In **Chapter 3**, we evaluated the bleeding phenotype and diagnostic characteristics of CPD patients. The most common diagnostic subgroups were ADP and thromboxane A2 pathway defects and isolated thrombocytopenia. The most common bleeding symptoms were heavy menstrual bleeding (HMB) and bleeds after a hemostatic challenge, such as surgery or childbirth. Glanzmann thrombasthenia and female sex were associated with a more severe bleeding phenotype. In **Chapter 4-6** we evaluated improved and advanced diagnostic tests. **Chapter 4** showed that the self-administered ISTH bleeding assessment tool (self-BAT) was sufficiently reliable and feasible to detect a bleeding tendency in patients with (suspected) CPDs. This supports the use of the self-BAT as a screening tool. A self-BAT completed at home would save valuable time during an outpatient clinic visit and enhance screening before referral to the hemoaphilia treatment center for a suspected CPD. The diagnostic accuracy of mepacrine fluorescence measured on the flow cytometer for storage pool disease was evaluated in **Chapter 5**. This test showed good discriminative ability and, although the sensitivity and specificity were moderate, we showed that this test can be used to exclude storage pool disease in patients suspected for a CPD. A DNA-based approach has been proposed to increase the diagnostic rate of CPDs. Genetic analysis can provide accurate diagnoses and is proposed to be performed as a first-line investigation. However, **Chapter 6** showed a limited diagnostic yield of genetic testing with a selected gene panel in patients suspected for a CPD. We recommended to not perform genetic analysis as a first-line investigation, but only in patients in whom a CPD is confirmed. **Chapter 7** reported the HR-QoL of patients with suspected or confirmed CPDs. We showed that HR-QoL is significantly impaired in suspected or confirmed CPD patients as compared to the general Dutch population and that this patient group is one to care for.

Taken together, this thesis contributed to our understanding of CPDs. We gained insight into the clinical characteristics and burden of disease of patients with CPDs and showed the advantages and limitations of advanced diagnostic tests. In the near future, we should continue

to put these patients in the spotlight in order to create awareness among health professionals and society and we should continue to gain insight into platelet pathophysiology and molecular mechanisms in order to improve the diagnostic work-up for CPDs.

## DISCUSSION

### Clinical features

CPDs comprise disorders of platelet production, resulting in a low platelet count and alterations in platelet morphology, disorders of platelet function, resulting in a defective platelet response to stimuli, or a combination of both. As a result, platelets fail to fulfil their critical role in primary hemostasis. The term 'congenital' implies that the condition is present at birth. However, not all patients present with bleeding symptoms in childhood. In fact, patients with mild CPDs typically present only after an appropriate hemostatic challenge[1,2].

In our study population of patients with CPDs, bleeds after a hemostatic challenge frequently occurred, as is shown in **Chapter 3**. This chapter also shows the clinical heterogeneity of CPDs. Although HMB and post-partum hemorrhage occured most frequently, a wide variety of bleeding symptoms was reported: eight different bleeding symptoms were reported with a frequency exceeding 40%. This clinical heterogeneity hampers the identification of patients and requires close collaboration between all health professionals involved in the care of these patients. The first step towards better identification of these patients is to create awareness and knowledge of abnormal bleeding symptoms. Studies showed that about 20% of women with HMB have an underlying bleeding disorder[3] and that in 15% of adult patients with a bleeding disorder, HMB is the only symptom reported[4]. The use of a bleeding assessment tool (BAT) is strongly encouraged to standardize evaluation and documentation of bleeding symptoms and to distinguish normal from abnormal bleeding. However, to our knowledge the BAT is not routinely used by doctors other than hematologists, so there is much to be gained there. Preferably, the BAT is completed for all patients presenting with bleeding symptoms in primary and secondary care to detect a bleeding tendency early on. Further research is required to assess the feasibility and the discriminating power of the BAT in these settings.

### Burden of disease

Evaluating a patient's health status-related quality of life (HR-QoL) has become increasingly important, because it will help to comprehend the patient's perspective on the disease. Even so, little is known about the burden of disease of CPDs. In **Chapter 7**, we performed the first study on HR-QoL in a large cohort of patients with suspected or confirmed CPDs. HR-QoL was assessed with the Short Form (SF)-36 survey. Our patients had decreased scores for several domains of the SF-36, including general health status, vitality and social functioning, as compared to the general Dutch population. This observation was independent of a confirmed CPD, indicating that a bleeding tendency is a considerable health problem on its own.

Several factors may contribute to this high burden. First, the knowledge regarding CPDs is limited, in contrast to hemophilia and von Willebrand disease. Although at least 42 types of CPDs have been identified[5], only a few types have been well characterized. The most well-known disorders are Bernard Soulier syndrome (BSS), Glanzmann thrombasthenia (GT) and storage pool disease (SPD). Other types of CPDs are not well defined and a precise definition is only available for a minority. Even the website from the World Federation of Hemophilia only addresses BSS, GT and SPD as types of CPDs[6]. Lack of awareness in primary and secondary care might cause late or missing referrals, resulting in underdiagnosis and undertreatment.

Second, due to underdiagnosis and insufficient platelet function assays, there is a significant diagnostic delay. For some patients it takes months or even years before a final diagnosis is made and many patients remain without a definitive diagnosis, even after extensive laboratory testing, due to the complexity of platelet function testing. Without a proper diagnosis, there is uncertainty about the correct treatment options and prognosis of the disease. Insecurity about their current and future health status and fear of bleeds could result in a decreased HR-QoL in patients with CPDs. In addition, patients might feel unheard and misunderstood. Furthermore, misdiagnosis of patients might lead to unnecessary or potentially harmful treatments. Improvement in the diagnostic work-up for CPDs is therefore much needed.

Third, there is a burden to the patients' health status. Frequent bleeds may require frequent hospital visits. In addition, some patients are frequently calling in sick to work and may become socially isolated. Activities of daily living and educational achievements may be hindered. Thus, CPDs take a toll on the patient, their families and their careers.

Finally, we found that patient's HR-QoL was influenced by their illness perceptions. Patients perceiving more consequences of bleeds or more concerns about bleeds experienced a lower general well-being. This is a clinically important finding, since illness perception may be modifiable through patient counseling. Accurately informating patients about their condition and treatment options may be helpful to improve their quality of life.

We assessed the burden of disease with the SF-36, a generic questionnaire. The advantage of a generic questionnaire is that data can be used in comparison with other conditions and with the general population. A disadvantage is that we were not able to assess the disease-specific impact on HR-QoL. Since most of the SF-36 domains assess complaints over a short period of time (during the past 4 weeks), impact of bleeds on HR-QoL is not well taken into account. Unfortunately, no disease-specific questionnaire is available for CPDs or disorders of primary hemostasis.

## Diagnostic tests

Identification of CPDs is challenging due to the complexity of platelet function and consequently, the large diversity of platelet defects. Diagnosing severe CPDs is relatively straightforward, because these patients present with distinct clinical and laboratory features that can readily be detected with the currently available diagnostic tools (**Chapter 2**). Diagnosing mild CPDs

is more challenging due to the heterogeneous phenotype. Furthermore, the etiology is often multifactorial and the current tests lack sensitivity to detect these mild disorders[7].

In 2015, the International Society for Thrombosis and Hemostasis (ISTH) developed an international guideline for the diagnosis of CPDs containing several sequential steps[8]. The first step in the diagnostic approach is to evaluate whether bleeding symptoms are present. The ISTH-BAT was designed to aid in distinguishing normal from abnormal bleeding, to grade the bleeding severity and to guide further laboratory investigation in patients with suspected bleeding disorders[9,10]. A few studies have assessed the performance of the ISTH-BAT in patients with a bleeding tendency and they reported that patients with platelet disorders had significantly higher BAT scores compared to those without [11-14]. The ISTH-BAT displays a high negative predictive value[11,12], which makes it useful in excluding a bleeding disorder. However, high bleeding scores should be interpreted with caution. One study showed that almost 25% of young healthy women experienced two or more bleeding symptoms, thus generating a high bleeding score in the absence of clinically important bleeds[15]. In addition, high scores cannot differentiate between CPDs and von Willebrand disease or between different types of CPDs. Currently, the ISTH-BAT is used as a screening tool to assess whether additional laboratory testing is warranted.

The ISTH-BAT is a physician-administered questionnaire and completing the questionnaire requires time and expertise. If patients can complete the BAT at home, this would save valuable time during an outpatient clinic visit and would enhance screening for a suspected CPD. **Chapter 4** shows that the self-BAT is reliable to detect a bleeding tendency, with a sensitivity of 96.9%, which supports its use as a screening tool. The self-BAT cannot completely replace the assessment of bleeding symptoms by a physician, since patients can interpret normal bleeds as abnormal and vice versa. To overcome this problem, the self-BAT can be discussed during consultation and can be adjusted accordingly. This would still be more time efficient than the current ISTH-BAT. To date, the ISTH-BAT is mostly used by hematologists in tertiary centers. The next step would be to expand its use to primary and secondary care centers and possibly avoid unnecessary referrals to a tertiary hemophilia treatment center. However, further research is needed to assess the potential use of the self-BAT in these settings and to evaluate the possible overdiagnosis and medicalization due to the moderate specificity of the self-BAT.

Laboratory diagnostics for CPDs entail many different methods, including assessment of platelet count and morphology, platelet function assays and methods evaluating platelet surface markers and platelet granule content[16]. Light transmission aggregometry (LTA) is currently the most used method to assess platelet function[7]. The main advantage of LTA is that platelet aggregation in response to multiple concentrations of different agonists can be studied. However, LTA has several diagnostic disadvantages: there is, despite considerable efforts, poor consensus about which agonists and concentrations should be used, explaining the poor inter-laboratory agreement[17,18]. In addition, it is labor intensive and time-consuming, requires large volumes of blood and is not feasible nor reliable in thrombocytopenic samples[19]. Moreover, it is not

sensitive for mild platelet disorders[20] and can provide false negative results in patients with storage pool disease (SPD)[21,22].

Since LTA can display normal results in patients with SPD, additional methods are required to evaluate patients with possible platelet secretion disorders. Currently, the most used method is the measurement of ADP and ATP using bioluminescence, either with lumiaggregometry or in platelet lysate[7]. Both methods are time-consuming and cannot be performed in patienst with thrombocytopenia. In **Chapter 5** we evaluated the performance of flow cytometric mepacrine fluorescence as a diagnostic test for SPD. Advantages of this method are that it requires only a small amount of blood and thrombocytopenia is not a significant barrier to accurate testing. We reported that mepacrine fluorescence has good discriminative ability. In addition, the test showed a high negative predictive value and a low negative likelihood ratio, indicating that mepacrine fluorescence can be used to exclude SPD in patients with suspected CPDs. Many diagnostic laboratories do not measure platelet nucleotide content routinely, potentially resulting in underdiagnosis of SPD[23,24]. By adding this rapid flow cytometry method to the diagnostic work-up for CPDs, the diagnostic rate of CPDs could be increased, especially if this test can be performed in local laboratories.

Despite costly and lengthly laboratory evaluation, a significant percentage of patients with a history of abnormal bleeding has normal or nondiagnostic results[25,26]. Genetic investigation with high- throughput techniques, such as next-generation sequencing, has facilitated the identification of previously unknown disease-causing genes, has improved the molecular characterization of CPDs and has contributed to a better understanding of the complexity of platelet function. DNA-based analysis is evolving from a primarily confirmatory test to a powerful tool for the detection of CPDs[27]. It could become the first diagnostic approach and increase the diagnostic rate of CPDs without the need for complex platelet function assays. Main advantages are the requirement of only a small amount of blood and little risk of pre-analytical artefacts. Unfortunately, **Chapter 6** showed that the diagnostic yield of genetic analysis is limited in patients suspected for CPDs and is not suitable as a first-line investigation in patients referred with abnormal bleeding symptoms. Identifying the gene and variant contributing to the bleeding phenotype among multiple possible variants remains challenging and clinical characterization and functional studies are essential for the interpretation of genetic test results. In addition, in mild CPDs, the bleeding tendency is often multifactorial and caused by additive effects of multiple genetic variants. Thus, the added value of upfront genetic analysis is limited and phenotypic characterization of platelets by non-genetic methods might be sufficient to guide clinical management. For now, we recommend to consider performing genetic analysis for:

- Patients with clear phenotypic disorders, such as GT and BSS. Genetic analysis can be used to confirm the diagnosis.
- Patients with disease manifestations beyond the hemostatic system, such as MYH9-related disorders, gray platelet syndrome and Hermansky-Pudlak syndrome. Molecular characterization of these disorders can aid in detecting serious consequences in a timely

manner. For example, progression of renal disease in MYH9-related disorders can be delayed by treatment with angiotensin converting enzyme inhibitors[28].

- Families with a bleeding tendency and a distinct platelet phenotype, in order to identify the underlying (novel) genetic mutation. Preferably, affected and unaffected family members are taken into account.
- Patients with a familial increased risk of hematologic malignancies, caused by mutations in genes *RUNX1*[29] and *ANKRD26*[30] among others. Due to these mutations, cell differentiation at early stages of hematopoiesis is affected with increased risk for myelodysplastic syndrome and leukemia. However, it is unclear which variants in these genes are associated with an increased leukemia risk and identification of mutations in these genes may cause major psychological distress, while it has no impact on disease management. Therefore, testing for mutations in these specific genes should only be considered after appropriate counseling and explicit informed consent from the patient.

## Future perspectives

Due to the complexity of platelet function, it is unlikely there will ever be one universal assay to test platelet function. Resources should be put towards a combination of near bedside tests with the highest diagnostic yield and the least amount of blood required.

The first step towards better platelet diagnostics is to determine universal diagnostic criteria for CPDs and reference values for several diagnostic tests. Various attempts have been made to standardize the diagnostic approach[8,17,31-33], yet these reports lack uniform criteria for interpreting the test results. The greatest struggle in platelet diagnostics is the lack of a robust gold standard test. Without it, it is impossible to determine whether an abnormal result should be considered true-positive or false-positive. Ideally, genetic analysis will be used to confirm the presence of a CPD and diagnostic tests will be validated based on this reference test. However, it will take years of research to identify the majority of underlying genetic variants for CPDs and to unravel the pathophysiology of platelet defects. In the mean time, we have to settle for less.

For now, it is important to define when test results are considered abnormal. Reference values could be determined based on responses in the healthy population. A commonly used cut-off value is the 2.5$^{th}$ percentile of healthy donors. This indicates that 2.5% of healthy donors will have abnormal test results, while having no symptoms or complaints. The use of several agonists in LTA will increase the number of false-positives when using the 2.5$^{th}$ percentile as a cut-off value; with 4 agonists, a healthy person will have a 90% (97.5%^4) chance of having a normal test result. Therefore, it seems justifiable to use a more stringent cut-off value, for instance the 1.0$^{th}$ percentile, to correct for multiple testing.

Preferably, a large cohort of healthy individuals of varying age, sex and race without an objective bleeding tendency will be used for this purpose. The number of false-positives can be verified by repeated measurements. Then, universal diagnostic criteria for CPDs can be proposed. The next step could be to use standardized criteria for the coding of cases, with for

instance human phenotype ontology (HPO)[34]. HPO was created to accommodate coding of phenotypic data derived from diverse sources, such as clinical interpretation and laboratory assays. It can be used to perform cluster analysis in patients with similar phenotypes likely to share genetic variants. This may guide the discovery of new genetic variants and further unravel the pathophysiology of platelet defects. Then, functional or structural studies should be performed to confirm that these new variants indeed disrupt platelet function and contribute to the bleeding phenotype.

Flow cytometry has been suggested as a viable alternative for currently available diagnostic tools. In addition to its use as a diagnostic tool for SPD, platelet surface receptor expression can be quantified and fibrinogen binding and P-selectin expression can be measured as platelet reactivity markers in response to agonist stimulation. There are several advantages over LTA: the sample volumes required are significantly lower, it allows reliable assessment of platelet function in thrombocytopenic patients, and standardization is no more complicated than that of LTA. Studies have demonstrated that a flow cytometry-based platelet function test has added value in diagnosing patients with suspected CPDs compared with LTA alone[35] and that it allows the subclassification of CPDs in patients with previously categorized platelet abnormalities[36]. Efforts should be made to further validate and standardize this test as a diagnostic tool for CPDs. In addition, research should be put towards standardizing assays to investigate platelet function in flowing blood or platelet procoagulant activity, since these are currently lacking.

The platelet proteome can be described as the complete set of proteins expressed within a platelet at a given time and circumstance[37]. Since platelets are anucleate cell fragments, protein synthesis is limited. Hence, defects in platelet function are almost completely attributable to altered protein expression and post-translational modifications. Proteomics can provide insight into the protein composition of platelets and changes in protein levels, can be used to characterize the fundamental processes that regulate platelets and can contribute to a better understanding of the pathogenesis of CPDs[38]. The proteomics field is still in its early stages and further validation and clinical studies are necessary to use this tool in clinical practice.

Although individually rare, CPDs together account for a significant proportion of inherited mild bleeding disorders. It is important to continuously gain insight into platelet pathofysiology and molecular mechanisms, in order to optimize diagnostic and treatment options. As novel genes are often identified in a single family, broad data sharing of previously unrecognized entities is essential. Many countries in Europe already have a regional or national expertise center and collaboration between these centers to exchange patient data and expertise would greatly be encouraged.

## Closing remarks

None of this work could have been possible without the help of the patients described in this thesis. We hope this thesis made a small contribution towards better understanding of CPDs and towards creating awareness of their impact on everyday life.

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# ADDENDUM

# NEDERLANDSE SAMENVATTING

Dit proefschrift is het resultaat van het promotieonderzoek van drs. M.W. Blaauwgeers naar aangeboren bloedplaatjesstoornissen, met de naar het Nederlands vertaalde titel: Aangeboren bloedplaatjesstoornissen: klinische kenmerken en diagnostische testen.

Bloedplaatjes spelen een belangrijke rol bij de bloedstolling. Als een bloedvat beschadigd raakt, worden bloedplaatjes geactiveerd en klonteren ze aan elkaar om het beschadigde vat af te dichten. Bij patiënten met bloedplaatjesstoornissen duurt het langer voordat een beschadiging van een bloedvat gedicht wordt. Bij bloedplaatjesstoornissen kan er sprake zijn van een verlaagd aantal bloedplaatjes, een gestoorde functie van bloedplaatjes of een combinatie van beiden. De stoornissen kunnen aangeboren of verworven zijn. In dit proefschrift focussen we op aangeboren stoornissen van de bloedplaatjes.

Patiënten met aangeboren bloedplaatjesstoornissen hebben een bloedingsneiging. Dit uit zich vooral in het optreden van bloedneuzen, hevige menstruaties en nabloedingen na een bevalling of ingreep, zoals kiezen trekken. Het is echter niet bekend welke bloedingen het vaakst voorkomen en welke impact deze bloedingen hebben op de kwaliteit van leven van deze patiënten.

De huidige diagnostiek naar bloedplaatjesstoornissen bestaat uit een anamnese, gevolgd door screeningstesten en specifieke trombocytenfunctietesten. Met deze testen worden met name de ernstige vormen gediagnosticeerd. Voor de milde vormen is deze diagnostiek vaak ontoereikend. Een goede diagnose is echter zeer belangrijk. Levensbedreigende of invaliderende bloedingen kunnen mogelijk voorkomen worden door goede voorlichting van de patiënt en tijdige behandeling, zoals profylactische behandeling voorafgaand aan een operatie. Daarnaast is een juiste diagnose van belang voor het instellen van de juiste behandeling en het voorkómen van onnodige en mogelijk schadelijke behandelingen ten gevolge van een verkeerde diagnose. Er is derhalve behoefte aan vernieuwde diagnostische testen.

Het doel van dit proefschrift was om inzicht te verkrijgen in het bloedingsfenotype van patiënten met een aangeboren bloedplaatjesstoornis en de impact van de bloedingen op hun kwaliteit van leven. Daarnaast hebben we de diagnostische waarde van verscheidene geavanceerde diagnostische testen geëvalueerd. De studie populatie bestond uit patiënten die geïncludeerd zijn in de ‘ Trombocytopathie in Nederland’ (TiN) studie, de eerste grote nationale studie naar aangeboren bloedplaatjesstoornissen.

In **Hoofdstuk 2** bespreken we de huidig beschikbare testen voor de diagnostiek naar bloedplaatjesstoornissen en hun voor- en nadelen. Ondanks een verscheidenheid aan laboratorium testen zijn de meesten insufficiënt om milde bloedplaatjesstoornissen aan te tonen. Flow cytometrie en genetisch onderzoek zijn mogelijk veelbelovende aanvullende diagnostische testen. In **Hoofdstuk 3** evalueren we het bloedingsfenotype en diagnostische kenmerken van patiënten met aangeboren bloedplaatjesstoornissen. De meest voorkomende diagnostische subgroepen zijn ADP defecten, tromboxaan A2 defecten en geïsoleerde trombocytopenie.

Bloedingen die het vaakst worden gerapporteerd, zijn hevig menstrueel bloedverlies en bloedingen na operaties en bevallingen. Patiënten met Glanzmann thrombasthenie en vrouwen hebben een ernstiger bloedingsfenotype. In **Hoofdstuk 4-6** wordt de waarde van geavanceerde diagnostische testen beschreven. In **Hoofdstuk 4** beschrijven we de waarde van de self-administered bleeding assessment tool (self-BAT). Normaal gesproken wordt de 'bleeding assessment tool' (BAT), een vragenlijst over verschillende bloedingssymptomen, door de arts ingevuld tijdens een polibezoek. De BAT wordt gebruikt als screeningsinstrument om te beoordelen of er bij patiënten sprake is van een bloedingsneiging en of verder onderzoek geïndiceerd is. De self-BAT bevat dezelfde vragen als de BAT met als verschil dat de self-BAT door patiënten zelf ingevuld kan worden. Als patiënten deze vragenlijst thuis invullen, wordt kostbare tijd tijdens een polibezoek bespaard. In dit hoofdstuk tonen we aan dat de self-BAT voldoende betrouwbaar en uitvoerbaar is om een bloedingsneiging vast te stellen bij patiënten met een verdenking op een aangeboren bloedplaatjesstoornis. De self-BAT kan dus, net zoals de BAT, gebruikt worden als screeningsinstrument en kan helpen bepalen welke patiënten doorverwezen moeten worden naar een gespecialiseerd hemofilie behandelcentrum voor nadere diagnostiek. In **Hoofdstuk 5** evalueren we de toepassing van mepacrine op de flow cytometer voor de diagnostiek van storage pool disease. Mepacrine is een fluorescente stof die bindt aan de 'dense granules' in bloedplaatjes. In patiënten met storage pool disease zijn deze dense granules verminderd aanwezig of zelfs afwezig. In dit hoofdstuk laten we zien dat deze methode goed gebruikt kan worden om storage pool disease uit te sluiten, waardoor deze test geschikt is als screeningstest. Genetisch onderzoek kan worden ingezet om de onderliggende mutatie van aangeboren bloedplaatjesstoornissen aan te tonen. Genetica wordt nu vooral gebruikt om de diagnose te bevestigen, maar er zijn discussies dat genetisch onderzoek eerder in het diagnostisch stappenplan ingezet moet worden. Genetisch onderzoek is echter kostbaar en tijdrovend en het is onduidelijk of het eerder verrichten van dit onderzoek toegevoegde waarde heeft in het aantonen van bloedplaatjesstoornissen. In **Hoofdstuk 6** laten we zien dat slechts bij een zeer gering aantal patiënten een onderliggende genetische mutatie aantoonbaar is en wij adviseren derhalve om alleen genetisch onderzoek te verrichten bij patiënten met een bewezen bloedplaatjesstoornis. **Hoofdstuk 7** beschrijft de kwaliteit van leven van patiënten met (een verdenking op) een bloedplaatjesstoornis. We tonen aan dat bij deze patiënten de kwaliteit van leven significant verminderd is ten op zichte van de algemene Nederlandse populatie en dat deze groep patiënten onze aandacht verdient.

Alles bij elkaar genomen, heeft dit proefschrift bijgedragen aan onze kennis over aangeboren bloedplaatjesstoornissen. We hebben inzicht gekregen in de klinische kenmerken en ziektelast van patiënten met aangeboren bloedplaatjesstoornissen en we hebben inzicht gekregen in de voordelen en beperkingen van geavanceerde diagnostische testen. Meer onderzoek naar bloedplaatjesstoornissen is belangrijk om bewustzijn te creëren bij artsen en in de maatschappij en om meer inzicht te krijgen in de pathofysiologie van bloedplaatjesstoornissen om op die manier de diagnostiek voor deze aandoeningen te verbeteren.

# LIST OF PUBLICATIONS

Blaauwgeers MW, Kruip MJHA, Beckers EAM, et al. Congenital platelet disorders and health status–related quality of life. Res Pract Thromb Haemost. 2020;4:100–105.

Blaauwgeers MW, van Asten I, Kruip MHJA, et al. The limitation of genetic testing in diagnosing patients suspected for congenital platelet defects. Am J Hematol. 2020 Jan;95(1):E26-E28

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## Awards and grants

CSL Behring Heimburger Award 2017: Improved diagnostics in patients with inherited platelet function disorders

European Hematology Association Abstract Achievement Award 2018: Laboratory characterization of patients with (suspected) inherited platelet disorders: results from the 'Thrombocytopathy in the Netherlands' study

## Presentations and posters

2019 Oral presentation, 13th Dutch Hematology Congress in Papendal, the Netherlands: "Diagnostics of inherited platele defects"

2018 Poster presentations, 23rd Congress of European Hematology Association in Stockholm, Sweden: "Laboratory characterization of patients with (suspected) inherited platelet disorders: results from the 'Thrombocytopathy in the Netherlands' study"; "Performance of the ISTH bleeding assessment tool in predicting the presence of inherited platelet function disorders"; "Quality of life is reduced in patients with (suspected) congenital platelet function disorders"

2018 Oral presentation, NVTH symposium in Koudekerke, the Netherlands: "Performance of the ISTH bleeding assessment tool in predicting the presence of inherited platelet function disorders"

2018 Poster presentation, 11th Congress of European Association for Haemophilia and Allied Disorders in Madrid, Spain: "Performance of the ISTH bleeding assessment tool in predicting the presence of inherited platelet function disorders"

2017 Poster presentations, 26th Congress of International Society on Thrombosis and Haemostasis in Berlin, Germany: "Quality of life is reduced in patients with (suspected) congenital platelet function disorders"; "Hemorrhagic diathesis in four patients with the heterozygous Pro258Thr mutation of the P2RY12 gene"; "Delta storage pool deficiency in a patient with a R216Q mutation of GATA1"

2016 Oral presenation, ISTH Advanced Training Course in Oxford, England: "Thrombocytopathy in the Netherlands' (TiN) study"

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**Ruurd**, jij kon al mijn problemen en frustraties altijd heerlijk relativeren en je weet mij elke dag weer aan het lachen te maken. Ik hou van je.

# CURRICULUM VITAE

Maaike Willemijn Blaauwgeers was born in December 1988 in Woerden, the Netherlands. After graduating secondary school *cum laude* at Goois Lyceum in Bussum (2006), she moved to Amsterdam to attend medical school at the University of Amsterdam. Maaike combined her studies with playing softball on the highest competitve level in the Netherlands. With the Dutch junior national team she participated in the World Championship (Enschede, the Netherlands) in 2007 and the Kingdom Games (Willemstad, Curacao) in 2009. In 2011 she spent four months in Coolidge (Arizona, USA) to play softball at a collegiate level. In 2013 she spent 6 weeks in Surinam for a clinical rotation in Neurology. After graduating *cum laude* in 2014, Maaike started her clinical career at the department of Internal Medicine at the Flevoziekenhuis in Almere.

In March 2015, Maaike started her PhD project described in this thesis at the Van Creveldkliniek at the University Medical Center Utrecht under supervision of prof. dr. R.E.G. Schutgens, dr. R.T. Urbanus and prof. dr. G. Pasterkamp. For her research she received the 2017 CSL Behring Heimburger Award and 2018 European Hematology Association Abstract Achievement Award. During her PhD project, she continued to play softball. She became Dutch National and European Champion and participated in several European Championships with her softballteam Sparks Haarlem.

Since May 2019, Maaike has been working as a nursing home physician at Zorgbalans in IJmuiden, the Netherlands. She starts her residency for nursing home medicine specialist in September 2020.