MAPPING THE DIAGNOSTIC MAZE OF PLATELET FUNCTION DISORDERS

Ivar van Asten

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MAPPING THE DIAGNOSTIC MAZE OF PLATELET FUNCTION DISORDERS

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Ivar van Asten
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PROMOTOREN

Prof. dr. R.E.G. Schutgens

Prof. dr. G. Pasterkamp

COPROMOTOR

Dr. R.T. Urbanus

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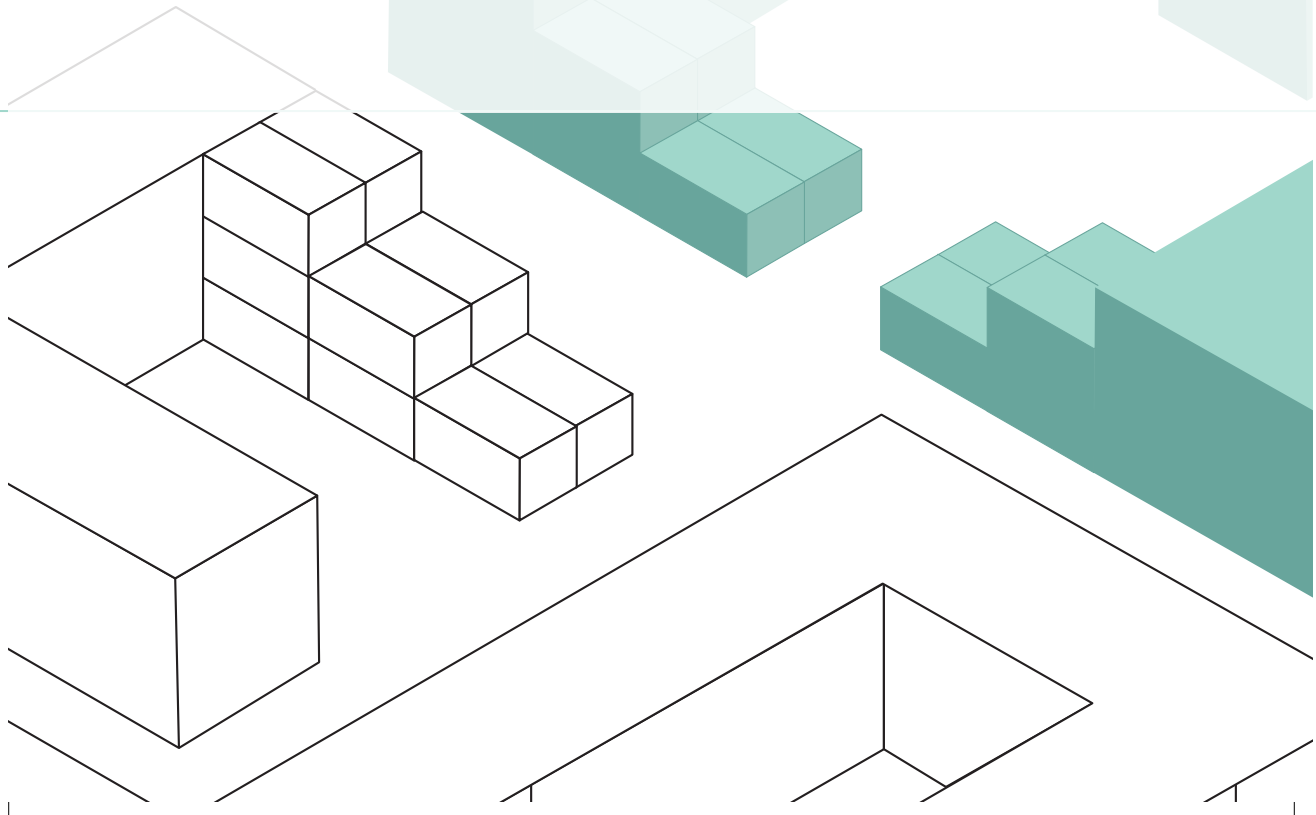

GENERAL INTRODUCTION

Platelets and hemostasis

Platelets are small cellular fragments with an average size of 2-3 µm. Platelets play an important role in maintaining normal blood flow after vascular injury by the formation of a stable platelet plug, a process called hemostasis[1]. Hemostasis can be divided in primary and secondary hemostasis. In primary hemostasis, formation of a platelet plug is initiated when von Willebrand Factor (vWF) binds to subendothelial collagen that is exposed in the damaged vessel wall. Due to bloodflow, vWF unfolds and captures circulating platelets trough an interaction with platelet GPIb-V-IX complex, leading to platelet arrest. Subsequently, platelets spread to cover the site of vessel injury and bind collagen via GPVI and α2β1 integrins[2]. Platelets become activated on collagen and will secrete the content of their α- and δ- granules and produce thromboxane A2 to enhance platelet activation (Figure 1). Simultaneously, during platelet activation, integrin αIIbβ3 changes conformation, which allows fibrinogen binding[3]. Fibrinogen crosslinks activated platelets to form a platelet aggregate. In secondary hemostasis, the formed platelet aggregate is stabilized by insoluble fibrin fibers, the end product of a cascade of enzymatic reactions, a process called coagulation (Figure 2). The coagulation cascade is initiated by the exposure of blood to extravascular tissue factor (TF). Although most coagulation factors circulate as inactive proenzymes, a small proportion of total coagulation factor (F)VII circulates as the active enzyme (FVIIa). Binding of FVIIa to TF leads to formation of the extrinsic tenase complex, which has full enzymatic activity. The TF:FVIIa complex activates both FIX and FX. Activated FX (FXa) subsequently activates its cofactor, FV, and forms the prothrombinase complex. This enzymatic complex converts prothrombin into the enzyme thrombin. Thrombin accelerates the clotting reaction by activating additional FV, as well as the cofactor of FIXa, FVIII. Together, FIXa and FVIIIa form the intrinsic tenase complex, which efficiently activates FX and amplifies the coagulation reaction even further[4]. Thrombin, the final enzyme of the coagulation reaction, converts the soluble plasma protein fibrinogen into insoluble fibrin. Furthermore, thrombin is responsible for the positive feedback loop in the clotting cascade by the activation of Factor XI, but is also important for further p latelet activation via the binding to platelets thrombin receptors (PAR1 and PAR4)[5].

Platelet function disorders

Disorders in primary hemostasis include von Willebrand disease and platelet function disorders. The prevalence of von Willebrand disease is 1:10.000[6], whereas platelet function disorders are rarer. The number of platelet function disorders might be underestimated because of misdiagnosis or failure to recognize the presence of a platelet function disorders[7]. Platelet function disorders are characterized by prolonged bleeding episodes, mucocutaneous bleeds, menorrhagia and spontaneous bruising. Bleeding symptoms can vary from mild to life threatening and depend on the type of platelet function disorder. Generally, platelet function disorders can be divided into qualitative disorders (platelet function disorders (PFD) or thrombocytopathy) and in quantitative disorders (thrombocytopenia), but some disorders are associated with both features[8]. There are 3 classical and easy to diagnose PFDs, namely Glanzmann Thrombasthenia, δ-Storage Pool disease

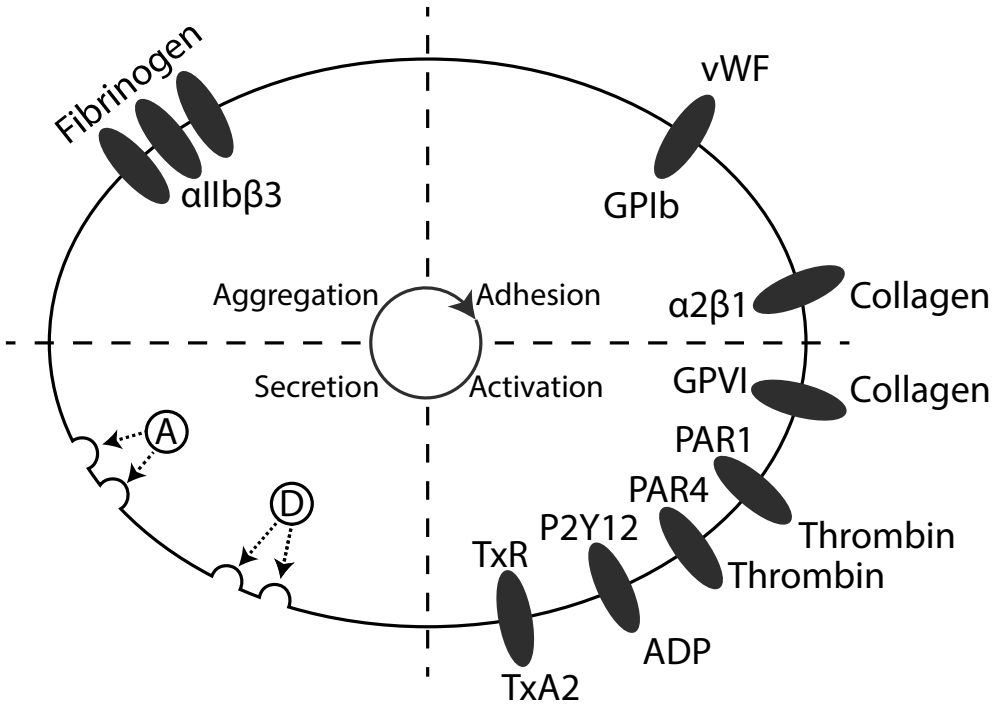

Figure 1. platelet function in hemostasis. vWF: von Willebrand factor, TxA2: Thromboxane A2, TxR: Thromboxane A2 receptor, D: dense granules, A: alpha granules

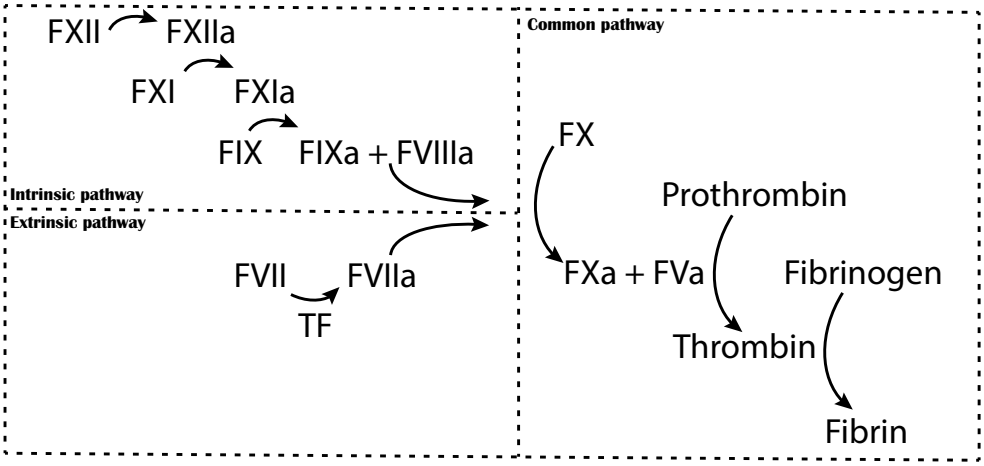

Figure 2. overview of the coagulation cascade.

and Bernard Soulier syndrome. Glanzmann Thrombasthenia is characterized by the absence of αIIbβ3 integrin which precludes the formation of platelet aggregates. Generally, Glanzmann Thrombasthenia gives rise to a severe bleeding tendency. Storage Pool disease is characterized by absent or empty dense granules. Here, platelet degranulation does not result in further platelet activation, which results in mild to severe bleeding symptoms. Finally, Bernard Soulier syndrome is characterized enlarged platelets and by the absence of the GPIb-V-IX receptor complex, which is important for platelet adhesion to vWF. Bernard Soulier syndrome is also accompanied by thrombocytopenia and bleeding symptoms are often mild to moderate. There are many more platelet function disorders that give rise to different degrees of bleeding problems. However, these are more difficult to diagnose, because specific laboratory tests for these disorders are missing. Correct identification of PFDs is important, because the increased risk of bleeding associated with a particular disorder might be life threatening, they may affect patients' quality of life and the disorder determines the treatment strategy.

Diagnosis of platelet function disorders

Severe PFDs are often diagnosed in early life, because patients present with severe and frequent bleeding symptoms. In contrast, mild PFDs are often diagnosed in adulthood, when patients have undergone more hemostatic challenges. Accurate diagnostics of mild PFDs are hampered by the low sensitivity of currently available diagnostic tests for mild platelet dysfunction[9]. Nevertheless, diagnosing mild platelet function disorders is equally important as diagnosing more severe platelet function disorders, since failure to recognize the presence of a platelet function disorder might result in the underestimation of bleeding risk during hemostatic challenges, for instance during surgery or trauma[10], and can result in life threatening bleeds.

The first step in the diagnosis of platelet function disorders is to determine whether symptoms of bleeding are present. To objectify the bleeding symptoms, a bleeding assessment tool is used. A commonly used tool is the International Society of Thrombosis and Hemostasis Bleeding Assessment Tool (ISTH-BAT)[11]. With this questionnaire the severity, frequency and treatment of different bleeding symptoms are quantified and this results in a bleeding score. This questionnaire has been developed for the screening of Von Willebrand disease, but is also applicable for platelet function disorders. It is very useful in excluding a bleeding disorder, but not very specific. Therefore, functional platelet assays are required to confirm the presence of platelet function disorder[12,13].

To provide more direction to the cause of bleeding symptoms, screening tests are performed. Those tests include the PT and aPTT to exclude clotting factor deficiencies, the Von Willebrand cofactor activity (vWF:RCo) assay to screen for Von Willebrand disease and a complete blood count (CBC) to determine platelet number and size. The PFA-100/200 is performed to screen for disorders in primary hemostasis, but is not present in all routine diagnostic laboratories[14].

If a platelet function disorder is still suspected after the anamnesis and the first screening, patients might be referred to a tertiary reference center in which platelet function assays are performed. The most commonly used test to measure platelet function is light transmission aggregometry (LTA)[15]. It measures the platelet aggregation in plasma in response to stimulation with different agonists. After platelet activation, platelet aggregates are formed and will drop to

the bottom of the test tube. As a consequence, the light transmission through plasma increases. The amount of increase in light transmission is a measure for platelet aggregation function. By adding different agonists, several platelet activation pathways are studied and this allows the discrimination of certain platelet function disorders, including Glanzmann Thrombasthenia, Storage Pool disease and Bernard Soulier syndrome[16]. Although this test is the most commonly used test for platelet function disorders, it is not sensitive for mild platelet function disorders, cannot be performed in samples with low platelet counts, is labor intense and requires a large blood volume[17].

Another, more specific, platelet function test is the measurement of platelet ADP content. This test measures the total amount of ATP and ADP in platelet lysate and is used for the identification of Storage Pool disease[18]. The measurement of platelet ADP content has similar limitations as LTA; it is labor intense, requires large blood volume and cannot be performed in samples with low platelet count[19].

Finally, whole exome sequencing in a selected panel of genes can be used in the diagnosis of platelet function disorders. The diagnostic yield of whole exome sequencing is limited, since it has been shown that a genetic defect was found in around 60% of the patients with excessive bleeding symptoms[8]. This low number can be explained because a selected gene panel is analyzed. Causative mutations in genes that are not included in the panel are missed. Moreover, genetic variations only have diagnostic value when the consequence of a single variant is known. The effect of new variants, even if they are within the selected gene panel, should be investigated before they can be used for diagnosis. Genetic testing becomes more complex if a bleeding disorder is caused by multiple variants, especially if those variants are distributed over multiple genes[20].

Aim of the thesis

The hemostatic system is a well-organized and complex mechanism to prevent blood loss upon vascular injury. Covering all aspects of hemostasis in laboratory tests would be ideal, but is currently impossible. LTA is the most commonly used platelet function test and has been developed since the 1960s. In the meantime, no other tests have shown to be superior to LTA in diagnosing PFDs, although LTA can be used for the severe platelet function disorders, it is not sensitive for mild platelet function disorders[7,21]. There is an urgent need for improved platelet function tests, because the exact cause of mucocutaneous bleeds remains unclear in more than 50% of the patients[22]. Genetic testing for platelet function disorders is promising and has been suggested as a first-line diagnostic tool[23,24], but still genetic testing is still inconclusive in at least 50% of patients with a platelet function disorder[25]. Therefore, the aim of this thesis is to improve the diagnostic field of platelet function disorders and to gain more insight in the pathophysiological mechanism of these platelet function disorders.

THESIS OUTLINE

Chapter 2 describes the several applications and the introduction of a flow cytometry based platelet function test in the diagnostic strategy of platelet function disorders. Flow cytometry has been recommended as a diagnostic tool, but its use is currently limited to measurements of platelet receptor expression in a diagnostic setting. Although the technique is highly suitable for

measuring platelet reactivity, this application is primarily used in research rather than in diagnostics. The introduction of new diagnostic tests in the field of platelet function disorders is challenging and is discussed in this review.

In **chapter 3**, a flow cytometry based platelet function test is validated and compared with the most commonly used platelet function test: light transmission aggregometry (LTA). Flow cytometry has practical advantages over LTA, because it requires a small amount of blood, can be performed in thrombocytopenia and is less labor-intense. In addition, we have shown that flow cytometry has added value to LTA in diagnosing platelet function disorders, but cannot replace LTA in the diagnostic strategy.

Chapter 4 describes another application of flow cytometry in the diagnosis of platelet function disorder. Mepacrine staining of platelet dense granules has been suggested as a tool for the diagnosis of platelet storage pool disease. In this chapter, the use of mepacrine fluorescence measured on the flow cytometer is validated in a cohort of patients which are suspected for a platelet function disorder. Mepacrine fluorescence is not superior to platelet ADP content, but could be used as a screening test for the exclusion of storage pool disease.

In **chapter 5,** the diagnostic value of upfront genetic testing in patients with (suspected) platelet function disorders is investigated. Recent studies have suggested that genetic testing should be performed earlier in the diagnostic work-up to increase diagnostic efficiency. In our study, patients with a definite, a possible or without a platelet function disorder based on laboratory platelet function testing, were analyzed with a whole exome sequencing gene panel. Our data showed that a genetic cause was identified in only a minority of patients with a platelet function disorder and no genetic variants were identified in patients with normal platelet function tests.

In **chapter 6** the pathophysiological mechanism of GNE-related thrombocytopenia is investigated. The GNE gene has recently been associated with thrombocytopenia, but the mechanism remained unclear. GNE is the rate-limiting enzyme in sialic acid synthesis and platelet desialylation has been described as trigger for platelet clearance. In our study, 2 siblings with a homozygous GNE variant were investigated. With both in vitro and in vivo experiments, we showed that GNE-thrombocytopenia in those siblings is characterized by increased hepatic platelet clearance, most likely via macrophages, as a consequence of platelet hyposialylation.

Chapter 7 focuses on 4 different cases that have an acquired bleeding tendency. In all cases a monoclonal antibody was present, which was shown to interfere with the hemostatic system, thus explaining the bleeding symptoms. Normally, an asymptomatic monoclonal gammopathy of unknown significance does not require treatment, but eradication of the monoclonal antibody in these 4 cases with chemotherapy successfully solved their bleeding symptoms.

In **Chapter 8** the content of this thesis is placed in a broader perspective.

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Ivar van Asten, MSc[1,2,3], Roger EG Schutgens, PhD[3], Rolf T Urbanus, PhD[1]

[1] Center for Circulatory health, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands
[2] Van Creveld Laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands
[3] Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands

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TOWARDS FLOW CYTOMETRY BASED PLATELET FUNCTION DIAGNOSTICS

ABSTRACT

The laboratory diagnostics of (inherited) platelet function disorders mainly comprises aggregation and secretion assays, which may be suitable for diagnosing some specific severe platelet function disorders, but not reliable enough for diagnosing mild platelet function disorders or disorders associated with low platelet count. Flow cytometric assessment of platelet reactivity will expectedly provide additional value during the diagnostic work-up of platelet function disorders, because it only requires small volume of whole blood and allows the measurement of platelet function in thrombocytopenic samples. Flow cytometry has frequently been used to evaluate platelet function in the research setting, so that these assays will require clinical validation before they can be used as routine diagnostic tools. The main challenge in the validation of innovative platelet function diagnostic tests is the lack of a gold standard test for mild platelet function disorders. This review aims to address the many applications of flow cytometry in the current diagnostic work-up of platelet function testing and discuss the challenges in introducing new tools for diagnosing platelet function disorders.

INTRODUCTION

Platelets are the main players in response to vascular injury and for preventing excessive blood loss. These small blood elements rapidly adhere to the site of injury and form a hemostatic plug finalized to limit abnormal blood loss. Platelet function in hemostasis is roughly divided into four different processes, namely platelet adhesion, activation, secretion and aggregation. The first step in platelet adhesion is the binding of the GPIb-V-IX complex to immobilized von Willebrand Factor (VWF)[1]. The subsequent interaction of α2β1 and GPVI to subendothelial collagen, along with integrin αIIbβ3 (previously named GPIIb/IIIa) binding to VWF, results in platelet activation and firm adhesion[2]. Platelet activation results in alpha and dense granule secretion, generation of thromboxane A2 and αIIbβ3 activation. The secretion of alpha and dense granules, along with production of thromboxane A2, further promote platelet activation, whereas activation of αIIbβ3 results in fibrinogen binding to crosslink activated platelets.

Platelet function disorders (PFDs) are characterized by easy or spontaneous bruising, mucocutaneous bleeds, or prolonged bleeding time and have a heterogeneous phenotype[3]. They can be inherited or acquired, and can be associated with a low platelet number (i.e., thrombocytopenia)[4,5]. Qualitative PFDs include Glanzmann Thrombasthenia (GT), Storage Pool Disease (SPD) and Bernard Soulier Syndrome (BSS). Other PFDs include Gi-receptor disorders, collagen receptor disorders and secretion disorders[6].

An accurate diagnosis is vital for quality of life and prevention of life-threatening bleeding episodes using prophylactic therapy. It is also essential to distinguish between congenital platelet deficit and other forms of thrombocytopenia, such as immune thrombocytopenia. The laboratory diagnostics of PFDs entails many different techniques. The currently available diagnostic tools may be suitable for identifying severe PFDs, especially GT; however, identification of mild PFDs including SPD and BSS remains challenging. There is therefore an unmet need for improved platelet function diagnostics, especially in thrombocytopenic samples. Flow cytometry appears suitable for assessing platelet function in samples with low platelet count and has been suggested as a viable alternative for currently available tools. However, flow cytometry needs both validation for mild PFDs and standardization before it can be used in a routine diagnostic setting. In this review, we will discuss the current diagnostic strategy for PFDs, the potential role of flow cytometry in platelet diagnostics and the challenges of introducing new diagnostic tests for PFDs.

DIAGNOSTIC WORK-UP OF SUSPECTED PFDS

The first step in the diagnosis of PFDs is based on evaluation of symptoms, bleeding and family history (figure 1). The accurate assessment of a bleeding disorder encompasses the possible use of standardized questionnaires, also known as Bleeding Assessment Tools (BATs), which allow quantification of the severity and frequency of bleeding episodes, finally expressed as a bleeding score. A commonly used bleeding score is the Tosetto-score, which has been designed for the assessment of patients with von Willebrand disease (VWD)[7]. A score ≤3 almost completely rules out VWD, exhibiting a negative predictive value (NPV) as high as 99%[8]. Conversely, a bleeding score >3 suggest that laboratory testing for a bleeding disorder should be initiated. Nevertheless, the Tosetto-score has not been validated for PFDs. The International Society on Thrombosis and

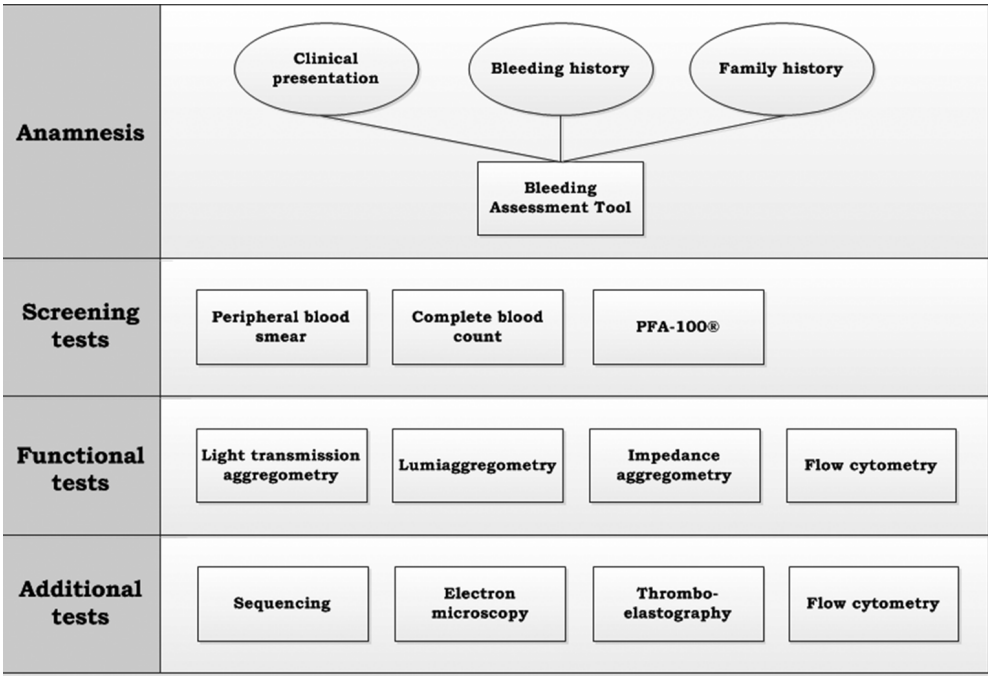

Figure 1. The diagnostic flowchart of platelet function testing.

Haemostasis (ISTH) has developed an alternative bleeding score, the ISTH-BAT, that can be used for both VWD and PFDs[9]. The ISTH-BAT bleeding score also displays a high NPV, but is characterized by low specificity and positive predictive value (PPV)[10]. The normal score is ≤3 for men and women ≤5 for women, respectively[11].

Before performing specific platelet function analyses, screening tests should be performed to confirm a suspicion of PFDs and to exclude VWD and coagulation factor deficiencies. In case of a high bleeding score and exclusion of VWD or coagulation factor deficiencies, a PFD is very likely[12]. The Platelet Function Analyzer-100 or 200 (PFA-100/200) is a sensitive analyzer for screening patients with primary haemostasis disorders, but is not sensitive enough for distinguishing PFDs from VWD[13]. A Complete Blood Cell Count (CBC), including platelet number, mean platelet volume (MPV) and number of reticulated platelets can help identify and differentiate thrombocytopenias. For example, the number of reticulated platelets may help distinguish between disorders characterized by decreased platelet production or enhanced platelet turnover[14]. A peripheral blood smear can also be helpful for diagnosing May-Hegglin disease, Gray Platelet Syndrome or pseudo-thrombocytopenia (e.g., due to EDTA)[15,16].

Among all different platelet function tests, the most frequently used test for the diagnosis of PFDs is light transmission aggregometry (LTA)[17]. A reliable alternative is whole blood impedance aggregometry, which is less time consuming and can be performed in whole blood, but still requires more validation for use in the diagnostic workout of inherited PFDs[18]. LTA measures the light transmitted through platelet rich plasma after stimulation with different agonists, used in

several concentrations. Upon platelet stimulation, light transmission through the sample increases over time as a result of platelet aggregation and can hence be considered a reliable measure of platelet function. Ristocetin-induced platelet agglutination is a measure of platelet adhesion. The most frequently used parameters include maximum aggregation amplitude, final amplitude and curve reversibility. By using a different subset of agonists, different platelet activation pathways can be studied. The most commonly used are adenosine diphosphate (ADP), collagen, arachidonic acid, epinephrine and ristocetin[19]. The diagnosis of GT and BSS can only be made with LTA, although some cases may be missed due to low platelet count. Impaired aggregation after stimulation with all platelet agonists except ristocetin is observed in GT, whilst normal platelet aggregation with only impaired ristocetin-induced platelet agglutination is found in BSS. Not all patients with SPD display decreased platelet aggregation with LTA, which explains why SPD cannot be ruled out when LTA is normal[20,21]. Therefore, the diagnosis of SPD requires additional testing. The diagnosis of mild heterogeneous PFDs with only LTA becomes even more challenging. Although LTA is an important test for diagnosing different severe PFDs, this test lacks sensitivity for the diagnosis of mild PFDs[6]. The overall sensitivity of LTA for PFDs is estimated between 34-60%[22], which is probably an underestimation due to the under-diagnosis of mild forms of PFDs[12]. Moreover, a major drawback of LTA is that it is not feasible, or reliable, to investigate platelet function in thrombocytopenic samples. Nevertheless, measuring platelet function in thrombocytopenic samples is vital since a platelet count <50x10^9/L is not necessarily associated with a bleeding diathesis[23]. According to general consensus, the minimum platelet count of 150x10^9/L platelets is necessary for obtaining reliable LTA data[24]. The assessment in samples with a lower platelet count is still feasible, but should be interpreted with caution, especially when the platelet count falls below 75x10^9/L platelets[25]. In addition to the mild sensitivity for PFDs, LTA exhibits additional drawbacks. LTA is poorly standardized and there is poor consensus about the concentration and number of agonists, thus explaining the poor inter-laboratory agreement[17,26]. The recommended panel of agonists for LTA is extensive, since it is necessary to gather the larger possible information on platelet function. LTA is hence labor intensive and time-consuming and requires a high volume of whole blood, which often precludes the diagnosis of PFDs in neonates and young children[27].

Due to drawbacks and lack of sensitivity of currently available tests, we need new methods for diagnosing PFDs, preferably encompassing collection of a minimal amount of blood. Flow cytometry has been recommended by the ISTH SSC as a reliable diagnostic tool for diagnosing PFDs[6]. The main advantage of flow cytometry over routine platelet function tests is the minimum amount of whole blood needed and its possible use in patients with thrombocytopenia[28,29]. The use of whole blood makes this technique less time-consuming and less vulnerable to pre-analytical problems. However, flow cytometry is also has drawbacks, just as for LTA; shear stress forces are not taken into account during analysis of platelet function and reagents are relatively expensive. Moreover, flow cytometry is only available in a minority of diagnostic laboratories as a first-line laboratory test[12]. The current role of flow cytometry in PFD diagnostics entails measuring surface receptor expression to confirm the diagnosis of BSS or GT, but can also use be used to test additional receptors, such as αIIβ1, GPIV and GPVI, for diagnosing collagen receptor deficiencies[6]. Moreover, agonist-induced platelet reactivity can be measured with a flow cytometer, although this analyzer

is mainly used in experimental settings, and lacks validation and standardization to broaden its use in routine diagnostics so far.

FLOW CYTOMETRIC TESTING OF PLATELET FUNCTION

Practical issues

Flow cytometry allows the rapid analysis of multiple cell markers on single cells with fluorescent antibodies, and has several advantages compared to LTA (table 1). The use of multiple excitation lasers and excitation or emission filters allows detecting different platelet markers within one sample after compensation of fluorescent intensity. In order to identify platelets by flow cytometry in whole blood samples, platelet rich plasma (PRP) or washed platelets, logarithmic scaling of forward (FSC) and sideward scatter (SSC) is needed. With these settings, normal sized platelets are easily separated from red blood cells (RBCs) and leucocytes, thus allowing analysis of specific receptors or activation markers with fluorescent dyes or antibodies. When platelet identification is only based on FSC and SSC, caution should be taken to avoid an analysis of hemolytic specimens, samples with large amount of cell debris, or those with air bubbles, as these will partly overlap with platelets. It should be noted that the FSC/SSC characteristics of large platelets, as found in patients with macrothrombocytopenia, overlap with those of other blood cells, thus hampering accurate platelet identification (Figure 2A). To prevent these issues, antibodies against a platelet-specific marker, such as GP1bα or CD41, should be added to help distinguish platelets from RBCs or cellular debris[30].

Antibodies against platelet activation markers can be added to the test samples to assess response to stimulation with agonists. Whole blood should be diluted >1:10 to prevent generation of platelet aggregates[31]. Rigorous sample mixing (e.g. on a vortex mixer) should be avoided to prevent platelet activation and aggregation.

Table 1. Advantages and disadvantages of flow cytometry and LTA

	Flow cytometry	LTA
Advantages	Whole blood test Requires low amount of whole blood Allows platelet reactivity testing and measurement of surface expression	Measures platelet function over time
Disadvantages	Expensive Difficult to standardize Requires validation Only end-point measurements Does not measure platelet function under shear	Requires large amount of whole blood Not possible in samples with low platelet counts Labor intensive Time consuming Many pre-analytic variables Difficult to standardize Does not measure platelet function under shear

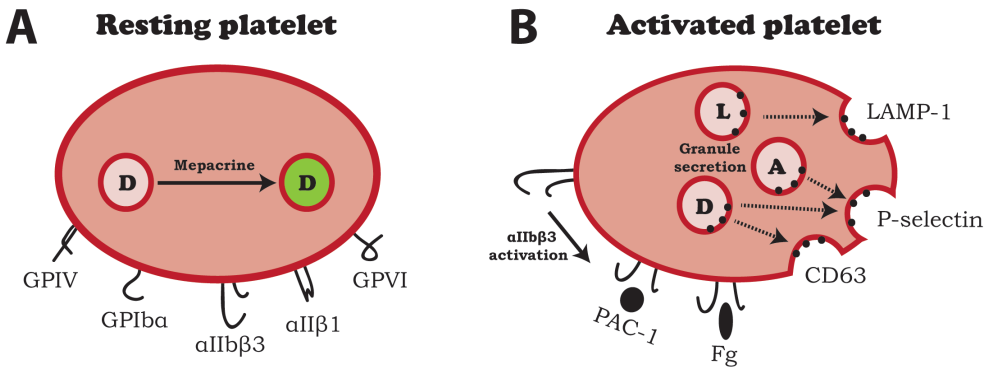

Figure 2. Markers for platelet function. In resting platelets, the most abundant platelet receptors can be quantified. These receptors include GPIV, GP1bα, αIIbβ3, αIIβ1 and GPVI. Also, the ADP content in dense granules (D) can be quantified using the fluorescent acridine derivative mepacrine. (B) After platelet activation, integrin αIIbβ3 activation results in binding of PAC-1 or fibrinogen (Fg). Upon platelet stimulation, dense granules, alpha granules (A) and lysosomes (L) will fuse with the outer membrane and platelet secretion markers, including CD63, P-selectin and LAMP-1, can be quantified.

Sample analysis cannot be carried out immediately, since samples should be fixed after incubation with agonists or antibodies against surface markers. Fixation can be performed with either formaldehyde, glutaraldehyde or methanol solutions, but standardized fixation reagents are also available. The main advantage of fixation is that samples can be collected prior to analysis, that samples can be transported to centers where flow cytometry is available, and that further ex-vivo activation events can be avoided.

Platelet function can be assessed in terms of either percentage positive events or median fluorescent intensity (MFI). The "percentage positive events" parameter is only useful when the threshold for positivity is based on unstimulated cells, since pre-activated platelets already express activation markers such as P-selectin. To sidestep underestimation of positive cells, thresholds should be determined in a sample with an isotype control antibody of the same immunoglobulin class, conjugated with an identical fluorophore. The "percentage positive events" parameter is particularly useful when activation markers are assessed in samples in which part of the population is not expected to express this marker. The major advantage of this method is that these semi-quantitative data are exchangeable between different laboratories, because the settings of flow cytometer and the evaluation software have modest influence on test outcome. Nevertheless, the drawback is that the percentage positive platelets parameter does not provide information about the degree of activation above the arbitrarily chosen negative threshold. Fully quantitative measures, such as MFI, provide more information on the degree of activation (Figure 2B), and are therefore more suited to quantify receptor expression than percentage positive events. The major drawback of the MFI is its dependence on the type of flow cytometer, laser quality and alignment, sensitivity and compensation settings of the flow cytometer, as well as on the analysis software. This makes it difficult to standardize flow cytometry, and compare

MFI among different laboratories. In diagnostics or long-term clinical studies, changes in laser quality and batch to batch variations could also introduce unwarranted variation. An internal quality control is advisable to achieve good reproducibility and allows a major degree of inter-laboratory exchangeability.

Platelet reactivity testing

Platelet function testing should include the assessment of the most common platelet signaling pathways leading to platelet activation. These include the collagen pathway, stimulated via GPVI, the thrombin pathway, in which protease activated receptor (PAR)-1 and PAR-4 play a role, the ADP pathway, in which the role of the purinergic receptor P2Y12 is deemed very important, and the thromboxane A2 pathway. These pathways can easily be assessed with a standardized agonist panel, similar to that used in LTA, and thus including ADP and peptide agonists for the thrombin receptors (PAR1-AP and PAR4-AP). However, the preferred agonist to test the collagen pathway in flow cytometry differs from the most commonly used source of collagen used in LTA. Whilst fibrillar equine collagen type I ('Horm collagen') is commonly used in LTA, this agonist is unsuitable for flow cytometry due to its fibrillar structure, which induces formation of platelet aggregates. Crosslinked collagen-related peptide is hence preferable in flow cytometry[32]. Another difference between LTA and flow cytometry is the relative insensitivity of platelets to exogenous arachidonic acid when platelets are not stirred, thus precluding direct appraisal of cyclooxygenase-1 (COX-1) activity with flow cytometry. Synthetic thromboxane A2 analogues, such as U46619, can be used to assess thromboxane A2-mediated platelet activation.

The optimal agonist concentration for assessing platelet reactivity differs among laboratories and depends on the assay purpose. For example, in case of platelet function testing for bleeding disorders, a high agonist concentration is needed to detect decreased platelet reactivity[33]. Since flow cytometry allows a fully-quantitative analysis of platelet reactivity based on median fluorescent activity, which cannot be made with semi-quantitative LTA, the true activation potential of platelets can be determined. On the other hand, a lower agonist concentration or a concentration gradient is more suitable in the presence of increased platelet reactivity.

Platelet reactivity can be measured using platelet activation markers (Figure 3), especially including those targeting or reflecting αIIbβ3 activation and granule secretion[34,35]. The measurement of αIIbβ3 activation can be performed with PAC-1 antibody or by measurement of fibrinogen binding[36]. PAC-1 binds the activated αIIbβ3 receptor, but fails to provide information on actual fibrinogen binding. On the other hand, fibrinogen binding assessment does not allow to distinguish between impaired αIIbβ3 function and fibrinogen deficiency.

Platelet secretion testing

The enumeration of dense granules per platelet using whole mount electron microscopy is the gold standard for diagnosing SPD[37]. Unfortunately, this technique is only available in few laboratories and is time consuming. The most commonly used tests are based on luminescence measurement of platelet ADP and ATP levels in platelet lysate [38] or on lumiaggregometric assessment of ATP

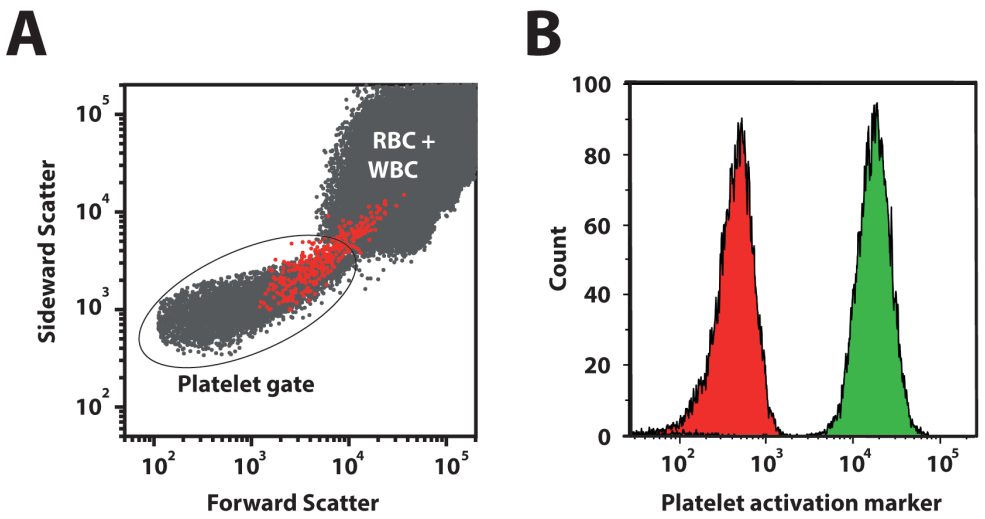

Figure 3. Whole blood platelet function testing on the flow cytometer. Typical plot of a 10-fold diluted whole blood sample (grey) on the flow cytometer. Platelets from healthy controls are identified based on Forward Scatter (FSC) and Sideward Scatter (SSC) using a platelet gate. Large platelets from a patient with macrothrombocytopenia (red; MPV of 20 fL) are hidden behind red blood cells (RBC) and white blood cells (WBC). (B) The median fluorescent intensity of a platelet activation marker in resting platelets (red) shifts to the right (green) in response to platelet stimulation.

secretion during platelet aggregation[39]. The assessment of ADP concentration in platelet lysate is a measure of potential platelet secretory capacity[40], whilst lumiaggregometry only measures ATP but not ADP secretion. Both tests cannot be performed in thrombocytopenic samples, and also encompass collecting a large volume of blood.

With flow cytometry, platelet secretion markers can be measured upon platelet stimulation. P-selectin, CD63 expression and LAMP-1 are intracellular markers expressed on platelet surface upon platelet activation. They can hence be used as markers of both platelet activation in general and, more specifically, of platelet secretion[41,42,43]. Although all of these proteins are present in intracellular granules and are exposed after activation, they are not completely interchangeable, as they provide information on different granule types. P-selectin, for example, is located in both alpha and dense platelet granules[44], whereas CD63 is only present in the dense granules and is hence a better marker for diagnosing SPD[45]. A low CD63 expression can result from impaired platelet activation, secretion or absence of platelet dense granules, and is thus not specific enough for diagnosing SPD. LAMP-1 is a lysosomal and dense granule specific protein, which is secreted upon activation[46].

The assessment of mepacrine uptake in resting platelets is an alternative approach for flow cytometric quantification of dense granule content. Mepacrine is a fluorescent acridine derivative with high affinity for adenine nucleotides, which are primarily stored in platelet dense granules. Therefore, mepacrine-labeled dense granules may allow their quantification with flow

cytometry. Although mepacrine uptake is low in patients with SPD, this technique has not been validated and its diagnostic performance has not been compared with that of other SPD specific diagnostic tests[47,48].

In addition to these classical markers of platelet activation, flow cytometry can be used assess the expression of anionic phospholipids on activated platelets, a method also known as platelet procoagulant activity. Anionic phospholipids, such as phosphatidylserine, are essential for propagation of coagulation reaction, and platelets are considered the main source of these phospholipids at the site of injury. In Scott syndrome, platelets lack the membrane phospholipid scramblase necessary for phosphatidylserine expression, thus resulting in decreased procoagulant activity and bleeding diathesis[49]. Procoagulant activity can be measured with fluorophore-conjugated annexin A5,[50] which specifically binds to phosphatidylserine in the presence of Ca^{2+} ions, or lactadherrin,[51] a calcium-independent phosphatidylserine-binding protein.

Finally, ristocetin-induced VWF binding to platelet can be measured in LTA to assess GP1b function. This can be mostly used for investigating BSS, type 2B VWD or platelet-type VWD. The ristocetin-induced VWF binding assay can also be performed with flow cytometry to assist the diagnosis of VWD or BSS[52,53]. Notably, quantification of GP1b receptor remains the gold standard for diagnosis BSS.

Other applications of flow cytometry based platelet function testing

Beside the potential application of flow cytometry in diagnosing bleeding disorders, there are other platelet-related applications in which flow cytometry may be useful.

One of these applications is in transfusion medicine. Long-term storage of platelet concentrates causes platelet storage lesion, characterized by decreased platelet function and viability[54]. The in vitro quality of platelet transfusion concentrates is therefore frequently assessed using flow cytometry. The expression of platelet surface markers like GP1b and αIIbβ can be investigated to monitor platelet quality[55], but the expression of P-selectin or CD63 in resting platelets is also used to assess pre-activated platelets[56]. PS-exposure can be determined via Annexin V binding or lactadherin to identify the amount of apoptotic platelets in stored platelet concentrates[55]. Nevertheless, the correlation of these markers with in vivo viability and function of platelets remains poor[57]. The residual platelet activation potential, or the agonist-induced platelet reactivity of residual platelet activation potential, may better predict in vivo viability of stored platelets[58]. However, this approach has not been validated so far.

Although thrombotic risk estimation and anti-platelet drug monitoring are other potential applications of flow cytometry in platelet function testing, the clinical usefulness of platelet function testing in patients receiving anti-platelet drugs remains uncertain. The vasodilator-stimulated phosphoprotein (VASP) assay is an already available flow cytometric assay for measuring P2Y12 function and quantifying the phosphorylation state of VASP after ADP stimulation[59]. A good correlation was demonstrated with LTA after in vitro P2Y12 inhibition[60], but a poor correlation was instead observed for measurement of antiplatelet efficacy in patients receiving anti P2Y12 therapy [61].

VALIDATION OF FLOW CYTOMETRIC ASSAY IS CHALLENGING

The validation of a flow cytometry-based test using clinical presentation as gold standard is unfeasible. The hemostatic system is complex, and its disorders are associated with overlapping clinical signs and symptoms. Only based on signs and symptoms, it is hence challenging to classify bleeding disorders into those affecting vascular wall integrity, PFDs, VWD, coagulation factor deficiencies or fibrinolytic dysfunction[62]. A validated bleeding score like the ISTH-BAT can be useful to assess whether a patient has an increased bleeding risk, but the results of the bleeding scores should always be interpreted with caution. Almost 25% of young healthy women report at least 2 bleeding symptoms, thus generating a high bleeding score in the absence of clinically significant bleeding[63]. Moreover, a high ISTH-BAT bleeding score does not allow to differentiate PFDs and VWD, and cannot be used to classify a particular type of PFD. Therefore, the ISTH-BAT is mainly used as a screening tool to determine whether additional laboratory testing is necessary. When diagnostic tools do not display sufficient sensitivity and specificity, the distinction between platelet dysfunction and other defects is challenging.

There is no single gold standard test for PFDs. Although LTA is the most commonly used platelet function test, and is an important diagnostic aid, it cannot be used to diagnose all PFDs. LTA measures agonist-induced aggregation, and the test is hence sensitive to both functional defects and platelet quantity. Due to this limitation, LTA is relatively insensitive to diagnose BSS, which is frequently associated with thrombocytopenia. Moreover, patients with an overt bleeding diathesis due to SPD might display normal platelet aggregation measured with LTA[20,64]. Additional diagnostic tools, such as flow cytometry, or platelet nucleotide content, are necessary in these cases to make an accurate diagnosis.

The biggest challenge in validating a new platelet function test, such as flow cytometry, is defining the patient population. Without a gold standard, which can be a single test or a combination of several tests, it is impossible to determine whether an abnormal flow cytometric measurement should be considered a true-positive or a false-positive. An ideal population to validate new diagnostic tests consists of a patient group in which the presence of the disorder can be confirmed or excluded based on a gold standard (reference) test. SPD is an example of a primary hemostasis disorder in which these studies are possible, because the diagnosis SPD is based on platelet ADP content, for which well-defined and reproducible tests are available[6]. In a SPD population, the diagnostic accuracy of both LTA and flow cytometry can be compared with the gold standard (i.e., platelet ADP content). Nevertheless, SPD is only one example of the many different causes of PFDs, and represents a minority of all patients with a defect of primary hemostasis. The majority of patients presenting with a bleeding diathesis will have a (mild) platelet function disorder of unidentified cause, and will have a heterogeneous laboratory phenotype[22]. If a test with known limitations such as LTA would be considered a gold standard test in this study population, new tests are likely to be non-superior, because abnormal findings would then be considered false-positive, although they might be accurate.

Genetic analysis could help the diagnosis of patients with inherited PFDs. The candidate list of genes involved in platelet function and thrombopoiesis currently includes as many as 329 genes[65],

and they are expected to increase further in the future[66]. Nevertheless, a genetic abnormality is found in the minority patients with an identified PFD using laboratory testing[67]. Most of the causative genetic determinants of PFDs are still unknown, with exception of severe inherited platelet disorders such as GT or BSS. Genetic determinants of most other platelet disorders are less clear. Genetic analysis of patients with PFDs will hence yield genetic variants of unknown (even innocent) significance, and this will take years and extensive research to be validated[68]. Ultimately, this may allow prediction of bleeding severity based on a genetic profiling[69]. However, this will remain challenging in patients with bleeding disorders caused by multiple genetic abnormalities[70], or in those with mild PFD combined with other hemostatic disorders[71]. Unfortunately, genetic testing in acquired PFDs is not helpful, so that functional testing will remain always necessary.

An alternative to an observational design aimed to validate a diagnostic test is a (prospective) follow-up study design, in which outcome of the platelet function test under investigation is validated by association with future bleeding episodes in patients with suspected PFD. This design may be reliable in a cohort of patients with severe platelet disorders, due to the frequent bleeding episodes they are likely to suffer. In case of mild PFDs, however, the bleeding episodes are rare, thus making it challenging to perform a follow-up study with sufficient power to draw conclusions[72]. Moreover, prophylactic treatment of (mild) bleeding disorders will preclude the use of a follow-up study design[73].

Validation of a flow cytometry-based platelet function test needs a clearly defined patient population with an elevated bleeding score. In these patients, coagulation factor deficiencies, VWD and fibrinolysis disorders should be excluded. Preferably, disorders of vascular and connective tissue should also be ruled out, but this will be challenging. In this circumstance the 'gold standard' is the clinical presentation in combination with exclusion of other easily diagnosed hemostatic disorders. By validating flow cytometry in this cohort, the diagnostic accuracy can be determined. The disadvantage of such a design is that the risk of false-positive results cannot be completely excluded, but this risk is likely similar for both the clinical presentation and flow cytometry. The number of false-positive results can be verified by repeated measurements. Moreover, perfect diagnostic accuracy is not predictable, because the studied population may contain patients with normal platelet function, or the platelet function cannot be measured thoroughly with one static assay. Therefore, we should develop new tools providing added value to the currently available diagnostic workout for improving knowledge and detection of PFDs. This may especially encompass diagnostic tools allowing measurement of platelet adhesion function under flow conditions, which cannot be detected by either LTA or flow cytometry.

CONCLUSIONS

Platelets exert many different functions in the generation of a stable hemostatic plug. Different techniques are used for diagnosing PFDs in routine or specialized clinical laboratories, but they all have their own limitations. Diagnostics of PFDs might benefit from the use of flow cytometry because of its many practical advantages, its possible use in different disciplines and the ability to assess a vast array of different platelet functions. Nevertheless, validation is necessary to establish its diagnostic value compared to the currently available tools, but also for associating test outcome

with clinical presentation. Enhanced diagnostic opportunities may improve the quality of life or treatment strategy and might ultimately increase the ability to dissect complex genotype-phenotype correlations.

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Ivar van Asten[1,2,3], Roger E.G. Schutgens[2,3], Marije Baaij[1,2,3],
Judith Zandstra[1], Mark Roest[1], Gerard Pasterkamp[1], Albert Huisman[1],
Suzanne J.A. Korporaal[1],Rolf T. Urbanus[1]

[1] Center for Circulatory Health, Department of Clinical Chemistry and Haematology,
University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands.
[2] Van Creveld Laboratory, University Medical Center Utrecht,
Utrecht, the Netherlands.
[3] Van Creveldkliniek, University Medical Center Utrecht, Utrecht, the Netherlands.

3

VALIDATION OF FLOW CYTOMETRIC ANALYSIS OF PLATELET FUNCTION IN PATIENTS WITH A SUSPECTED PLATELET FUNCTION DEFECT

ABSTRACT

Background

Light transmission aggregometry (LTA) is the most commonly used test for the diagnosis of platelet function disorders (PFDs), but has moderate sensitivity for mild PFDs. Flow cytometry has been recommended for additional diagnostics of PFDs but is not yet standardized as a diagnostic test. We developed a standardized protocol for flow cytometric analysis of platelet function that measures fibrinogen binding and P-selectin expression as platelet activation markers in response to agonist stimulation.

Objectives

To determine the additional value of flow cytometric platelet function testing to standard LTA screening in a cross-sectional cohort of patients with a suspected PFD.

Methods

Platelet function was assessed with flow cytometry and LTA in 107 patients suspected of a PFD in whom Von Willebrand disease and coagulation factor deficiencies were excluded. Both tests were compared in terms of agreement and discriminative ability for diagnosing patients with PFDs.

Results

Out of 107 patients, 51 patients had an elevated bleeding score. 62.7% of the patients had abnormal platelet function measured with flow cytometry and 54.2% of the patients were abnormal based on LTA. There was fair agreement between LTA and flow cytometry (κ=0.32). The discriminative ability of flow cytometric analysis in patients with an elevated bleeding score was good (AUC 0.82, 0.74-0.90), but moderate for LTA (AUC 0.70, 0.60-0.80). Both tests combined had a better discriminative ability (AUC 0.87, 0.80-0.94).

Conclusion

Flow cytometric analysis of platelet function has added value in diagnostics of PFDs in patients with unexplained bleeding tendency.

INTRODUCTION

Platelets have an important role in the preservation of blood flow. Upon vascular damage, platelets prevent excessive blood loss by the formation of a stable platelet plug at the site of injury. Platelet function disorders (PFDs) are characterized by spontaneous mucocutaneous bleeding, easy bruising, menorrhagia, or an extended bleeding time[1]. PFDs are highly heterogeneous and may be inherited or acquired. Patients with PFDs may have an abnormal platelet count (thrombocytopenia) or impaired platelet function, but may also have both[2].

Mild PFDs are usually associated with mild bleeding symptoms that manifest after trauma or other hemostatic challenges. The prevalence of mild bleeding symptoms in the general population is high, hampering identification of patients with mild PFDs. Standardized bleeding scores, such as the Bleeding Assessment Tool of the International Society on Thrombosis and Haemostasis (ISTH-BAT)[3], have been developed to objectify bleeding symptoms and have shown high negative predictive value, but low specificity and positive predictive value[4]. One study showed that nearly 25% of healthy young women experienced two or more bleeding symptoms, indicating that these bleeding scores should be interpreted with caution[5].

Diagnosing severe inherited PFDs, such as Glanzmann Thrombasthenia (GT) or Bernard Soulier Syndrome (BSS), is relatively straightforward, since these disorders have a clear clinical presentation and the platelet function defects are readily detected with currently available diagnostic tools.

Laboratory diagnostics of mild PFDs are more challenging, as they often present with a heterogeneous phenotype. Phenotyping these disorders requires highly specialized laboratory techniques, which are not available at most diagnostic laboratories, precluding the diagnosis of a PFD[6,7]. If the diagnosis of a PFD can be made, the exact cause remains unclear in 34% to 60% of patients[6,8]. Nevertheless, a correct diagnosis in patients with a PFD is important for risk stratification, therapeutic intervention, and quality of life.

The most commonly used platelet function test for diagnosing PFDs is light transmission aggregometry (LTA)[9]. In combination with other functional tests, LTA is an important assay in the characterization of different (severe) PFDs, but lacks sensitivity for mild PFDs including Storage Pool Disease (SPD)[6,10,11]. Furthermore, LTA is time-consuming, operator dependent, labor intensive, needs large volumes of blood[9]. Although the test itself is not very reproducible, the diagnosis of a PFD based on LTA can be confirmed in 90% of the cases[12]. There is poor consensus about which agonists and concentrations should be used, resulting in low agreement between laboratories[9,13]. Finally, LTA requires a minimum platelet count of $150x10^9$/L in platelet rich plasma (PRP)[14]. Experiments of samples with a platelet count <$75x10^9$/L should be interpreted with caution, which is a problem when attempting to identify platelet function disorders in thrombocytopenic patients[15].

There is an unmet need for methods that improve the diagnostic accuracy of (mild) PFDs. An alternative approach to test platelet function is by means of flow cytometry. Here, platelet activation can be determined by quantifying fibrinogen binding to integrin αIIbβ3, or expression of P-selectin or CD63 on the platelet surface upon stimulation[16,17], but also ristocetin induced vWF-platelet binding can be determined[18,19]. Flow cytometry-based approaches for the assessment of platelet function have already been used for many years in research settings[20,21]. The major advantage of a flow cytometry based approach is that it requires a small amount of blood and allows

the analysis of platelet function in thrombocytopenia[22,23]. The ISTH SSC recommends the use of flow cytometry in the diagnostic workup of PFDs[9], but lack of standardization prevents the use of flow cytometry as a diagnostic test. Therefore, we optimized a flow cytometry-based platelet activation test (PACT) for diagnostic use, which measures P-selectin expression and αIIbβ3 activation after stimulation with a variety of different agonists.

In this study, we validated our assay in patients with a well-defined hereditary platelet function disorder. We subsequently determined the added value of flow cytometric analysis of platelet function in a cross sectional cohort of patients with unidentified bleeding tendency in whom VWD or coagulation disorders were excluded and who were referred for platelet function testing to a tertiary referral hospital.

METHODS

Participants

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

Patients. Two patient cohorts were used in this study. In cohort 1, patients with a previously diagnosed PFD were included. These patients were diagnosed with SPD, GT or BSS. Cohort 1 was used for the proof of principle of the flow cytometric PACT assay. In cohort 2, 143 consecutive patients with a mucocutaneous bleed pattern that were referred to a hemophilia treatment center for LTA analysis were included. After visiting the hematologist, a bleeding score was calculated using the ISTH-BAT[3]. Exclusion criteria were von Willebrand Disease, coagulation factor deficiencies, pregnancy and age < 18 years. Approval for this study was obtained from the medical ethics review board of the UMC Utrecht. Written informed consent was obtained from patients with hereditary platelet function disorders (cohort 1) in accordance with the declaration of Helsinki. Informed consent requirement was waived by the Institutional Review Board for patients included in cohort 2.

Blood collection

Peripheral venous blood from patients and controls was drawn by venipuncture into 109 mM trisodium citrate Sarstedt tubes in a 9:1 (v:v) blood to anticoagulant ratio. All blood samples were processed within 1-6 hours after blood collection.

Reagents

PE conjugated anti-P-selectin (AK4) and APC conjugated anti-GP1b (HIP1) antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA), FITC conjugated rabbit anti-human fibrinogen was obtained from DAKO (Glostrup, Denmark) and FITC conjugated goat anti-human VWF was

obtained from Bio-Rad laboratories (Veenendaal, the Netherlands). Adenosine diphosphate (ADP) and indomethacin was from Sigma-Aldrich (Zwijndrecht, the Netherlands), fibrillar equine collagen I (Horm collagen) was from Takeda (Linz, Austria), crosslinked collagen related peptide (CRP-xl) was a generous gift from professor Richard Farndale (University of Cambridge, Cambridge, UK), ristocetin was purchased from American Biochemical and Pharmaceuticals Ltd (Marlton, NJ, USA), arachidonic acid was from Bio/data corporation (Horsham, PA, USA), protease activating receptor (PAR)-1 activating peptide SFFLRN (PAR1-AP) was obtained from Bachem (Weil am Rhein, Germany) and PAR-4 activating peptide AYPGKF (PAR4-AP) was from the Netherlands Cancer Institute (Amsterdam, the Netherlands).

Light transmission aggregometry

LTA was performed at 37°C with the PAP-8E platelet aggregometer (Sysmex, Etten-Leur, Netherlands) within 3 hours after blood collection. PRP was obtained by centrifugation of whole blood at 160g for 15 minutes at 20°C. Platelet counts were adjusted to 250 x 10^9 platelets/L with platelet poor plasma, obtained by centrifugation of the remaining blood (2000 g, 15 minutes, 20°C). LTA is dependent on platelet count in PRP and can be inaccurate at lower platelet counts [24]. LTA data was not obtained when platelet count < 75x10^9/L. Aggregation was initiated with ADP (2.5 and 5.0 µM), Horm collagen (1.0 and 4.0 µg/mL), arachidonic acid (1.5 mM) or ristocetin (1.0 mg/mL). Samples were stirred at 900 rpm and aggregation traces were recorded for 15 minutes and the final amplitude (FA) of the aggregation curve was evaluated. Cut-off levels for differentiation between normal and abnormal responses were based on the 2.5th percentile of the FA of 58 healthy controls for each agonist.

Flow cytometric analysis of platelet activation markers in whole blood

Whole blood was diluted 1:10 in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$ x $6H_2O$, 5 mM KCl pH 7.4) which contained a platelet agonist and either FITC conjugated anti-fibrinogen (1:100) antibodies and PE-conjugated anti-P-selectin antibodies (1:25) or FITC conjugated anti-VWF antibodies (1:1000). We used a streamlined agonist panel similar to what was described for LTA[25]. To confirm BSS diagnosis, APC conjugated anti-GP1b (1:25) was diluted in HBS. Whole blood was stimulated for 20 minutes with either a single concentration of agonist (30 µM ADP, 100 µM PAR1-AP, 1500 µM PAR4-AP or 1µg/mL CRP-xl) or serial dilutions (0.008-125µM ADP, 0.153-2500 ng/mL CRP-xl, 0.038-625 µM PAR1-AP, 0.2-4000 µM PAR4-AP or 0.00-0.75 mg/mL ristocetin) at room temperature as indicated. Platelet count does not influence platelet responses to agonists [26]. Samples were fixed (0.148% formaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH_2HPO_4, 10.2 mM Na_2HPO_4, 1.15 mM KH_2PO_4, 4mM EDTA, pH 6.8) for 20 minutes and analyzed on a BD Accuri flow cytometer (BD Biosciences). Prior to analysis, the flow cytometer was calibrated using fluorescent beads. Platelets were identified with forward and sideward scatter and Median Fluorescent Intensity (MFI) data were obtained. MFI was normalized to correct for lot-to-lot variations. Area Under the Curve (AUC) and EC50 were calculated in samples where a concentration range of agonists was used to stimulate platelets.

Statistical analysis

Measurements were screened for normality with the Shapiro-Wilk normality test, and test reference values of the LTA (n=58) and the standardized flow cytometry based platelet reactivity test (n=202) were determined according CSLI guideline EP09A3 [27]. Percentage Final Amplitude (FA) in LTA and the MFI of P-selectin expression and fibrinogen binding to αIIbβ3 were considered abnormal below the 2.5th percentile of the control population.

A receiver operator curve (ROC) was based on a probability score created with multiple logistic regression analysis of the 9 variables for the flow cytometric platelet reactivity test (MFI of P-selectin expression and fibrinogen binding after stimulation by PAR1-AP, PAR4-AP, ADP and CRP-xl, and ristocetin induced vWF binding) and 4 variables for LTA (FA after ADP, arachidonic acid, collagen and ristocetin incubation). Sensitivity was plotted against the false positive rate to show discriminating ability of both tests for diagnosis.

All statistical analyses were performed using Graphpad Prism software version 6.0 (San Diego, CA, USA) and IBM SPSS statistics version 21 (Armonk, NY, USA).

RESULTS

Severe inherited platelet function disorders can be detected with a standardized flow cytometry-based platelet activation assay.

Dose response curves after platelet stimulation with ADP, CRP-xl, PAR1-AP, PAR4-AP, or ristocetin were obtained in 17 healthy controls. P-selectin expression was used as a marker for granule release (Fig. 1A), fibrinogen binding was used as a marker for αIIbβ3 activation (Fig. 1B) and VWF-binding was assessed as a marker for GPIb-IX-V functionality (Fig. 1C). All platelet activation markers increased with increasing concentrations of agonist and were maximal at the highest agonist concentrations used. To determine whether this flow cytometry based assay was able to discriminate between normal and abnormal platelet responses, dose response curves were obtained in patients with severe inherited PFDs, including 5 patients with δ-storage pool disease (SPD), 4 patients with Glanzmann Thrombasthenia (GT) and one Bernard Soulier Syndrome (BSS) patient. The AUC was calculated for patients and controls as a measure of total platelet reactivity. Cut-off values to discriminate normal from abnormal responses were based on the 5th-95th percentile of the response in 17 healthy controls (Fig 1D-F). All patients with SPD had an abnormal response to one or more platelet agonists, but VWF-binding was normal in all SPD patients. As expected, fibrinogen binding was absent in platelets from patients with GT in response to all agonists. In contrast, P-selectin expression in GT patients was normal for all agonists, with the exception of ADP. VWF-binding was normal in all GT patients. In the patient with BSS, all platelet responses were normal, except for VWF-binding, which was reduced. Taken together, these data indicate that the flow cytometric platelet reactivity assay PACT can discriminate between patients with severe PFDs and healthy controls.

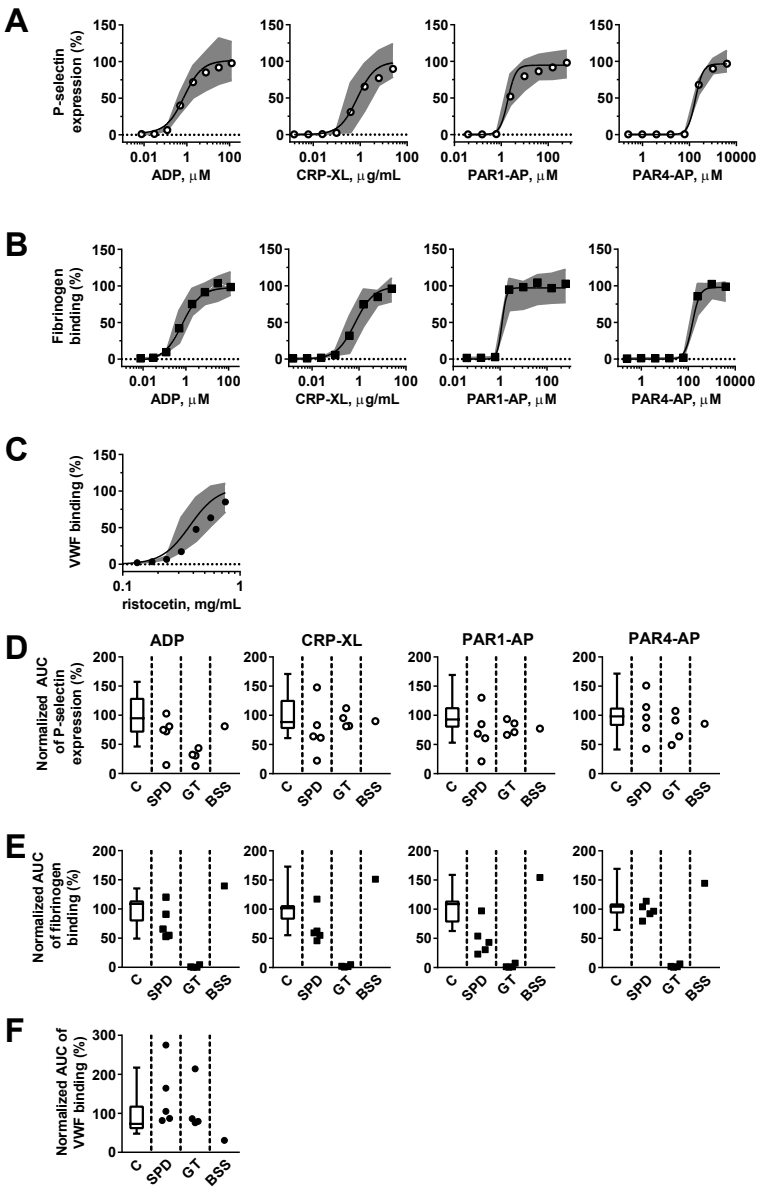

Figure 1. Platelet responses in healthy controls and patients with SPD, GT or BSS. Whole blood obtained from 17 healthy controls was stimulated with ADP, CRP-xl, PAR1-AP, PAR4-AP and ristocetin at the indicated concentrations for 20 minutes, fixed and subjected to flow cytometric analysis. P-selectin expression (A) was assessed as measure of granule release, fibrinogen binding (B) as a measure of αIIbβ3 activation, and VWF binding (C) to determine functionality of the GPIb-V-IX complex. Data were normalized on the median maximal result and expressed as median fluorescent intensity with 95-% confidence interval. The area under the curve (AUC) of normalized P-selectin expression (D), fibrinogen binding (E) and VWF binding (F) of 5 patients with δ-storage pool disease (SPD), 4 patients with Glanzmann Thrombasthenia (GT) and 1 Bernard Soulier Syndrome patient (BSS) was compared with the AUC in healthy controls (box and whiskers). The error bars indicate the 95%-confidence interval of the control population.

Performance characteristics of the standardized flow cytometry-based platelet reactivity test PACT

Analysis of the area under the dose-response curve yielded results that were similar to those obtained with analysis of the response to a high concentration of agonist (supplementary table 1). We therefore simplified the test by including a single concentration for each agonist derived from the shoulder region of the dose response curve: 30 μM ADP, 100 μM PAR1-AP, 1500 μM PAR4-AP, 1 μg/mL CRP-xl or 0.4 mg/mL ristocetin. Reproducibility was assessed with 6 repeated measurements from 2 healthy controls on three days, at least one week apart. Blood from 1 of these donors was treated with 100 μM indomethacin to mimic reduced platelet reactivity. The mean coefficient of variation of the response to each agonist was 7.4% in healthy platelets and 5.8% in platelets with decreased reactivity, indicating good reproducibility. All coefficients of variation for single agonists were < 10%.

Standardized flow cytometry based platelet reactivity testing has added value on top of LTA in identification of PFDs.

In cohort 2, 143 consecutive patients with a suspected PFD were enrolled (Fig. 2). 20 out of the 143 patients were below 18 years of age, 6 patients were pregnant, 7 patients had von Willebrand Disease and 3 patients had a coagulation factor deficiency. In the remaining 107 patients with a suspected PFD (supplementary table 2), we determined platelet reactivity with both LTA and PACT to determine whether flow cytometry can be used to identify patients with a PFD. The mean platelet count was $224x10^9$/L (range: 7-640). LTA data were obtained in 100 patients (93%); LTA data were missing in the remaining 7 patients due to platelet counts <$75x10^9$/L. Flow cytometric analysis of platelet function was performed in all patients. The reference values for the LTA were based on the 2.5th percentile in 58 healthy controls, but at 2.5 μM ADP and 1.0μg/mL collagen, the cut-off value was at 0% aggregation. Therefore, only 5.0 μM ADP, 1.5 mM arachidonic acid, 1.0 μg/mL collagen and 1.0 mg/mL ristocetin were used for analysis. Reference values for the PACT were established, based on the 2.5th percentile of P-selectin expression, fibrinogen binding and VWF binding in 202 healthy volunteers (supplementary table 3). As expected, platelet reactivity towards agonists varied substantially in the general population (Fig. 3A-D).

There was large variation between individuals in platelet reactivity measured with flow cytometry, especially when αIIbβ3 activation was measured in our patients (Fig. 3E-H). Out of 107 patients, 57 patients (53.3%) had at least one reduced response to a platelet agonist, of whom 26 patients (45.6%) showed both reduced fibrinogen binding and decreased P-selectin expression, 22 patients (38.6%) had reduced fibrinogen binding but normal P-selectin expression, and 9 patients (16%) had reduced P-selectin expression but normal fibrinogen binding.

Out of 107 patients with a suspected PFD, 51 patients had an elevated bleeding score [28] (>3 in men; >5 in women). Thirty-two out of these 51 patients showed decreased platelet reactivity with PACT, whereas 26 out of 48 patients showed decreased platelet reactivity with LTA (Table 1), and therefore had a PFD. LTA data were unavailable in three patients due to thrombocytopenia. Twenty of 26 patients with an abnormal LTA also had abnormal PACT results. Nine out of 32 patients with

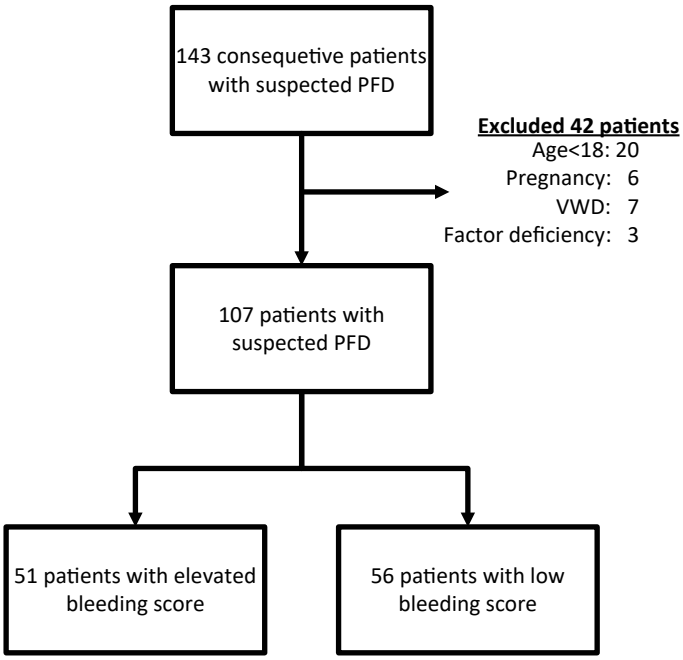

Figure 2. Inclusion of patients with suspected PFD in cohort 2.

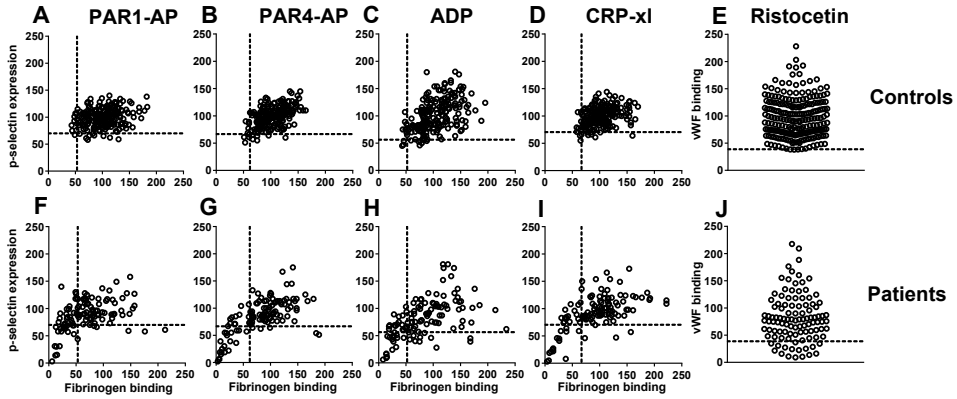

Figure 3. Platelet reactivity after stimulation of different agonists in healthy controls (n=202) and patients with suspected PFDs (n=107). Platelet reactivity was measured after stimulation with PAR1-AP (A&F), PAR4-AP (B&G), ADP (C&H), CRP-xl (D&I) and ristocetin induced vWF binding (E&J). Every dot represents the P-selectin expression (y-axis) and fibrinogen binding (x-axis) of one individual per agonist. The dotted line per agonist is based on the 2.5th percentile of the healthy control population. Healthy controls are plotted in A-E and the patient population is plotted in F-J.

abnormal PACT results showed normal platelet aggregation. Agreement between LTA and PACT was fair (κ=0.32; P<0.05)(Table 2).

Discriminative ability between patients and healthy controls

Next, diagnostic accuracy was estimated with ROC analysis. PACT and LTA data from all 107 patients, the 51 patients with a high bleeding score, or the 56 patients with a low bleeding score were compared with PACT and LTA results obtained in a cohort of 58 healthy controls (Fig 4). LTA data from 7 patients were missing due to a platelet concentration <75x10^9/L of whom 3 had a high bleeding score and 4 had a low bleeding score. Therefore, in 48 patients with a high bleeding score LTA data was available and in 52 patients with a low bleeding score was available. The flow cytometry based test data was obtained in all patients.

Performance of the PACT (AUC 0.74, 0.66-0.82) was similar to performance of LTA (AUC 0.65, 0.57-0.74; P=0.14) (Table 3). Combined, LTA and flow cytometry (AUC 0.80, 0.74-0.87) performed better than LTA alone (P<0.01), but performance of the combination of LTA and flow cytometry was similar to PACT alone (P=0.23). When analysis was limited to patients with an elevated bleeding score, the performance of PACT improved (AUC 0.82, 0.74-0.90) compared with LTA (AUC 0.70, 0.60-0.80; P=0.07). The combination of both tests performed better than LTA alone (AUC 0.87, 0.80-0.94; P<0.01). The better performance of PACT than LTA could be due to the use of an extra agonist in the PACT compared to LTA. A comparison of the performance of single agonists in PACT and LTA showed similar results in both patients with a high and with a low bleeding score (Table 4).

Table 1. Flow cytometry based analysis of patients with an elevated bleeding score and a suspected PFD compared to LTA.

Flow cytometric platelet reactivity (n=51)		Platelet aggregation with LTA (n=48)*	
Normal reactivity	19 (37.3%)	Normal aggregation	22(45.8%)
Abnormal	32 (62.7%)	Abnormal	26 (54.2%)
Reduced, 1 agonist	5 (9.8%)	Reduced, 1 agonist	14 (29.2%)
Reduced, 2 agonists	6 (11.8%)	Reduced, 2 agonists	5 (10.4%)
Reduced, 3 agonists	3 (5.9%)	Reduced, 3 agonists	7 (14.6%)
Reduced, 4 agonists	13 (25.5%)	Reduced, 4 agonists	0
Reduced, 5 agonists	5 (9.8%)		

*No data available in 3 patients due to thrombocytopenia

Table 2. Fair agreement between LTA and PACT

		Abnormal PACT		
		No	Yes	
Abnormal LTA	No	13	9	22
	Yes	6	20	26
	Missing	0	3	3
		19	32	51

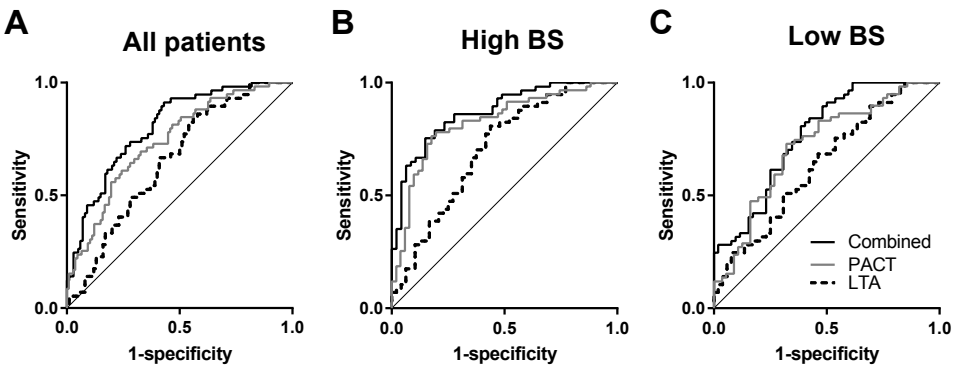

Figure 4. The discriminative ability of LTA and flow cytometry between healthy controls and patients with a suspected PFD. Probability scores for patients with a suspected PFD and 58 healthy controls were calculated with multiple logistic regression and plotted in an ROC curve to determine the discriminative ability of flow cytometric platelet function testing and LTA. The discriminative ability of the PACT and LTA was determined in all 107 patients (A), in 51 patients with a high bleeding score (B) and in 56 patients with a low bleeding score (C). LTA data was unavailable in 7 patients due to thrombocytopenia. LTA data was available in 48 patients with a high bleeding score and in 52 patients with a low bleeding score.

Table 3. AUC of discriminative ability in patients with suspected PFD

	LTA		PACT			Combined		
	Area	95% CI*	Area	95% CI	P-value†	Area	95% CI	P-value†
All	0.65	0.57-0.74	0.74	0.66-0.82	0.14	0.80	0.74-0.87	0.006
High BS	0.70	0.60-0.80	0.82	0.74-0.90	0.07	0.87	0.80-0.94	0.009
Low BS	0.64	0.53-0.74	0.72	0.62-0.81	0.27	0.77	0.68-0.86	0.06

*indicates the 95% confidence interval. † P-value in comparison with LTA
Abbreviations: LTA, Light Transmission Aggregometry; PACT, Platelet Activation Test.

Table 4. AUC of single variables in LTA and PACT

	LTA			PACT		
		Area	95% CI*		Area	95% CI
High BS	ADP	0.68	0.57-0.78	ADP	0.67	0.57-0.78
	Collagen	0.64	0.43-0.74	CRP-xl	0.58	0.47-0.69
	Ristocetin	0.62	0.53-0.75	Ristocetin	0.64	0.51-0.73
	AA	0.59	0.48-0.70	PAR1-AP	0.7	0.57-0.79
				PAR4-AP	0.62	0.51-0.73
Low BS	ADP	0.6	0.49-0.71	ADP	0.6	0.50-0.71
	Collagen	0.57	0.47-0.68	CRP-xl	0.57	0.47-0.68
	Ristocetin	0.56	0.44-0.67	Ristocetin	0.55	0.44-0.66
	AA	0.65	0.55-0.75	PAR1-AP	0.57	0.46-0.67
				PAR4-AP	0.57	0.46-0.67

*indicates the 95% confidence interval. † P-value in comparison with LTA
Abbreviations: LTA, Light Transmission Aggregometry; PACT, Platelet Activation Test.

DISCUSSION

This study shows that a standardized flow cytometric approach can be used to identify patients with a PFD. Patients with a severe inherited platelet disorder showed decreased platelet reactivity compared with healthy controls and flow cytometry could be used to discriminate between patients with a suspected PFD and healthy controls in a real life setting.

The ISTH/SSC guidelines recommend the use of flow cytometry in the diagnostic work-up of patients with PFDs[6]. Previous studies have shown that a flow cytometry based platelet function test corresponds well with the bleeding severity in patients with immune thrombocytopenia[22] and that it is a promising screening tool for patients with mild bleeding disorders. An additional advantage of flow cytometry compared with conventional platelet diagnostics is the small sample volume. Whereas conventional diagnostics require a large blood volume, flow cytometry can be performed with as little as 100 µL of whole blood, thus enabling platelet diagnostics in infants and small children[29]. Nevertheless, the technique needs standardization and validation before it can be implemented in a diagnostic laboratory. It has been shown that flow cytometry allows the sub classification of PFDs using CD63 expression and P-selectin expression in a patient population with previously categorized platelet abnormalities[16]. In our study, we demonstrated that flow cytometry has additional value to LTA in the diagnosis of patients with a suspected PFD by using fibrinogen binding instead of CD63 expression. As our population reflects the real-life patient population seen by the hematologist, our study shows that a standardized flow cytometry approach has diagnostic potential in patients with a suspected bleeding disorder.

Light transmission aggregometry is still considered the primary diagnostic tool during workup of PFDs, despite known limitations such as large variability in response in individual donors and low sensitivity for mild PFDs. Our study indicates that there is fair agreement between LTA and platelet function measured with flow cytometry. The differences between LTA and flow cytometry in our study can be explained in part by the agonist panels that were used in both tests. LTA was not performed with PAR1-AP and PAR4-AP, whereas the flow cytometric test did not contain arachidonic acid induced platelet activation, which is frequently impaired in patients with unidentified bleeding problems[30].

Interestingly, the diagnostic accuracy of flow cytometry for detection of patients with a suspected PFD was similar to that of LTA, with poor accuracy for LTA and moderate accuracy for flow cytometry. Accuracy was good when data obtained with LTA and flow cytometry were combined, suggesting both tests detect different patient populations and that a flow cytometric approach provides added diagnostic value. In patients with a high bleeding score, the diagnostic accuracy of the PACT appeared to improve more than LTA, but with only 51 patients with an increased bleeding score our study lacked sufficient power to attain statistical significance. However, in further support of the better performance of PACT, the combination of PACT and LTA performed similar to PACT alone. We cannot exclude that the better performance of PACT is caused by the number of agonists that were analyzed in each test. Whereas PACT investigated 5 agonists, LTA investigated only four. The equal performance of single agonists in both tests, combined with the added value of the PACT on top of LTA in our regression model indicates that the PACT is non-inferior to LTA in the identification of patients with decreased platelet reactivity.

Bleeding symptoms are rare in patients with a platelet count above 50 x 10^9/L, but normal platelet function. Therefore measuring platelet function in patients with thrombocytopenia is important[31]. Flow cytometry allows the measurement of platelet function in thrombocytopenia[32] and in our study all 7 patients with a platelet count lower than 75 x 10^9/L had decreased platelet reactivity, whereas platelet function could not be assessed in this population with LTA[15].

Validation of new diagnostic tests for PFDs is difficult because of the absence of a gold standard test. The increasing knowledge of the genetic background in PFDs might help in the evaluation of new approaches for platelet function testing. However, prediction of bleeding severity based on genetics is difficult[33] and is not helpful in acquired PFDs. Ideally, diagnostic tests can be compared to future bleeding episodes with a follow-up study design, but the low frequency of bleeding episodes in mild PFDs and prophylactic treatment of moderate to severe bleeding disorders preclude the possibility of such a strategy[34]. For this reason, we validated the flow cytometry based test in a population of patients with a positive bleeding history in whom a PFD is suspected, which corresponds to the real-life patient population seen by the hematologist. A drawback of this population is that there is no demonstrable PFD in some patients with a clear bleeding diathesis, making it impossible to determine whether a patient is true- or false-negative. Furthermore, we did not perform repeated measurements to verify the number of false-positive measurements.

The strength of this validation study is that a standardized assay with reference values was used to compare with LTA. The investigators were not blinded for case or control status in the determination of diagnostic accuracy, but data analysis was unbiased due to the use of a multiple logistic regression model.

With our study, we confirmed the utility of flow cytometric platelet function testing in severe PFDs and thrombocytopenia, but also showed that it provides added value to the routine diagnostic work-up in patients with a positive bleeding history and a suspected PFD. Therefore, flow cytometry-based platelet function testing should be considered as a promising tool in the diagnostic approach of PFDs and effort should be made to further validate and standardize flow cytometric tests for platelet function.

ADDENDUM

R.E.G. Schutgens, M. Roest, G. Pasterkamp and R.T. Urbanus designed the study. I. van Asten, M. Baaij and J. Zandstra performed the experiments. I. van Asten, R.E.G. Schutgens, M. Baaij, J. Zandstra, A. Huisman, S.J.A. Korporaal and R.T. Urbanus analyzed the data. I. van Asten, R.E.G. Schutgens, M. Baaij, J. Zandstra, M. Roest, G. Pasterkamp, A. Huisman, S.J.A. Korporaal and R.T. Urbanus wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

S.J.A. Korporaal and R.T. Urbanus are stock holders in U-PACT BV, a spin-off company from UMC Utrecht. The rest of the authors state that they have no conflict of interest.

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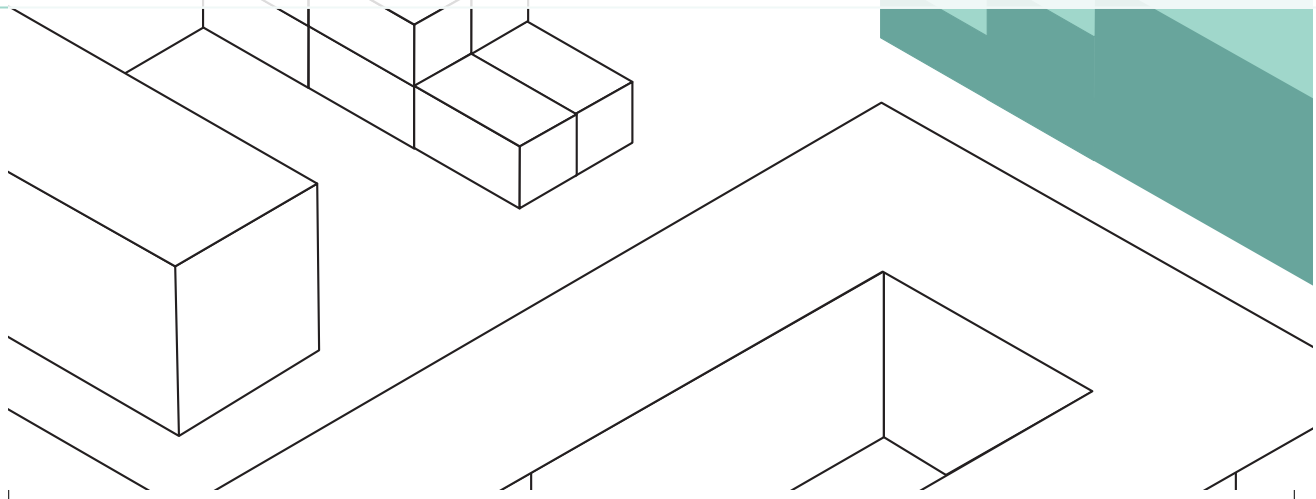
Ivar van Asten[1,2,3], Maaike Blaauwgeers[1,2], Lianne Granneman[1], Harry F.G. Heijnen[1], Marieke J.H.A. Kruip[4], Erik A.M. Beckers[5], Michiel Coppens[6], Jeroen Eikenboom[7], Rienk Y.J. Tamminga[8], Gerard Pasterkamp[1], Albert Huisman[1], Karin P.M. van Galen[2,3], Suzanne J.A. Korporaal[1,9] Roger E.G. Schutgens[2,3] and Rolf T. Urbanus[1,2,3]

[1] Van Creveld Laboratory, University Medical Center Utrecht, Utrecht, the Netherlands
[2] Van Creveldkliniek, University Medical Center Utrecht, Utrecht, the Netherlands
[3] Center for Circulatory Health, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands
[4] Department of Haematology, Erasmus University Medical Center, Rotterdam, the Netherlands
[5] Department of Hematology, Maastricht University Medical Center, Maastricht, the Netherlands
[6] Department of Vascular Medicine, Amsterdam Cardiovascular Sciences, Amsterdam University Medical Center, location AMC, Amsterdam, the Netherlands
[7] Department of Internal Medicine, division of Thrombosis and Haemostasis, Leiden University Medical Center, Leiden, the Netherlands;
[8] Department of Pediatric Hematology, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, the Netherlands.
[9] Laboratory of Experimental Cardiology, University Medical Center Utrecht, University Utrecht, Utrecht, The Netherlands.

4

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



ABSTRACT

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.

Patients/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

11/156 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 allows exclusion of δ-SPD with a limited amount of whole blood.

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 light transmission aggregometry (LTA) and the measurement of ADP and ATP using bioluminescence, the latter either in platelet lysate or with lumiaggregometry[9,10]. LTA is neither sensitive nor specific for platelet dense granule disorders[11,12]. 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[13], but is insensitive for secretion defects[14]. Interestingly, many diagnostic laboratories do not measure platelet nucleotide content, resulting in potential underdiagnosis of δ-SPD[15,16]. 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)[17,18]. 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[9,19]. 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[20], has been used to measure platelet dense granule content. Several studies showed decreased platelet mepacrine fluorescence in patients with δ-SPD[21-23]. Although these data are promising, performance of mepacrine fluorescence has not yet been compared with routine diagnostic tests for δ-SPD[24,25]. In the present study, we validated a flow cytometric mepacrine fluorescene 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. 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. 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 $MgSO_4x6H_2O$, 5mM KCl, pH 7.4), which contained 100 µM 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 (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 2.5th 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). Platelet ADP content (Figure 1A) was decreased in all patients with δ-SPD, whereas the platelet ATP/ADP ratio (Figure 1B) was increased in all patients with δ-SPD. In the 7 SPD 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 (2.5th 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 ($\kappa=1$) with platelet ADP content (Figure 2A) and mepacrine release after PAR1-AP activation was in good agreement ($\kappa=0.93$) with platelet ADP content (Figure 2B). Mepacrine fluorescence was also in good agreement ($\kappa=0.93$) with PAR1-AP induced mepacrine release (Figure 2C).

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 2.5th 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 (table 2) had a platelet ADP content below 1.4 µmol/10^{11} platelets and were diagnosed with δ-SPD (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

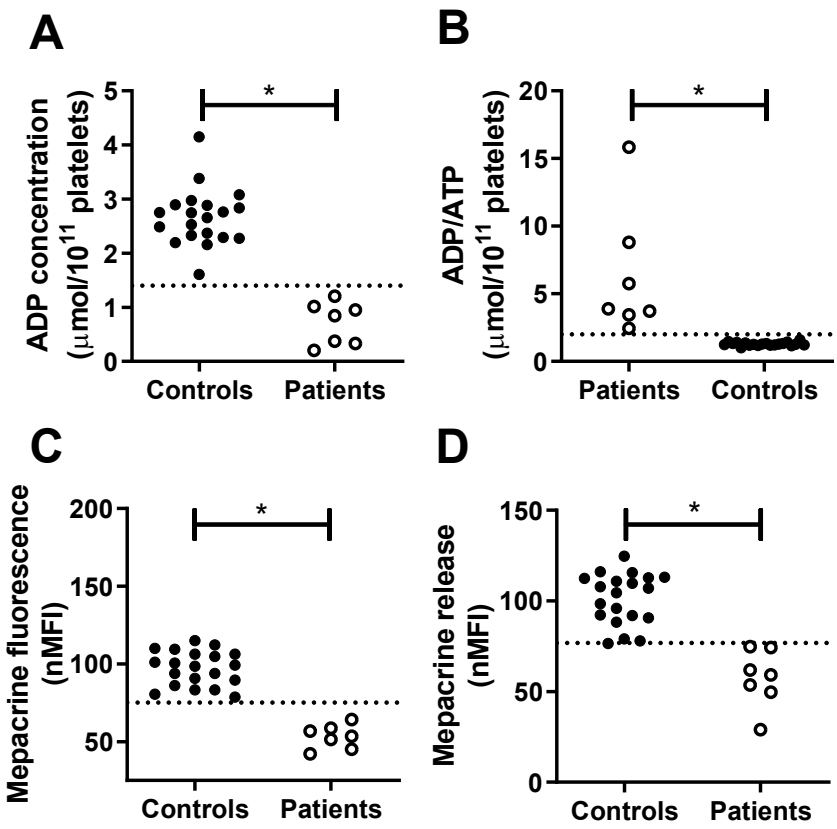


Figure 1. Good discriminative ability of flow cytometric measurement of platelet dense granule content in patients with previously diagnosed δ-SPD. (A) Platelet ADP content, expressed as µmol/10^{11} platelets measured with luminescence, (B) platelet ADP/ATP ratio, (C) Normalized mepacrine fluorescence 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. * indicates a p-value < 0.05.

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

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

IQR: inter-quartile range, MPV: mean platelet volume

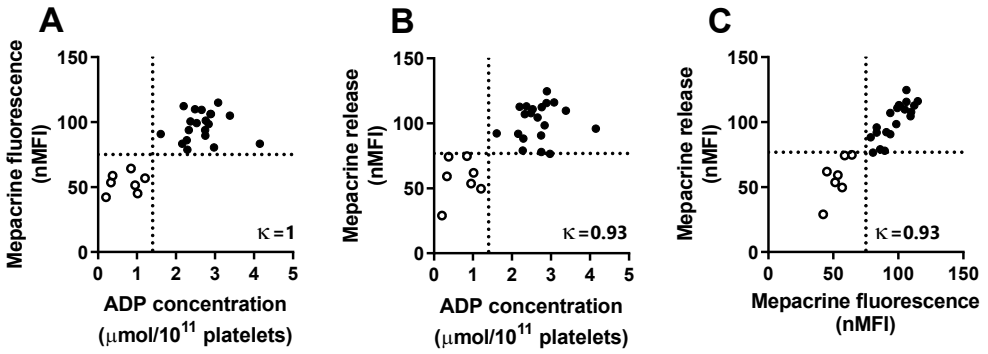


Figure 2. Good agreement between mepacrine fluorescence and platelet ADP content in diagnostic testing of δ-SPD. Cohen's kappa was calculated to determine the agreement between (A) mepacrine fluorescence and platelet ADP content (B) mepacrine release and platelet ADP content (C) mepacrine release and meapcrine fluorescence in 20 healthy controls 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 2.5th percentile of the healthy control population.

Table 2. Characteristics of patients with a suspected platelet function disorder

	Non-SPD (n=139)	SPD (n=17)	Reference value
Male sex (%)	22 (16)	7 (41%)	
Age (years), median (IQR)	38 (29-52)	41 (31-56)	
Bleedingscore, median (IQR)	9 (7-12)	10 (8-14)	Male <4, female <6[30]
Platelet count (10^9/L), median, (IQR)	235 (190-282)	200 (69-272)	150-450
MPV (fL), median, (IQR)	8 (7.2-8.7)	7.4 (6.5-8.2)	7.0-9.5
Platelet ADP content (μmol/ 10^{11} platelets), median, (IQR)	2.5 (2.1-2.8)	0.9 (0.5-1.2)	1.4-3.8

MPV= mean platelet volume

the area under a receiver-operator curve (AUC) (Figure 3F). 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 2.5th 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

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)

* 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

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.

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[24,27], but these studies did not compare the flow cytometric assays with routine diagnostic tests. Similar to these studies, we found that mepacrine fluorescence allows perfect discrimination between patients with confirmed δ-SPD and healthy controls. In

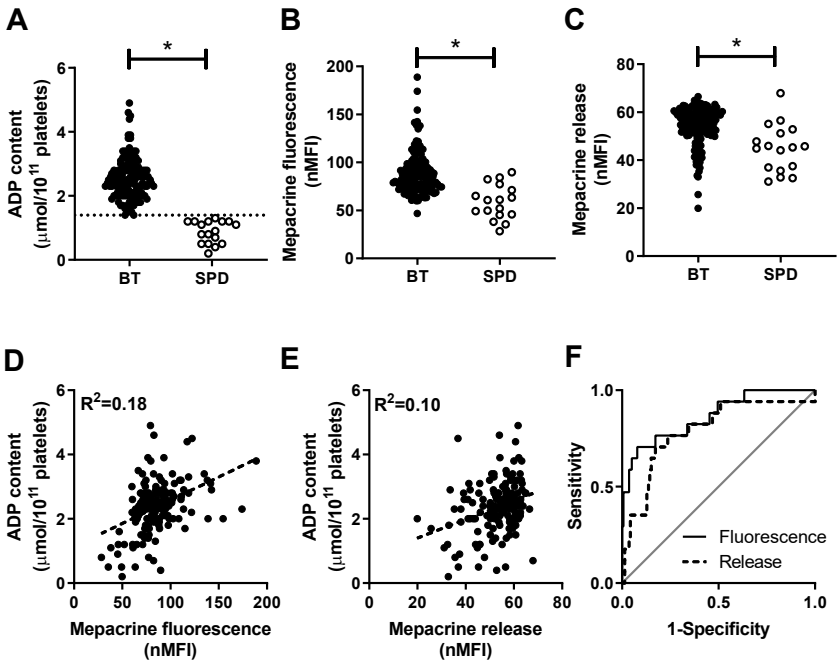


Figure 3. Diagnostic accuracy of flow cytometric mepacrine uptake and mepacrine release in patients with suspected platelet function disorders. (A) Platelet ADP content, expressed as µmol/10^{11} platelets, (B) mepacrine fluorescence expressed as normalized MFI, (C) normalized mepacrine release for all 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 156 patients with suspected platelet function disorder. (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. * indicates a p-value <0.05.

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.

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[28,29]. 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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Ivar van Asten[1,2,3*], Maaike W. Blaauwgeers[1*], Marieke J.H.A. Kruip[4], Erik A.M. Beckers[5], Michiel Coppens[6], Jeroen Eikenboom[7], Karin P.M. van Galen[1], Albert Huisman[3], Suzanne J.A. Korporaal[3,8], Hans Kristian Ploos van Amstel[9], Rienk Y.J. Tamminga[10], Rolf T. Urbanus[1,2], and Roger E.G. Schutgens[1]

[1] Van Creveldkliniek, University Medical Center Utrecht, University Utrecht, Utrecht, the Netherlands;
[2] Van Creveld Laboratory, University Medical Center Utrecht, University Utrecht, Utrecht, the Netherlands;
[3] Center for Circulatory Health, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, University Utrecht, Utrecht, the Netherlands;
[4] Department of Haematology, Erasmus University Medical Center, Rotterdam, the Netherlands;
[5] Department of Hematology, Maastricht University Medical Center, Maastricht, the Netherlands;
[6] Department of Vascular Medicine, Amsterdam Cardiovascular Sciences, Amsterdam University Medical Center, location AMC, Amsterdam, the Netherlands;
[7] Department of Internal Medicine, division of Thrombosis and Haemostasis, Leiden University Medical Center, Leiden, the Netherlands;
[8] Laboratory of Experimental Cardiology, University Medical Center Utrecht, University Utrecht, Utrecht, the Netherlands;
[9] Department of Medical Genetics, University Medical Center Utrecht, University Utrecht, Utrecht, the Netherlands;
[10] Department of Pediatric Hematology, Beatrix Children's Hospital, University Medical Center Groningen, Groningen, the Netherlands.
* Contributed equally

5

THE DIAGNOSTIC YIELD OF UPFRONT GENETIC TESTING IS LIMITED IN PATIENTS SUSPECTED FOR CONGENITAL PLATELET DEFECTS

ABSTRACT

Congenital platelet defects (CPD) are rare disorders of primary hemostasis caused by congenital defects in platelet production or function. Identification of CPDs is challenging and usually requires highly specialized tests and multiple hospital visits. DNA-based analysis has become increasingly important and recent studies have suggested moving genetic analysis 'upward' in the diagnostic approach. This study reports on the diagnostic value of upfront genetic testing in a prospective cohort of patients suspected for a CPD. In total, 175 patients were evaluated: 19 patients with a previously diagnosed CPD who served as positive control and 156 patients suspected for an CPD, of whom 96 previously had abnormal platelet count or function and 60 had a bleeding tendency suspected to be due to a CPD. Laboratory tests consisted of a complete blood count, light transmission aggregometry, nucleotide measurement, flow cytometry and whole exome sequencing with a selected 76 gene panel. In all control group patients, (likely) pathogenic genetic variants consistent with their CPD were identified. In the study group, a CPD was confirmed in 39% of patients and a possible CPD was identified in 13% of patients. A (likely) pathogenic mutation was identified in 13% of CPD patients. In patients with a normal platelet count and no objective platelet function defect (44%), no mutations were found. In conclusion, upfront genetic testing with our 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.

INTRODUCTION

Congenital platelet defects (CPD) are rare disorders of primary hemostasis. They can be due to congenital 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 signaling pathways.[1,2] Typical manifestations are mucocutaneous bleeds and extensive blood loss following a hemostatic challenge such as surgery and childbirth.[3]

Identification of CPDs is challenging due to the lack of a gold standard test. Some CPDs, such as Bernard-Soulier syndrome (BSS), Glanzmann thrombasthenia (GT) and MYH9-related disorders, are relatively easy to diagnose due to distinctive clinical and laboratory features, although a concomitant thrombocytopenia might hamper the assessment of platelet function. The remaining CPDs are often under- or misdiagnosed 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 these tests.[4] However, an accurate diagnosis is important for proper counseling and management of patients and to avoid ineffective and potentially harmful treatments due to ITP misdiagnosis.

Several studies have shown that DNA-based analysis, such as high-throughput sequencing, can improve the diagnostic yield for CPD patients.[5-9] 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, renal insufficiency and hematological malignancies associated with NBEAL2, MYH9 and RUNX1 variants. The International Society for Thrombosis and Haemostasis (ISTH) currently recommends to perform genetic analysis as a third-line investigation, i.e. after extensive phenotyping and functional analyses have confirmed the presence of a platelet disorder.[10] Recent studies on the efficacy of genetic testing in platelet disorders have suggested that genetic analysis could be moved 'upward' in the diagnostic approach in order to simply and hasten the diagnosis of IPDs.[5,9,11,12] However, it remains unclear whether genetic analysis should be performed as a first-line investigation, alongside initial functional analysis of platelet function in patients in whom a 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 for a CPD. Here, we report the results of this study.

METHODS

Participant selection

The TiN study is a nationwide cross-sectional study on CPDs, conducted in collaboration with the 6 hemophilia treatment centers in the Netherlands to collect data on clinical characteristics, functional assays and genetics in a real-life population of patients with a suspected platelet disorder. Patients of 18 years or older with a clinical or laboratory suspicion for a CPD were recruited from February 2016 to December 2017. The following patients were eligible to participate in the study:

1. Patients with a previously identified CPD, added as a validation group and used for the proof of principle of the selected gene panel
2. Patients suspected for a CPD based on previous abnormal platelet counts, LTA results or platelet ADP content without a molecular diagnosis
3. 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
4. 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

Exclusion criteria were use of antiplatelet drugs that could not be stopped prior to inclusion, a known acquired platelet disorder, von Willebrand disease (VWD), hemophilia or another disorder of secondary hemostasis or fibrinolysis, and inability to give informed consent. All patients were referred to one tertiary reference center (Van Creveldkliniek, UMC Utrecht) for documentation of baseline characteristics, the ISTH bleeding assessment tool (ISTH-BAT) and for all laboratory assessments. The study was approved by the Medical Ethical Committee of the University Medical Center Utrecht and all participants gave written informed consent in accordance with the declaration of Helsinki.

Healthy volunteers were obtained to determine reference values for flow cytometry and platelet ADP content 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 Utrecht. All participants gave written informed consent in accordance with the declaration of Helsinki. The donors did not have a bleeding tendency (ISTH-BAT <4 in men and <6 in women) and reported to be free from antiplatelet drugs or non-steroid anti-inflammatory drugs for at least ten days prior to blood donation. Healthy volunteers for reference values for LTA were obtained previously.[13]

Diagnostic methods

The diagnostic approach is depicted in Figure 1. Blood was collected into vacuum citrated tubes for platelet function testing and EDTA for whole blood count, blood smear and genetics through 21G needles, by a specialized research physician. LTA was performed between 1-3 hours after collection and flow cytometry between 1-6 hours, as described previously.[13] All reference intervals were determined according to the CSLI guideline EP28A3C.[14]

Assessment of bleeding tendency

Bleeding tendency was assessed with the ISTH-BAT[15] by experienced physicians. The frequency and severity of 14 different bleeding symptoms were scored on a scale ranging from 0 to 4 points. Since none of the study group patients had a definitive diagnosis of CPD, all symptoms prior to inclusion were reported. Bleeding scores were computed from the total of all items. Signs and symptoms suggestive for syndromic platelet defects (e.g. albinism, hearing loss) were routinely examined.

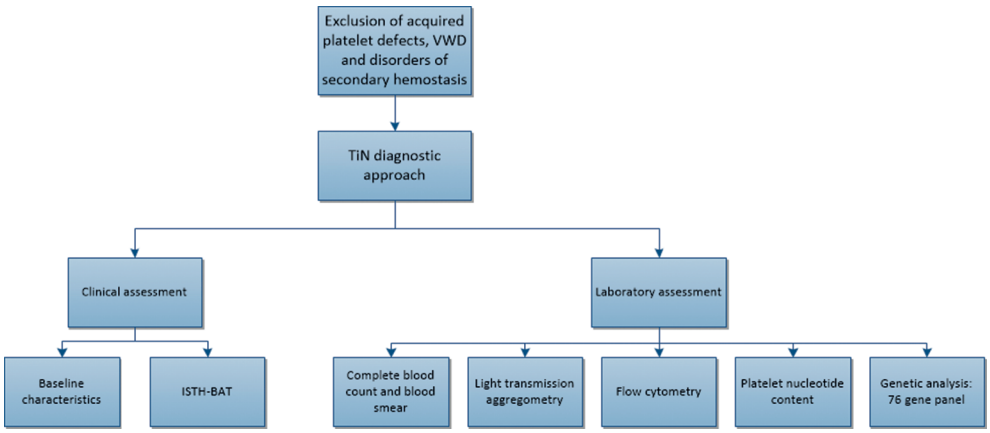

Figure 1. Diagnostic approach of the TiN study. ISTH-BAT, ISTH bleeding assessment tool; VWD, von Willebrand disease.

Complete blood count and morphology

Complete blood count was measured with a Cell-Dyn Sapphire hematology analyzer (Abbott Diagnostics, Santa Clara, CA, USA). Platelet morphology, grey platelets and leukocyte inclusion bodies were assessed in a peripheral blood smear(May-Grünwald-Giemsa staining).

Light transmission aggregometry

Platelet aggregation was performed at 37°C with the PAP-8E platelet aggregometer (Bio/Data corporation; Horsham, PA, USA). Platelet Rich Plasma (PRP) was obtained by 15 minutes centrifugation at 160g at room temperature. Platelet aggregation was initiated with 2.5 and 5.0µM ADP (Sigma-Aldrich; Saint Louis, MO, USA), 1.0 and 4.0µg/mL Horm collagen (Takeda; Tokyo, Japan), 1.5mM arachidonic acid (Bio/Data corporation; Horsham, PA, USA) and 0.6, 0.8 and 1.0mg/mL ristocetin (American Biochemical and Pharmaceuticals Ltd; Marlton, NJ, USA) according to the guideline.[16] Epinephrine was not used as an agonist, since aggregation in response to 5µM epinephrine was abnormal in over 30% of cases in our reference cohort and was therefore not distinctive. Platelet samples were stirred at 900 rpm and the maximal aggregation amplitude was determined after 15 minutes of aggregation. The cut-off value for abnormal platelet aggregation was determined for every agonist and was based on the 2.5th percentile minus the coefficient of variation of 52 healthy donors (Suppl. Table 1).[13] If aggregation was abnormal for only the lower concentration of one agonist, LTA was considered normal. In our laboratory, the within test variation of LTA was 7.6% in healthy platelets and 16.6% in platelets with decreased reactivity and the between test variation was 3.8% and 19.3%, respectively. As LTA is hampered by low platelet counts, it was not performed when platelet count in PRP was below 75x10^{9}/L.[10,17]

Flow cytometry

Platelet surface receptor expression was evaluated with 1:50 (v:v) diluted PE-conjugated anti-CD41α (Thermo fisher scientific; Waltham, MA, USA), 1:25 (v:v) diluted eFluor660 conjugated anti-GPVI (Thermo fisher scientific; Waltham, MA, USA), 1:25 (v:v) diluted FITC-conjugated anti-CD49b (BD biosciences; Franklin Lakes, NJ, USA) and 1 µg/mL of an in-house PE-conjugated anti-GPIbα single domain antibody (clone GPIb-17). After 10 minutes of incubation, samples were fixed with 0.148% formaldehyde, 137mM NaCl, 2.7 mM KCl, 1.12mM NaH_2HPO_4, 1.15mM KH_2PO4, 4mM EDTA, pH 6.5. Samples were analyzed on a BD FACSCanto II (BD biosciences, Franklin Lakes, NJ, USA) that was routinely checked and calibrated with fluorescent beads. Cut-off values for abnormal platelet receptor expression were determined based on the 2.5th percentile of 49 healthy donors (Suppl Table 2).

Platelet nucleotide content

PRP with a platelet count between 100 and 250 x 10^9/L was diluted 1:3 (v:v) in ice cold 10 mM EDTA, 86.4% ethanol, pH 7.4. Platelets were lysed by a freeze/thaw cycle. ADP content was determined in platelet lysate as previously described.[18] ADP and ATP levels were expressed in µmoles / 10^{11} platelets. Dense granule deficiency was diagnosed when the ADP content was lower than 1.4 µmoles / 10^{11} platelets, based on the 2.5th percentile of 49 healthy donors.

Genetic analysis

High molecular weight DNA was isolated from whole blood according to established procedures. Whole-exomes were enriched from genomic DNA using the SureSelect Clinical Research Exome (Cre) V2- kit (Agilent, CA, USA) and sequenced in rapid run mode on the HiSeq2500 sequencing system (Illumina, CA, USA) at a mean target depth of at least 100X. The exome target was defined as all coding exons of UCSC and Ensembl +/- 20bp intron flanks. At this depth ~99% of the target gene panel was covered at least 20X. Reads were aligned to hg19 using BWA (BWA-MEM v0.7.5a) and variants were called using the GATK haplotype caller (v2.7-2). Seventy-six genes associated with CPDs or predisposing for other disorders with similar symptoms were selected based on literature search (Table 1).[7,19-23] Identified variants were classified according to consensus guidelines.[24] Filtering of variants was performed using the Cartagenia BENCHlab NGS module (V.5.0) and Alissa Interpret (V 5.1) (Agilent Technologies, Belgium), with a validated 'classification tree'. Variants with (possible) clinical relevance were subsequently analyzed in the Alamut Visual mutation interpretation software program (V.2.9) (Interative Biosoftware, France) using among others Polyphen2, SIFT, GERP and Grantham scores, and multiple splice-site prediction programs. Variant confirmation and segregation analyses were performed using standard Sanger sequencing.

A genetic variant was stated to be causal when a (likely) pathogenic variant (class 4 respectively 5)[24] was identified in one or more of the selected genes that corresponded to the platelet phenotype.

Table 1. Genes included in the WES gene panel for molecular screening of congenital platelet defects.

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		
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

Table 1. (continued)

Target protein	Gene	Description	Gene	Description
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		
Procoagulant disorders	*ANO6*	Scott syndrome		
Collagen disorders	*COL1A1*	Ehlers-Danlos syndrome	*COL5A1*	Ehlers-Danlos syndrome
	COL3A1	Ehlers-Danlos syndrome	*COL5A2*	Ehlers-Danlos 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;

Definitions

A CPD was diagnosed when an abnormal platelet count or function was found on at least 2 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
- abnormal platelet function tests were found on at least 2 separate occasions, but results were inconsistent

An inherited platelet defect (IPD) was diagnosed when a causal genetic variant was identified in patients with a CPD.

RESULTS

A total of 200 patients were included in the TiN study. For the current analysis, 25 patients were excluded: 14 patients were related to other study participants and genetic analysis was not performed in 11 patients. Thus, 175 patients were included for analysis.

The validation group consisted of 19 unrelated patients, 11 women and 8 men, with an established CPD: 14 patients with GT, 2 with BSS, 2 with a MYH9-related disorder and 1 with a P2Y12 dysfunction. The median age was 49 years (IQR 30-68) for women and 57 years (IQR 47-64) for men. The median bleeding score was 21 (IQR 9-22) for women and 13 (IQR 6-19) for men. In all 19 patients, functional abnormalities were consistent with the original diagnosis and causal pathogenic genetic variants consistent with their CPD were identified (Suppl. Table 3).

The study group consisted of 156 unrelated patients in whom a CPD was suspected. The majority of patients were women (127/156, 81%). The median age was 39 years (IQR 30-52) for women and 37 years (IQR 22-55) for men. The median bleeding score was 10 (IQR 7-13) for women and 7 (IQR 3-10) for men. In 96 of 156 patients (62%), a CPD was suspected based on previously abnormal platelet counts, LTA results or platelet ADP content. Sixty of 156 patients (38%) had a mucocutaneous bleeding tendency suggestive for a CPD, of whom 39 previously had normal LTA results and 21 were newly referred for platelet function testing (Figure 2).

Diagnosis of CPD

LTA with 4 agonists, platelet receptor expression with flow cytometry and platelet ADP content were determined in all patients. Based on this approach, a CPD was confirmed in 61/156 patients (39%), of whom 30 had thrombocytopenia (Table 2). A possible CPD was diagnosed in 20/156 patients (13%). In these patients, an abnormal platelet function was found once in our diagnostic laboratory or abnormal platelet function tests were inconsistent with previous findings. Seventy-five of 156 patients (48%) did not meet our criteria for a CPD. Six of 75 patients (8%) met the criteria for an acquired and not congenital defect based on positive antiplatelet antibodies or increased platelet counts in response to treatment with prednisone or IVIG.

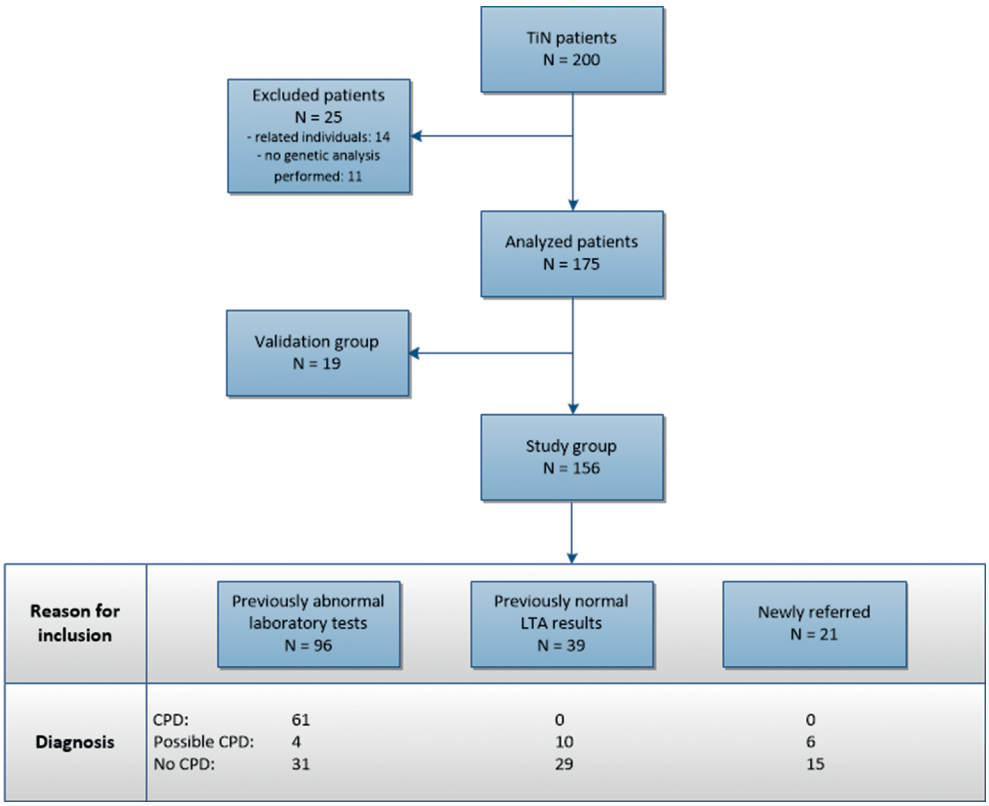

Figure 2. Flow chart of TiN patients. CPD, congenital platelet defect.

Table 2. Classification of study group patients.

Classification	Number of patients	% of patients
CPD	61	39%
Isolated thrombocytopenia	14	23%
Thrombocytopenia and platelet function defect	16	26%
Isolated platelet function defect	31	51%
Possible CPD	20	13%
No CPD	75	48%
Acquired platelet defect	6	8%
Bleeding tendency of unknown cause	69	92%

CPD, congenital platelet defect .

Genetic analysis is only useful in patients with a confirmed CPD

Genetic analysis with a selected 76 gene panel was performed in all patients, of whom 61 were diagnosed with a CPD. We identified 5 pathogenic variants and 4 likely pathogenic variants in five genes (Table 3), as well as 15 variants of unknown significance in eight genes. Variants of

Table 3. Identified genetic variants in the study group.

Gene	Cases	Zygosity	Variant[a]	Protein change	Variant assessment[b]	MAF GnomAD	Platelet phenotype
GATA1 (NM_002049)	1	Hemi	c.647G>A[33]	p.Arg216Gln	Pathogenic	No data	Thrombocytopenia, ↓ADP content;
GP9 (NM_000174)	1	Homo	c.182A>G[34]	p.Asn61Ser	Likely pathogenic	0.0005	Macrothrombocytopenia, ↓ ristocetin aggregation, ↓GP1b-V-IX expression
	1	Compound het	c.182A>G[34]; c.70T>C[35]	p.Asn61Ser; p.Cys24Arg	Likely pathogenic (both)	0.0005 0.00008	Macrothrombocytopenia, ↓ristocetin aggregation, ↓GP1b-V-IX expression
P2RY12 (NM_176876)	1	Het	c.772C>A[36]	p.Pro258Thr	Pathogenic	0.00003	↓ADP aggregation
	1	Het	c.293_294del[37]	p.Gln98fs	Likely pathogenic	0.000004	↓ADP aggregation
RUNX1 (NM_001754)	1	Het	c.610C>T[38]	p.Arg204*	Pathogenic	No data	Microthrombocytopenia, ↓ADP content;
	1	Het	c.602G>A[38]	p.Arg201Gln	Pathogenic	No data	Thrombocytopenia, ↓ADP, AA, collagen aggregation, ↓FG and PS to PAR4 and U46619
SLFN14 (NM_001129820)	1	Het	c.657A>C[39]	p.Lys219Asn	Pathogenic	No data	Macrothrombocytopenia, ↓FG and PS to CRP, ↓FG to ADP

a. References for previously reported genetic variants are depicted with numbers in square brackets. b. Pathogenicity was assessed following the guidelines of the American College of Medical Genetics and Genomics.24 AA, arachidonic acid; ADP, adenosine diphosphate; CRP, collagen related peptide; FG, fibrinogen binding; GnomAD, The Genome Aggregation Database; Hemi, hemizygous; Het, heterozygous; Homo, homozygous; MAF, Minor allele frequency; PAR, protease-activated receptor; PS, p-selectine expression; ↓, decreased.

unknown significance have no current diagnostic value. Therefore, these variants were not used in subsequent analyses. As a result, an IPD was diagnosed in 8/61 CPD patients (13%) (Table 4). It was just as likely to identify an IPD in CPD patients with thrombocytopenia as in CPD patients with normal platelet counts (OR 0.3; 95% CI 0.1 to 1.5). No genetic variants were identified in patients with normal platelet count and function. The ISTH-BAT did not significantly differ between patients with (median 13, IQR 7-15) and without a genetic defect (median 10, IQR 7-13).

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. This study reports the diagnostic value of upfront genetic testing in a prospective cohort of patients suspected for a CPD. We identified a genetic defect in 13% of patients, all of whom had a definite CPD. Our data show that upfront genetic testing with a selected gene panel currently has limited diagnostic yield in patients suspected for a CPD and support the recommendation of the ISTH to perform genetic analysis as a third line investigation.

We included several subgroups of patients suspected for a CPD to properly assess when genetic analysis should be performed in the diagnostic procedure. As LTA results can be false negative in patients with storage pool disease[25] and platelet ADP content was not previously determined in most patients, we also included a subgroup of patients with a mucocutaneous bleeding pattern in whom previous LTA results were normal.

A challenging clinical problem is differentiating immune thrombocytopenia (ITP) from congenital thrombocytopenia. The diagnosis of ITP is one of exclusions and it is not recommended to routinely perform assays for antiplatelet antibodies[26]. Moreover, antiplatelet antibody assays have a moderate sensitivity for ITP[26-28], indicating that a negative result does not exclude the presence of ITP. At the time of inclusion, we were not able to distinguish patients with ITP from patients with congenital thrombocytopenia. Therefore, we included patients with (chronic) thrombocytopenia suspected for a congenital defect based on clinical and laboratory characteristics.

We identified 15 probably damaging variants of unknown significance in 8 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

Table 4. Identified genetic variants per classification.

Classification	Causal genetic variants (n)	VUS (n)
CPD	8	13
Possible CPD	0	1
No CPD	0	2

VUS, variants of unknown significance.

whether these variants actually affect platelet function and contribute to the bleeding phenotype of these patients.

Since diet and supplements may impair platelet aggregation, LTA abnormalities should be consistent on at least 2 separate occasions before a definitive diagnosis of a CPD can be made. We identified LTA abnormalities consistent with previous findings in 51% of patients. This is in contrast to a previously reported reproducibility rate of 90%[29]. In the latter study, defective or absent arachidonic acid induced aggregation was attributed to effect of drugs, foods or other substances and cases with initial defective arachidonic acid aggregation were excluded from analysis if one or more subsequent LTA tests were normal. We included all patients with previously abnormal LTA results, which can explain our lower reproducibility rate.

In a large proportion of patients with a suspected CPD (69/156, 44% we were unable to identify a platelet defect with the diagnostic tools employed in our study. However, we cannot completely rule out a CPD in these patients. For instance, LTA is documented to have low sensitivity for platelet disorders[30,31]. Moreover, we did not investigate platelet function in flowing blood, or look at platelet procoagulant properties. Standardized assays to determine these parameters are currently lacking.

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[32]. 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.

The strength of our study is the large number of patients included. In addition, our study group reflects the population of patients suspected for a CPD referred to outpatient clinics of hemophilia treatment centers for additional platelet function testing. Furthermore, we used healthy controls to determine clear reference values and criteria for abnormal test results for all laboratory assays and we incorporated explicit criteria for the diagnosis of a CPD. Finally, this study demonstrates the feasibility of genetic analysis with a selected gene panel. We used a positive control group of patients with a previously identified CPD, including BSS, GT and MYH9-related disorders and all patients were correctly diagnosed with this gene panel.

In conclusion, upfront 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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SUPPLEMENTARY TABLES

Supplementary Table 1. 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

Cut-off values are depicted as % maximal aggregation. CV, coefficient of variance

Supplementary Table 2. Cut-off values for platelet receptor expression.

Platelet receptor	GP1b-V-IX	αIIbβ3	α2β1	GPVI
2.5th percentile	60	32	57	33

Cut-off values are depicted as % of normal expression.

Supplementary Table 3. Identified genetic variants in the validation group.

Gene	Cases	Zygosity	Variant[a]	Protein change	Variant assessment[b]
GP9 (NM_000174)	2	Homo	c.182A>G[34]	p.Asn61Ser	Likely pathogenic
ITGA2B (NM_000419)	2	Homo	c.3060+2T>C[40]	p.V951-K989del	Pathogenic
	1	Compound het	c.1787T>C[41]; c.2841+1G>T	p.Ile596Thr; p.?	Pathogenic (both)
	1	Compound het	c.3060+2T>C[40]; c.916C>T	p.V951-K989del; p.Gln306*	Pathogenic; Likely pathogenic
	1	Compound het	c.3060+2T>C[40]; c.586C>T	p.V951-K989del; p.Arg196Cys	Pathogenic ; VUS
	1	Compound het	c.3060+2T>C[40]; c.2943+1G>A	p.V951-K989del; p.?	Pathogenic (both)
	1	Compound het	c.3060+2T>C[40]; c.2326_2331dup	p.V951-K989del; p.Glu745_Ala746dup	Pathogenic (both)
	1	Compound het	c.3060+2T>C[40]; c.2348+1G>C	p.V951-K989del; p.?	Pathogenic; Likely pathogenic
	1	Compound het	c.2348+1G>C; c.800-1G>A	p.?	Likely pathogenic (both)
	1	Compound het	c.526C>G[42]; c.2929C>T[43]	p.Pro176Ala; p.Arg977*	Pathogenic (both)
	1	Compound het	c.3060+2T>C[40]; c.2349-21G>A	p.V951-K989del ; p.?	Pathogenic; Likely pathogenic
ITGB3 (NM_000212)	1	Compound het	c.709_710del[44]; c.1637A>G	p.Ser237fs; p.Tyr546Cys	Pathogenic; VUS
	1	Compound het	c.262C>T[45]; c.450G>A	p.Arg88*; p.Met150Ile	Pathogenic; VUS
	1	Compound het	c.353T>A[44]; c.719G>A[46]	p.Leu118His; p.Arg240Gln	Pathogenic (both)
MYH9 (NM_ 002473)	1	Het	c.579C>T[47]	p.Arg1933*	Pathogenic
	1	Het[c]	NA	NA	NA
P2RY12 (NM_176876)	1	Homo	c.772C>A[36]	p.Pro258Thr	Pathogenic

a. References for previously reported genetic variants are depicted with numbers in square brackets. b. Pathogenicity was assessed following the guidelines of the American College of Medical Genetics and Genomics.24 c. Detailed data was not available. Het, heterozygous; Homo, homozygous NA, not available; VUS, variant of unknown significance; ? protein change is not reported/unknown.

I van Asten[1,2,3], REG Schutgens[1,2], AJ Lakerveld[3], T Noordermeer[3], CA Koekman[3], KY Hage[2,3], S Sebastian[2,3], G Pasterkamp[1], A Huisman[3], DJ van den Heuvel[4], HC Gerritsen[4], SJA Korporaal[3,5], M Bierings[6,7], JJ van der Smagt[8], ME van Gijn[8], RT Urbanus[1,2,3]

[1] Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[2] Van Creveld laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[3] Center for Circulatory Health, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands
[4] Department of Molecular Biophysics, Utrecht University, The Netherlands
[5] Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.
[6] Department of Stem Cell Transplantation, Utrecht University Children's Hospital, Utrecht University, Utrecht, The Netherlands
[7] Princess Maxima centre for Pediatric oncology, Utrecht, The Netherlands
[8] Department of Genetics, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands

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PREMATURE PLATELET CLEARANCE VIA CLUSTERING OF THE HYPOSIALYLATED GPIBα RECEPTOR IN GNE-RELATED THROMBOCYTOPENIA

ABSTRACT

Congenital thrombocytopenia due to hyposialylated platelets is associated with mutations in the Glucosamine (UDP-N-Acetyl)-2-Epimerase and N-Acetylmannosamine Kinase (GNE) gene. However, the exact mechanism of GNE-related thrombocytopenia remains unclear. Here, we investigated the molecular mechanism underlying GNE-related thrombocytopenia. We identified 2 female siblings with a severe macrothrombocytopenia from birth, a severe bleeding pattern and a homozygous R420Q mutation in the GNE gene. Patient platelets were strongly hyposialylated. Overexpression of GNE-R420Q in GNE-deficient HEK293 cells confirmed the association between the R420Q GNE variant and hyposialylation. Indium-labeled autologous platelet scanning in one patient showed hepatic sequestration of platelets and a decreased platelet half-life. Consistent with these findings, uptake of patient platelets by THP-1 macrophages and HepG2 liver cells was increased up to 6-fold. Uptake of desialylated platelets by THP-1 macrophages depended on GPIbα and the presence of αMβ2 on macrophages. Patient platelets, as well as sialidase-treated platelets, showed substantial GPIbα clustering. Preventing GPIbα clustering in desialylated platelet attenuated platelet uptake by THP-1 macrophages. GPIbα was not involved in uptake by HepG2 liver cells. Taken together, this study showed that GNE-related thrombocytopenia is characterized by increased hepatic platelet clearance, largely attributable to clustering of the hyposialylated GPIbα receptor.

INTRODUCTION

The average platelet life span is 8-10 days and depends on the platelet glycosylation status. Platelets contain many N- and O-linked glycans, which normally contain a terminal sialic acid group. The most abundantly glycosylated protein on platelets is the GPIbα receptor. Loss of terminal sialic acid from carbohydrates (desialylation) is a clearance signal for senescent platelets. Clearance of desialylated platelets is thought to take place in the liver and the spleen. Both macrophages and hepatocytes are reported to phagocytose desialylated platelets[1].

Platelet desialylation has been reported to occur in diverse pathologies associated with thrombocytopenia. For instance, desialylation has been described in immune thrombocytopenia (ITP) due to the release of sialidases by activated $CD8^+$ T-cells.[2] In addition, thrombocytopenia has been reported in response to viral infections and septicemia, whit pathogen-associated sialidases causing platelet desialylation and inducing thrombocytopenia[3,4,5]. Several cases of congenital thrombocytopenia in association with impaired sialylation have been described, although the responsible molecular pathway remained unclear at the time[6]. In one case, the transmembrane transporter of sialic acid in the Golgi (CMP-sialic acid transporter) was inactivated, which was associated with macrothrombocytopenia[7,8]. Recently, 4 genetic variants in the Glucosamine (UDP-N-Acetyl)-2-Epimerase/N-Acetylmannosamine Kinase (GNE) gene were identified in patients with congenital macrothrombocytopenia[9,10]. One of the identified variants was associated with platelet hyposialylation, which might explain the observed thrombocytopenia. In the other variants, platelet sialylation was not measured. GNE is the rate-limiting enzyme in the synthesis of endogenous sialic acid. The most common pathology associated with mutations in GNE is adult-onset myopathy. Low platelet counts have been reported in isolated cases of GNE myopathy[11], but platelet counts appear to be normal in most patients with GNE-related myopathy.

Several pathways have been described to mediate the clearance of desialylated platelets. In vitro data and murine studies suggest hepatocytes and liver resident macrophages, or Kupffer cells, bind desialylated platelets via the Ashwell Morell Receptor (AMR)[12]. Clearance of desialylated platelets has also been described to occur after prolonged exposure to low temperatures. Here, cold storage triggers the release of endogenous sialidases, causing desialylation and clustering of the GPIbα receptor. Clustered GPIbα is subsequently recognized by macrophages, which phagocytose the platelets in an αMβ2 integrin dependent manner[13,14]. The mechanism that applies to GNE-associated macrothrombocytopenia remains to be determined. Here, we investigated the molecular mechanism behind GNE-related thrombocytopenia in two patients with a novel homozygous mutation in the GNE gene.

METHODS

Participants

Healthy volunteers

Healthy volunteers were recruited via the Mini Donor service, an in-house blood donation facility for research purposes that is approved by the medical ethics committee of the University Medical Center Utrecht. All donors gave written informed consent, in accordance with the declaration of Helsinki.

Patients

Both index patients with a severe bleeding tendency and macrothrombocytopenia received extensive diagnostic follow-up. Both patients provided informed consent in accordance with the declaration of Helsinki.

Blood collection

Peripheral venous blood was collected with venipuncture into 3.2% trisodium citrate vacuum tubes. Tubes were gently inverted to mix the anticoagulant with the blood. All samples were processed within 6 hours after blood collection.

Whole exome sequencing

Genomic DNA was isolated from whole blood according to established procedures. Whole-exomes were enriched from DNA using the SureSelect XT Human All Exon V5 kit (Agilent) and sequenced in rapid run mode on the HiSeq2500 sequencing system (Illumina) at a mean target depth of 100×. Reads were aligned to hg19 using BWA (BWA-MEM v0.7.5a) and variants were called using the GATK haplotype caller (v2.7-2). Detected variants were annotated, filtered and prioritized using the Bench NGS Lab platform (Agilent, Leuven, Belgium). Reported variants were validated by Sanger sequencing. Primer sequences are available upon request.

Platelet isolation, labeling and enzymatic treatments

Washed platelets were obtained as previously described[15]. Due to the giant sized patient platelets, PRP was obtained by 2 hours of sedimentation. For platelet uptake experiments, platelets were labeled with 1μM CellTracker Deep Red (Invitrogen, Carlsbad, CA, USA) for 30 minutes at 37°C and subsequently desialylated with 10mU/mL sialidase from Clostridium perfringens (Sigma Aldrich, Zwijndrecht, The Netherlands) for 15 minutes at 37°C in HEPES Tyrode (HT) buffer (145mM NaCl, 5 mM KCl, 500μM Na_2HPO_4, 1mM $MgSO_4$, 10mM HEPES, 5mM glucose), pH 6.5. Labeling and sialidase reactions were stopped by washing the platelets with HT buffer, pH 7.3. GPIbα receptors were removed with 40μg/mL o-sialoglycoprotein endopeptidase (OSE) (Cedarlane Labs, Burlington, Canada) for 1 hour at 37°C in HT buffer, pH 7.3. GPIbα clustering was inhibited with 50μM monosialoganglioside 3 (GM3) (Sigma Aldrich) for 30 minutes.

Cell culture

Human Embryonic Kidney (HEK)293 cells and Human hepatocarcinoma (HepG2) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)(Thermo Fisher Scientific). The human mononuclear cell line THP-1 was cultured in Optimem (Thermo Fisher Scientific), all supplemented with 100 U/mL penicillin, 100μg/mL streptomycin (Thermo Fisher Scientific) and 10% fetal calf serum (Thermo Fisher Scientific). Cells were cultured at 37°C and 5% CO_2.

Flow cytometry

Platelet sialylation status and GPIbα expression were measured on a BD FACS Canto II (BD Biosciences, San Jose, CA, USA). 5µL whole blood or HEK293 cells were added to 50µL HBS containing carbohydrate specific lectins and incubated for 20 minutes at room temperature. Galactose residues were measured with 5 µg/mL Fluorescein conjugated Ricinus Communis Agglutinin (RCA)-1 (Vector Labs, Burlingame, CA, USA) and sialic acid residues were measured with 5 µg/mL fluorescein-conjugated Sambucus Nigra Lectin (SNA)(Vector Labs) in HEPES buffered saline (HBS; 10mM HEPES, 150mM NaCl, 1mM $MgSO_4x6H_2O$, 5mM KCl, pH 7.4). Specific lectin binding was determined by subtracting the background signal, which was measured in the presence of an excess of galactose (500 mM; RCA-1) or lactose (200 mM; SNA). Platelets and cells were incubated for 20 minutes at room temperature prior to fixation with fixative solution (0.148% formaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH_2HPO_4, 10.2 mM Na_2HPO_4, 1.15 mM KH_2PO_4, 4mM EDTA, pH 6.8).

Expression of GPIbα was measured by adding 5µL whole blood to 50µL HBS containing 1:50 diluted FITC-conjugated anti-GPIbα antibody (clone SZ2; Beckman Coulter, Brea, CA, USA). Samples were incubated for 20 minutes at room temperature prior to fixation.

Platelets and cells were gated based on their forward scatter and sideward scatter. Receptor expression and sialylation status of platelets and cells were expressed as median fluorescent intensity (MFI).

Plasmid constructs.

A Guide (g)RNA targeting exon 2 of the GNE gene was designed with the CRISPR design tool http://crispr.mit.edu/ in order to maximize CAS9 efficiency and to minimize off-target effects. gRNAs used in this study were: gRNA1: 5'-CACCGTTTAATGCCAAACATGATCG-3' and gRNA2: 5'-AAACCGATCATGTTTGGCATTAAAC-3'. Both gRNA oligos were hybridized and cloned into the pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid, which was a gift from prof. Feng Zhang (Addgene plasmid #62988), generating PX459-GNE, as previously described[16]. The protein encoding sequences of GNE1, based on NM_005476.6, and the GNE-R420Q variant were synthesized by Integrated DNA Technologies (IDT, Leuven, Belgium) as a gBlock with a 5' Kozak sequence and cloned into the pcDNA 6/V5-His A expression vector with 5'BAMHI and 3'NotI (both New England Bioloabs, Ipswich, MA, USA) restriction sites.

GNE knockout in HEK293 cells

HEK293 cells ($2x10^5$) were transfected with 1µg/mL PX459-GNE plasmid with lipofectamin 2000 (Thermo Fischer Scientific), according to the instructions of the manufacturer. After 48 hours, culture medium was supplemented with 2.5 µg/mL puromycin (Thermo Fischer Scientific) to select transfected cells. Subsequently, HEK293 GNE knock-out cells were transfected with a pcDNA 6-GNE(WT) or pcDNA 6-GNE(R420Q) plasmid with lipofectamin 2000. HEK293 GNE knock-out

cells expressing either the wild-type or the R420Q GNE variant were selected with 10µg/mL blasticidin. GNE expression was monitored in cell lysate with SDS-PAGE followed by western blotting. Polyvinylidine fluoride membranes were incubated with rabbit-anti GNE (1:250; Atlas Antibodies, Bromma, Sweden) and mouse anti-GAPDH (1 µg/mL; Chemicon International, Billerica, MA, USA) antibodies, washed and incubated with IRDye700 goat anti-rabbit and IRDye 680 donkey anti-mouse (both from LI-COR Biosciences, Lincoln, NE, USA) antibodies. Bands were visualized on an Odyssey infrared scanner (LiCor, Lincoln, NE, USA).

SiRNA silencing of CD11b on macrophages

$5x10^5$ undifferentiated THP-1 cells were transfected with 10nM anti-CD11b siRNA or scrambled siRNA (negative control) from the TriFECTa siRNA kit (IDT, Coralville, IA, USA) with Lipofectamin RNAiMAX (Thermo Fischer Scientific), according to the manufacturer's protocol. After 3 days, THP-1 cells were differentiated with 0.1µM PMA (Sigma Aldrich) for 24 hours with a density of $5x10^5$ cells/well in a 24-wells plate. CD11b expression was measured on the flow cytometer with 1:25 diluted APC-conjugated anti-CD11b (BD Biosciences).

In vitro platelet binding assays with HepG2 cells and THP-1 macrophages

HepG2 cells were seeded at a density of $5x10^5$ cells/well and cultured for 24 hours in a 24-well plate. THP-1 cells were seeded at a density of $5x10^5$ cells/well and differentiated into macrophages with 0.1µM PMA for 24 hours. Serum-free medium was added to HepG2 cells or THP-1 macrophages and incubated for 30 minutes, before adding $8x10^6$ CellTracker Deep Red labeled platelets/well to HEPG2 cells or THP-1 cells. HepG2 cells were incubated with 100µM asialofetuin (Sigma Aldrich) to study the platelet binding via the AMR. Platelets were mixed every 10 minutes by gentle agitation of the plate. After 30 minutes, unbound platelets were washed away with PBS and cells were detached with 0.5% trypsin-EDTA (Thermo Fischer Scientific) in PBS. Cells were harvested and analyzed on the flow cytometer. HepG2 or THP-1 cells were gated based on forward and sideward scatter. Platelet binding was defined as the percentage platelet-positive HepG2 or THP-1 cells. In total, 10^5 events were analyzed for each sample.

GPIbα clustering

Platelets were fixed with 2% paraformaldehyde for 30 minutes at RT and concentrated on a glass slide by cytospin (500rpm, 5 minutes). Slides were dried for 10 minutes at room temperature, washed three times with PBS and blocked with 1% BSA in PBS for 1 hour. GPIbα was stained with 1 µg/mL Alexa Fluor-488 conjugated 6B4-Fab fragments (donor), or Alexa Fluor-594-conjugated 6B4-Fab fragments (acceptor) in HBS supplemented with 1% BSA for 1 hour. 6B4 Fab-fragments were kindly provided by Dr Hans Deckmyn (KU Leuven, Kortrijk, Belgium). Samples were washed with water and imbedded with mowiol (Sigma Aldrich). GPIbα clustering was measured with fluorescence lifetime imaging (FLIM) as described[17]. The fluorescence lifetime values were calculated into Förster resonance energy transfer (FRET) efficiency, which is a measure for GPIbα clustering. FRET efficiency was defined as: , in which τ is the lifetime in the absence () and presence) of the acceptor.

Statistical analysis

All data are plotted as mean values ± standard deviation. Statistical analysis were performed in GraphPad Prism 7 (San Diego, CA, USA). P-values<0.05 (*) were considered statistically significant.

RESULTS

Association between GNE R420Q variant and macrothrombocytopenia

Two female siblings (P1 and P2) presented with spontaneous mucocutaneous bleeds and menorrhagia, which required frequent hospitalization. Their ISTH-BAT bleeding scores[18] were 29 for P1 and 27 for P2. Their history showed a persistent severe macrothrombocytopenia from 1 week of age (table 1), with giant platelets in a peripheral blood smear (Figure 1A). Both patients received platelet transfusions on demand to restore hemostasis. Flow cytometric evaluation of their platelets showed normal surface receptor expression of α2β1, α2bβ3 and GPVI (Figure 1B). Platelet reactivity could not be determined due to the low platelet count. Their non-consanguineous parents did not have a bleeding tendency and had normal platelet counts (207x10^9/L and 341x10^9/L). Clinical quadruple whole exome sequencing was performed to search for potential causative genetic variants. Whole exome data from both siblings were compared with data from their parents, which yielded a homozygous missense variant (c.1259G>A) in the GNE gene transcript NM_005476.5 as the only likely candidate (Figure 1C). Both parents are heterozygous carriers of this variant (Figure 1D). This variant leads to a p.R420Q amino acid substitution in the ATP binding region of the N-acetylaminosamine kinase domain of the GNE protein (Figure 1E). The allele frequency of this variant is 8x10^{-6} in the total population according to the gnomAD browser[19], and its pathogenicity is predicted to be "probably damaging".

The GNE R420Q enzyme is functionally impaired

Since GNE is the rate-limiting enzyme in sialic acid synthesis, the platelet glycosylation status was assessed with flow cytometry. Both patients showed up to 10-fold increase in RCA-1 lectin binding

Table 1. clinical parameters probands

	Proband 1	Proband 2	Reference
Current Age (y)	20	17	
ISTH-BAT	29	27	<5
PLT count (10^9/L)	13	14	150-450
MPV (fL)	18	21.2	7.0-9.5
TPO (E/mL)	8	17	4-32
vWF:rco (IU/dL)	137	121	40-150
Hb (g/dL)	8.5	8.3	7.4-9.6
RBC (10^{12}/L)	4.6	4.8	3.7-5
WBC (10^9/L)	6.5	4.7	4-10
Urinary Sialic Acid (mmol/mol creatinine)	30	35	19-43
Sialylation APOCIII	Normal	Normal	
Sialylation Transferrin	Normal	Normal	

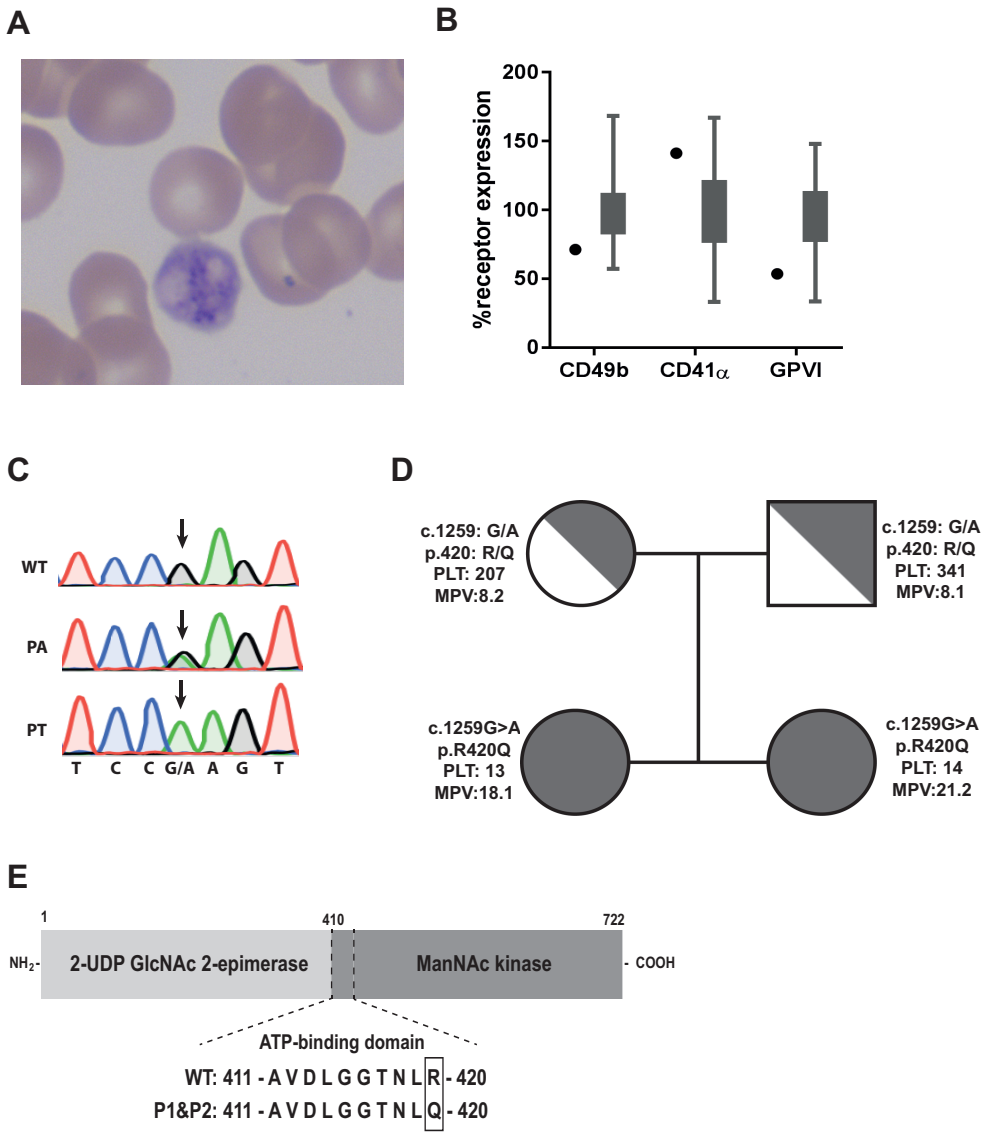

Figure 1. Mutation in GNE is associated with congenital macrothrombocytopenia. (A) A peripheral blood smear of P1 showed giant sized platelets. (B) Relative expression of CD49b, CD41α and GPVI of P1 (black dots) compared with the 95% confidence intervals of 49 healthy controls measured with flow cytometry. (C) Both parents are asymptomatic and are carriers of the R420Q GNE variant. (D) Schematic illustration of the guanine (black) substitution by adenosine (green) on location 1259 of GNE, confirmed with sanger sequencing. Reference (WT), Parent (PA) and P1 (PT) are illustrated. (E) Schematic illustration of the bifunctional GNE protein. The R420Q mutation is located in the ATP-binding region of the ManNAc kinase domain of GNE.

compared with healthy controls, their parents and other thrombocytopenic patients (Figure 2A), which indicates substantial terminal galactose expression on glycans and supports the potential pathogenicity of the GNE variant. In contrast, the sialylation of red blood cells, and plasma proteins APO-CIII and transferrin was normal, as was the urinary sialic acid concentration (data not shown), indicating that only specific tissues are affected by GNE dysfunction.

To confirm causality between the GNE-R420Q variant and platelet hyposialylation, GNE-deficient HEK293 cells were engineered with CRISPR-Cas9. Subsequently, GNE-R420Q and wild-type GNE were overexpressed in GNE-deficient cells (Figure 2B). Expression levels of recombinant GNE-R420Q and recombinant wild-type GNE were similar to expression levels of native GNE in HEK293 cells. Next, glycosylation profiles were analyzed to determine the contribution of the R420Q variant on sialylation. Knock-down of GNE resulted in a 33% (P=0.01) reduction of sialic

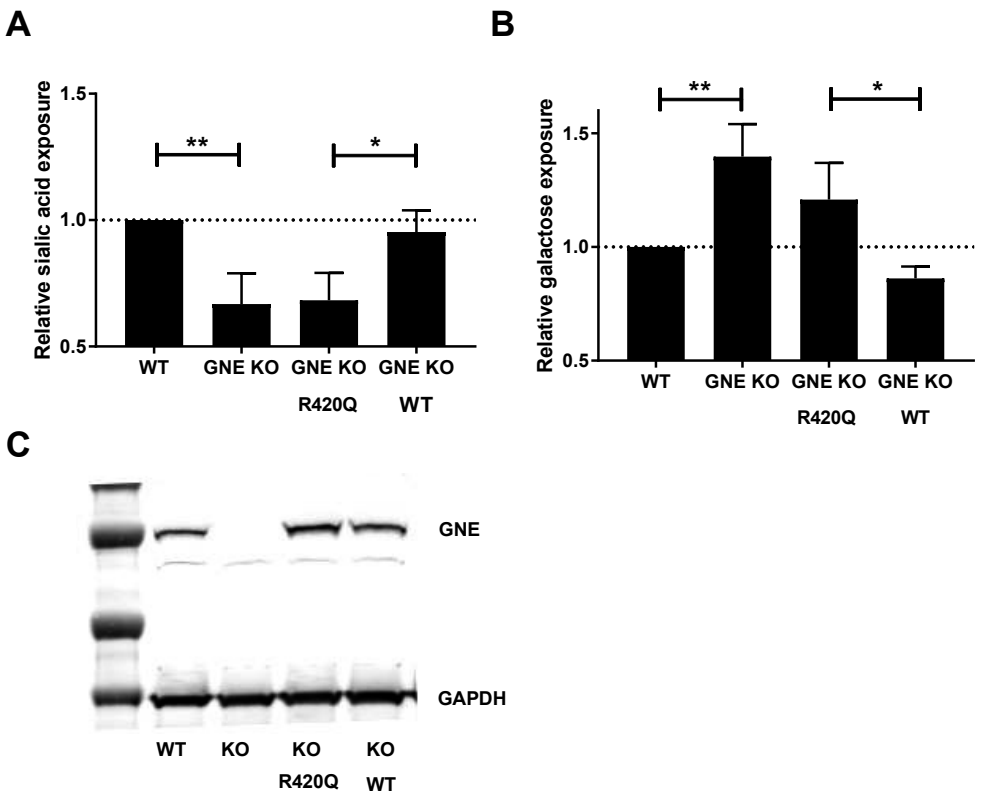

Figure 2. R420Q GNE variant results in hyposialylation. (A) Platelet galactose exposure was determined as a measure of platelet sialylation. Galactose exposure was measured with FITC-conjugate RCA-lectin on the flow cytometer in 68 healthy controls (CO), 4 thrombocytopenic patients (TP), the parents (PA) and in both P1 and P2 (PT). (B) Wild-type (WT), GNE-deficient (KO), and GNE-deficient HEK293 cells overexpressing either recombinant wild-type GNE, or overexpressing recombinant GNE R420Q were lysed and subjected to SDS-PAGE and western blot. GNE and GAPDH were probed. Sialylation (C) and galactose exposure (D) of the generated HEK293 cells were measured on the flow cytometer. * indicates P-value<0.05, error bars represent the standard deviation.

acid surface expression. Reconstitution of R420Q GNE protein did not increase cellular sialic acid expression, but reconstitution of wild-type GNE fully restored sialic acid expression (Figure 2C). Conversely, galactose expression increased by 39% (P=0.01) after disruption of the GNE gene. Galactose expression remained high after overexpression of GNE R420Q, but normalized after overexpression of wild-type GNE (Fig 2D). These data confirm that the GNE R420Q variant is functionally impaired.

GNE-associated thrombocytopenia is due to increased hepatic platelet clearance

The observed thrombocytopenia could either be due to increased clearance or decreased platelet production. Thrombopoietin (TPO) levels were normal in both patients, suggesting normal platelet production. To investigate the possibility of platelet clearance, ^{111}In-labeled autologous platelet scanning was performed in P1, which revealed a substantially shortened platelet half-life (16 hours) compared with platelets from healthy subjects (60-100 hours) (Figure 3A). In addition, there was hepatic sequestration of platelets, based on a liver:spleen ratio of 3:1 (normal ratio 1:4) (Figure 3B). The platelet count in P1 gradually increased to 50x10^9/L upon treatment with the TPO-mimetic Romiplostim (Figure 3B), indicating normal bone marrow functionality. Combined, these data suggest that GNE-related thrombocytopenia is caused by increased hepatic platelet clearance.

Uptake of hyposialylated platelets by macrophages depends on GPIbα-clustering

Hepatic clearance of desialylated platelets has been attributed to both hepatocytes and liver-resident macrophages[20,21]. To further investigate the hepatic clearance mechanism for the hyposialylated platelets in GNE-associated thrombocytopenia, we assessed platelet binding by human hepatocytes and macrophages with THP-1 macrophages and HepG2 cells. Platelets from P1 showed 6-fold more binding to both THP-1 macrophages and HepG2 cells than control platelets (Figure 4A,B). Sialidase treatment of normal platelets, which resulted in a 3-fold increase in galactose expression (Figure 4C), also caused increased HepG2 binding and THP-1 macrophage binding, confirming a role for desialylation in platelet uptake by these cells. Uptake of desialylated platelets could not be inhibited with an excess of asialofetuin (Figure 4D), suggesting an Ashwell-Morell receptor independent uptake mechanism.

Clustering of GPIbα is reported to occur upon platelet desialylation due to prolonged cold storage[17]. Indeed, platelets from patient P1 showed substantial clustering of GPIbα compared with control platelets, as indicated by an increase in FRET efficiency measured with FLIM (Figure 5A,B). Sialidase-treated control platelets showed GPIbα clustering to a similar extent. Combined, these data suggest that GPIbα-clustering is caused by hyposialylation due to impaired GNE function. Next, we investigated the role of GPIbα-clustering in platelet uptake. Removal of GPIbα with OSE (Figure 5C) from the platelet surface fully negated the effects of sialidase treatment on platelet binding to THP-1 macrophages, but did not influence binding of untreated platelets, suggesting that the uptake of hyposialylated platelets by macrophages depends on GPIbα (Figure 5D). In

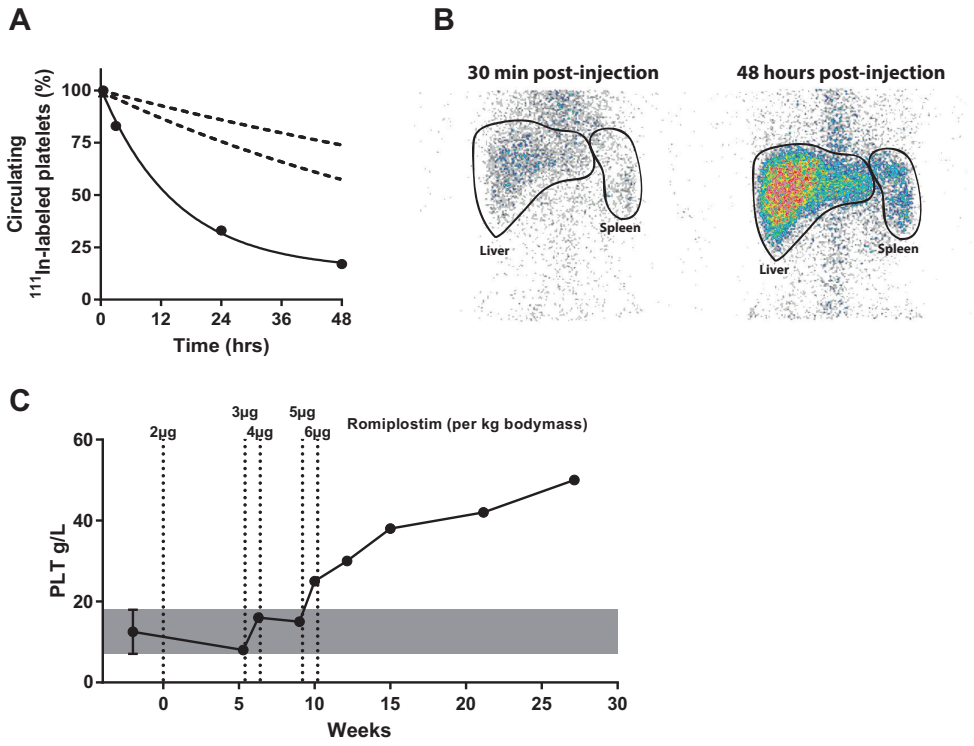

Figure 3. GNE thrombocytopenia is associated with increased platelet clearance. (A) To measure the platelet clearance rate, platelets were labeled with 111-Indium and injected into the patient. Platelets were collected at different time points to determine platelet half-life time. The fraction 111Indium labeled platelets at 30 minutes post injection was set at 100%. The connected line represents the % 111-Indium-labeled platelets of P1, whereas the dotted lines represent the reference values for platelet life span. (B) A PET-scan of P1 of the liver and spleen at 30 minutes and 48 hours post-injection of 111-Indium-labeled platelets. A normal platelet clearance results in a spleen-liver ratio of 1:4. (C) The platelet count (G/L) over time after different concentrations of Romiplostim.

contrast, GPIbα removal with OSE lead to an increase in binding of normal and desialylated platelets to HepG2 cells, indicating a GPIbα-independent uptake mechanism in HepG2 cells (Figure 5E). Prevention of GPIbα-clustering on sialidasetreated platelets with ganglioside GM3 fully reversed the effects of sialidase treatment on platelet binding to THP-1 macrophages, indicating that the uptake of hyposialylated platelets by macrophages is due to GPIbα clustering (Figure 5F). Silencing of the putative counter receptor for clustered GPIbα on macrophages, $\alpha_M\beta 2$ integrin, also reduced the binding of desialylated platelets (Figure 5G,H).

DISCUSSION

We identified two female siblings with congenital macrothrombocytopenia associated with a loss of function mutation in the GNE gene, causing platelet hyposialylation. This induces clustering of the hyposialylated GPIbα receptor and gives rise to increased hepatic platelet clearance.

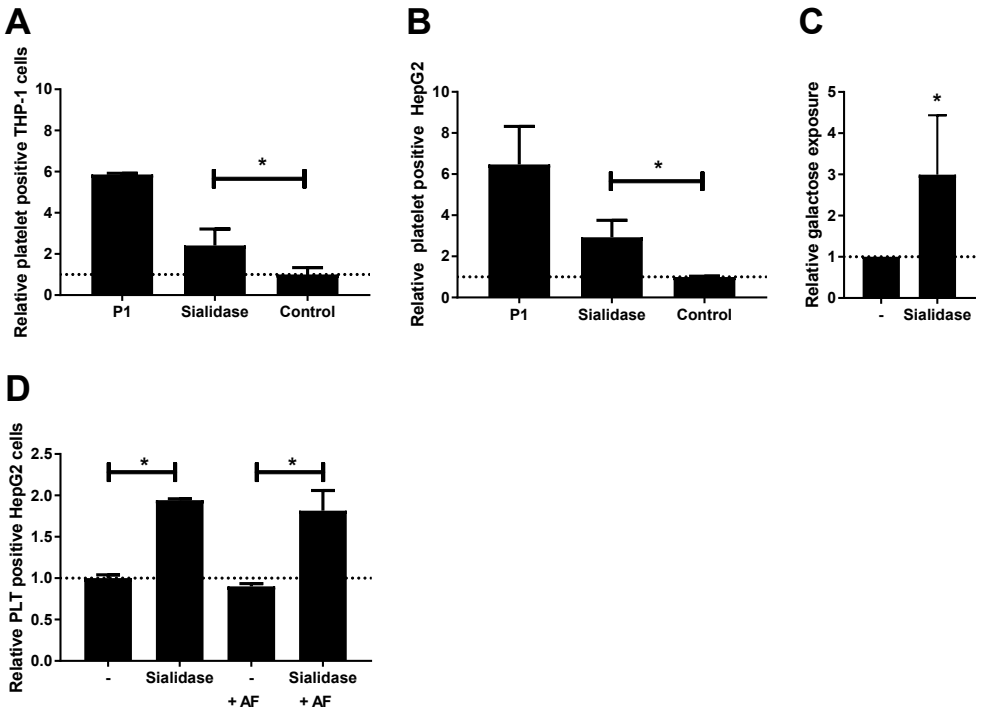

Figure 4. Increased in vitro platelet binding to THP-1 macrophages and HepG2 hepatocytes. Celltracker Red labeled platelets of P1, sialidase treated platelets and control platelets were incubated with THP-1 macrophages (A) and HepG2 hepatocytes (B) and the number of platelet positive cells were compared with control platelets, as a measure for platelet binding. (C) The relative galactose exposure of control platelets and sialidase treated platelets were measured on the flow cytometer with FITC-conjugated RCA-lectin. (D) 100μM asialofetuin was added to HepG2 cells to determine the role of the AMR in the uptake of desialylated platelets. * indicates P-value<0.05, error bars represent the standard deviation.

Sialylation of glycan structures is crucial in many biological processes and interactions.[22] Mice incapable of endogenous sialic acid synthesis due to knockout of the GNE gene die during embryonic development, which illustrates the importance of sialylation. The only known human pathologies that are associated with mutations in the GNE gene are sialuria, myopathy and thrombocytopenia[23]. GNE is expressed in many different tissues. The GNE gene encodes a bifunctional enzyme with two distinct regions. The R420Q mutation we identified in our patients results in loss of function of the ManNAc kinase domain, whereas the Glucosamine (UDP-N-Acetyl)-2-Epimerase function of GNE is probably normal. Loss of function due to amino acid substitutions at the R420 position have been reported, namely: R420M[24] and R420X[25]. The R420 is a well conserved amino acid within carbohydrate kinases, illustrating the importance of this amino acid[26]. Interestingly, our data showed that sialylation of plasma proteins and red blood cells was normal, as was the urinary secretion of sialic acids. There are indications for a partial redundancy of the ManNAc kinase domain in GNE, which could explain this observation. N-Acetyl-D-glucosamine kinase (NAGK) is an enzyme with similar activity as the ManNAc kinase domain of GNE. We

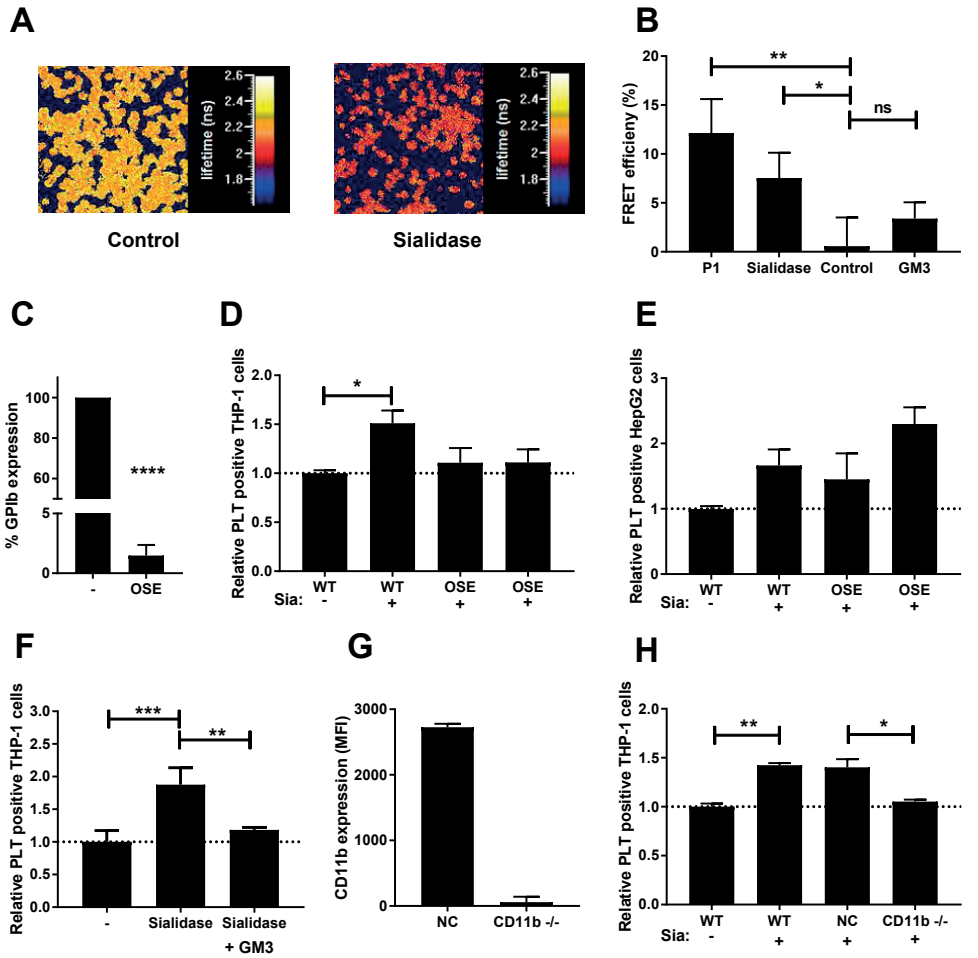

Figure 5. GNE deficiency results in platelet clearance via GPIbα clustering. (A) A representative example of GPIbα lifetime of control platelets (left panel) and sialidase treated platelets (right panel). The lifetime of the donor probe is expressed in nanoseconds (ns). (B) Quantification of the FRET efficiency in P1, sialidase treated platelets and control platelets. (C) Removal of GPIbα with 40μg/mL OSE was confirmed on the flow cytometer. The effect of GPIbα removal by OSE on the platelet binding to THP-1 macrophages (D) and HepG2 hepatocytes (E). (F) 50 μM GM3 was added to sialidase treated platelets to inhibit GPIbα clustering. (G) SiRNA treated THP-1 cells show downregulation of CD11b. (H) Platelet binding of control and desialylated platelets treated with or without siRNA targeted to CD11b on macrophages. *P-value<0.05, **P-value<0.01, ***P-value<0.005, error bars represent the standard deviation.

hypothesize that the expression of NAGK in other tissues prevents the susceptibility for impaired sialic acid synthesis in GNE-related pathologies[27]. Neither patient showed signs of myopathy as yet in their teens, but onset of GNE-related myopathy most often occurs later in adulthood.

111-Indium-labeled platelet scanning showed hepatic sequestration of the platelets of patient P1, indicating hepatic platelet clearance. Indeed, platelets of P1 showed increased uptake by both hepatocytes and macrophages. Both cell types have been implicated in the clearance of

desialylated platelets[28,29]. However, the endothelial lining of liver sinusoids forms a physical barrier between liver cells and blood cells. Although sinusoid endothelium is fenestrated, the size of the pores precludes the uptake of platelets by hepatocytes in vivo. This fits with data that showed hepatic uptake of desialylated platelets is driven by Kupffer cells[20,21]. Nevertheless, hepatocytes are able to form protrusions through the endothelial fenestrations and thereby trap desialylated platelets, which are then taken up by the hepatic macrophages[20]. Macrophage depletion in a mouse model with deficient sialyltransferase showed decreased platelet survival compared with control mice, indicating a macrophage independent component in the clearance of hyposialylated platelets[28]. Data from murine studies indicate that the Ashwell Morell Receptor on hepatocytes and macrophages recognizes desialylated GPIbα receptor[30]. In contrast to these studies, our data indicate that uptake of desialylated human platelets by human hepatocytes does not depend on GPIbα and is not inhibited by asialofetuin, a competitive inhibitor of the AMR, even at very high concentrations. In macrophages, we showed that platelet uptake depends on clustering of the desialylated GPIbα receptor and that platelet uptake can be reversed by down regulation of the $\alpha_M\beta 2$ integrin. A similar uptake mechanism has been observed in chilled platelets[17] and has been described in a guinea pig model, in which antibody induced GPIbα clustering resulted in macrophage dependent platelet uptake in the liver[21].

The pronounced hepatic sequestration of platelets in patient P1 rules out splenectomy as a treatment option for GNE-associated thrombocytopenia. However, the platelet count of P1 could be elevated by stimulation of megakaryopoiesis with the TPO mimetic Romiplostim. Although the platelet count remained below normal level, the most severe bleeding symptoms disappeared with the increase in platelet count, which improved quality of life substantially.

Increased platelet clearance due to hyposialylation contributes to the low platelet count in GNE-related macrothrombocytopenia, but we cannot exclude a role for impaired platelet production: both patients had giant sized platelets. Murine studies report giant platelets when platelet sialylation is impaired, suggesting sialylation is important for platelet size[31]. Our data show that desialylation results in clustering of the GPIbα receptor. Pathologies associated with GPIbα receptor dysfunction, such as Bernard Soulier syndrome[32] or platelet-type von Willebrand disease[33], are associated with large platelets, as are deficiencies in the GPIbα-associated cytoskeletal protein Filamin A.[34] Macro-thrombocytopenia has also been observed in type 2B von Willebrand disease[35], presumably due to clustering of the GPIbα receptor after spontaneous binding of vWF to megakaryocytes[36]. A similar mechanism may apply to GNE-related macrothrombocytopenia.

In conclusion, GNE-related macrothrombocytopenia is caused by increased platelet clearance by hepatic macrophages due to clustering of the hyposialylated GPIbα receptor. GNE-related thrombocytopenia can be managed with Romiplostim. Whether GNE is also responsible for enlarged platelets and why only platelets are affected deserves further investigation.

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Ivar van Asten[1,2,3], Maaike W Blaauwgeers[2,3], Karin PM van Galen[3],
Marc Durian[4], Sonja Zweegman[5], Rob Fijnheer[6],
Monique C. Minnema[7], Rolf T Urbanus,[1,2,3], Roger EG Schutgens[3]

[1] Center for Circulatory Health, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands.
[2] Van Creveld Laboratory, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands.
[3] Van Creveldkliniek, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands.
[4] Department of Hematology, ETZ Hospital, Tilburg, The Netherlands
[5] Department of Hematology, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands
[6] Department of Internal Medicine, Meander Medical Center, Amersfoort, the Netherlands.
[7] Department of Hematology, UMC Utrecht Cancer Center, Utrecht, The Netherlands

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HEMORRHAGIC DIATHESIS ASSOCIATED WITH MONOCLONAL GAMMOPATHY OF UNKNOWN SIGNIFICANCE; A NEW SPECTRUM OF MONOCLONAL GAMMOPATHY OF CLINICAL SIGNIFICANCE

ABSTRACT

Monoclonal Gammopathy of Undetermined Significance (MGUS) is a premalignant disease that is usually asymptomatic and does not require immediate treatment. However, in some patients, the presence of a monoclonal antibody is associated with adverse clinical events, justifying treatment with chemotherapy. These gammopathies are currently known as Monoclonal Gammopathy of Clinical Significance (MGCS). Here, we report four separate cases in which a monoclonal gammopathy was associated with a hemorrhagic diathesis. Two patients presented with a primary hemostasis defect, whereas the other two patients presented with an acquired coagulopathy. Three patients were treated with bortezomib/cyclophosphamide/dexamethasone (VCD), which resolved their bleeding symptoms, improved hemostatic laboratory parameters and lead to remission of the MGUS. In conclusion, similar to other manifestations of MGCS, anti-myeloma treatment can reverse clinical symptoms in patients with a bleeding tendency due to a monoclonal gammopathy.

INTRODUCTION

Monoclonal Gammopathy of Undetermined Significance (MGUS) is a premalignant disorder and is characterized by the presence of a monoclonal antibody (<3g/dL), <10% monoclonal plasma cells in bone marrow, and the absence of anemia, renal insufficiency, hypercalcemia or bone lesions[1]. The risk of developing multiple myeloma or another plasma cell dyscrasia is approximately 1% per year[2]. Therefore, annual diagnostic screening for malignant diseases has been recommended[3].

The prevalence of MGUS is around 3.2% in people >50 years[4] and 0.3% among patients <50 years[5]. Because of the high prevalence in the healthy population, MGUS is often diagnosed by coincidence during the diagnostic work-up of other diseases[6]. In many cases it remains unclear whether the MGUS is associated with the disease condition[7]. However, there can be a clear association between MGUS and renal dysfunction, which lead to the introduction of the term Monoclonal Gammopathy of Renal Significance (MGRS)[8]. Here, the monoclonal antibodies impair kidney function. As the recurrence rate after kidney transplantation is >80%, treatment of this monoclonal gammopathy is desired[9].

In addition to MGRS, there are other non-malignant diseases associated with MGUS, including dermatologic, neurologic and hemorrhagic disorders. These disorders are now called monoclonal gammopathy of clinical significance (MGCS)[10]. Cases in which a monoclonal gammopathy caused acquired von Willebrand Disease[11], acquired hemophilia[12] or acquired Glanzmann Thrombasthenia have been described[13,14] and can be classified as monoclonal gammopathy of hemorrhagic significance. Patients with acquired von Willebrand disease were treated with intravenous immunoglobulins, desmopressin or von Willebrand Factor infusion. Although this therapy was successful in managing the bleeding symptoms, it did not cure the monoclonal gammopathy.

Here, we describe the successful reversal of severe bleeding symptoms in 4 patients using anti plasma cell directed therapy with Bortezomib/Cyclophosphamide/Dexamethasone in patients with a 'monoclonal gammopathy of hemorrhagic significance'.

METHODS

Blood collection

Peripheral venous blood was drawn in sodium citrate Vacutainer® tubes (BD Biosciences, Franklin Lakes, NJ, USA). Blood from healthy volunteers was collected via the Mini Donor Service of the University Medical Center Utrecht, a blood donation facility for research purposes that is approved by the institutional ethics review board. All donors gave informed consent in accordance with the declaration of Helsinki.

Light transmission aggregometry

Platelet aggregation was measured at 37°C with a PAP-8E platelet aggregometer (Sysmex, Etten-Leur, the Netherlands). Platelets were washed as previously described[15]. After a final centrifugation step, the platelet pellet was resuspended in pooled normal plasma (PNP) or patient plasma (PTP). Platelet aggregation was measured in PNP or PTP and was initiated with 2.5μM ADP (Sigma Aldrich, Zwijndrecht, the Netherlands), or 4.0μg/mL Horm collagen (Takeda, Tokyo, Japan). Platelet samples were stirred at 900rpm and platelet aggregation traces were recorded.

Flow cytometry

Whole blood was diluted 10x in HEPES buffered saline (HBS; 10mM HEPES, 150mM NaCl, 1mM $MgSO_4$, 5mM KCl, pH 7.4) containing a platelet agonist and Alexa488-conjugated anti-fibrinogen nanobodies (clone Fb-C3), PE-conjugated anti-GPIbα nanobodies (clone GPIb-17) and Alexa647-conjugated anti-P-selectin nanobodies (clone B10.6), and was incubated for 10 minutes at 37°C. Platelet agonists were protease activating receptor (PAR)-1 activating peptide SFLLRN, PAR4-activating peptide AYPGKF, ADP, crosslinked collagen related peptide and U46619. αIIbβ3 receptor expression was evaluated with PE-conjugated anti-CD41α (Thermo fisher scientific; Waltham, MA, USA). After 10 minutes of incubation, samples were fixed with 0.148% formaldehyde, 137mM NaCl, 2.7 mM KCl, 1.12mM NaH_2HPO_4, 1.15mM KH_2PO4, 4mM EDTA, pH 6.5. Samples were analyzed on a BD FACSCanto II that was routinely checked and calibrated with fluorescent beads. Platelets were identified based on morphology (forward and sideward scatter) and the expression of GPIba. Fibrinogen binding and P-selectin expression were measured as platelet reactivity markers in response to agonist stimulation.

IgG isolation

Total IgG fractions from PNP and PTP were purified with Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Chicago, IL, USA). In short, plasma was passed through a column packed with protein G Sepharose. The column was subsequently washed with 5 column volumes of HBS with 0.5M NaCl and 5 column volumes of HBS. IgG was eluted with 0.1M glycine, pH 2.7, followed by immediate buffer exchange to HBS. IgG concentration was calculated by measuring the absorbance at 280 nm.

Coagulation assays

The prothrombin time (PT; Dade Innovin, Siemens, Erlangen, Germany), activated partial thromboplastin time (APTT; Actin FS, Siemens) and dilute Russell's viper venom time (dRVVT; LA screen, Stago, Asnières-sur-Seine, Paris) were performed on the MC10Plus (Merlin Medical, Lemgo, Germany) with PNP or with PTP supplemented with 100, 200 or 500µg/mL purified IgG from either patient or healthy control. In some experiments, clotting was initiated by adding 50 µL of HBS with 0.1% BSA, 16.6 mM CaCl2, 10 µM phospholipids (Coag reagent I; Avanti Polar Lipids, Alabaster, AL, USA) and either 10 µg/mL α-FXIIa (Haematologic Technologies, Essex Junction, VT, USA), 1 µg/mL FXIa (Enzyme Research Laboratories, South Bend, IN, USA), 60 ng/mL FXa (Enzyme Research Laboratories), or 2 µg/mL FIXa (Enzyme research laboratories) to 50 µL PNP spiked with normal IgG or patient IgG, as indicated. All clotting tests were performed at 37°C.

RESULTS

Case 1: acquired Glanzmann Thrombasthenia

A 52 year old woman presented at the van Creveldkliniek of the University Medical Center Utrecht for diagnostic work-up of her bleeding tendency (bleeding score 24 according the ISTH-Bleeding Assessment Tool[16]) that had existed for 2 years and was characterized by melena, extensive

control platelets was attenuated in patient plasma, but normal in pooled normal plasma (Fig 1B). Combined, these data indicated that the aggregation defect was caused by a plasma component.

To investigate whether an antibody was responsible for the GT-phenotype, flow cytometry was performed. This indicated a 6-fold increase in IgG binding to unstimulated normal platelets in patient plasma compared with normal plasma (Fig 1C). Based on these data, a relationship between the IgGκ paraprotein and the GT-phenotype was assumed.

Because of her severe clinical bleeding tendency, the patient was treated with 4 cycles of VCD therapy, consisting of 1.3mg/m^2 Bortezomib, 500mg/m^2 Cyclophosphamide and 40mg Dexamethasone. Four weeks after treatment, blood was drawn for re-evaluation of platelet function. Platelet aggregation had improved substantially after VCD therapy (Fig 1D), as did the platelet capacity to bind fibrinogen after stimulation with several agonists (fig 1E). Moreover, platelet aIIb3 integrin expression increased from 31% to 79% of normal. Combined, these data suggest that the acquired GT was caused by an anti-platelet paraprotein.

Case 2: acquired thrombocytopenia and spontaneous platelet aggregation

A 52 year old woman presented with an Hb<3 mmol/L, WHO grade 3 hematomas, rectal and vaginal blood loss. Because of her platelet count<1x10^9/L. She was treated with platelet transfusions when Hb<3 mmol/L. Her coagulation factor levels were normal (PT 11.3s, APTT 21s) and she had an IgG paraprotein of 18g/L. She did not respond to corticosteroids, splenectomy, gammaglobulin infusion and TPO agonists. To investigate whether the paraprotein affected platelet function, aggregation of washed platelets from a healthy donor was analyzed in the presence of either patient plasma or pooled normal plasma. In patient plasma, donor platelets showed spontaneous platelet aggregation, suggesting spontaneous platelet activation (Figure 2A). Platelet reactivity towards ADP and collagen was similar in patient plasma and PNP. After treatment with 4 cycles of VCD, the bleeding symptoms of the patient disappeared and the platelet count restored to 375x10^9/L.

Case 3 and 4: acquired coagulopathy

Patient 3 was a 51 year old woman who underwent pre-operative screening because of recent hematuria and rebleeding after dental extraction. Coagulation screening indicated a prolonged APTT (72 s), a slightly prolonged PT (14.8s) and a slightly prolonged thrombin time (19.0s; reference interval 12.6-18.6s). An APTT mixing test indicated the presence of an inhibitor. The patient had a IgGλ paraprotein of 10 g/L.

To confirm whether the prolongation of the APTT was caused by the monoclonal antibody, the effect of purified IgG from patient plasma was compared with the effect of normal IgG on coagulation tests. Patient IgG caused a dose-dependent prolongation of the clotting time in both APTT (Figure 3A) and dRVVT (Figure 3B) assays, while IgG from PNP did not. The PT (Figure 3C) was not affected by addition of IgG. No specific coagulation inhibitor could be identified. To determine which enzymatic reaction was affected by the inhibitor, coagulation was initiated with either FIXa, FXa, FXIa or FXIIa. Whereas prolonged clotting times were observed upon initiation with FIXa, FXIa and FXIIa, clotting times were normal upon initiation with FXa (Figure 3D). Combined, these data

hematomas and epistaxis. Her family history was negative for a bleeding tendency or easy bruising. Based on the clinical presentation, an acquired primary hemostasis defect was suspected.

Laboratory evaluation revealed a slightly increased white blood cell count (11.1x10^9/L), strongly reduced platelet aggregation measured with Light Transmission Aggregometry (LTA), decreased platelet aIIbb3 expression and the presence of an IgGκ paraprotein that could not be quantified. This patient did not have hematological malignancies (Table 1). Based on these data, an acquired Glanzmann Thrombasthenia (GT) was suspected. Immune thrombocytopenia (ITP) was deemed unlikely, since the platelet count was normal. To establish whether the GT-like phenotype was caused by a plasma protein, mixing studies were performed. Aggregation of patient platelets was absent in autologous plasma, but normal in PNP (Fig 1A). Conversely, aggregation of healthy

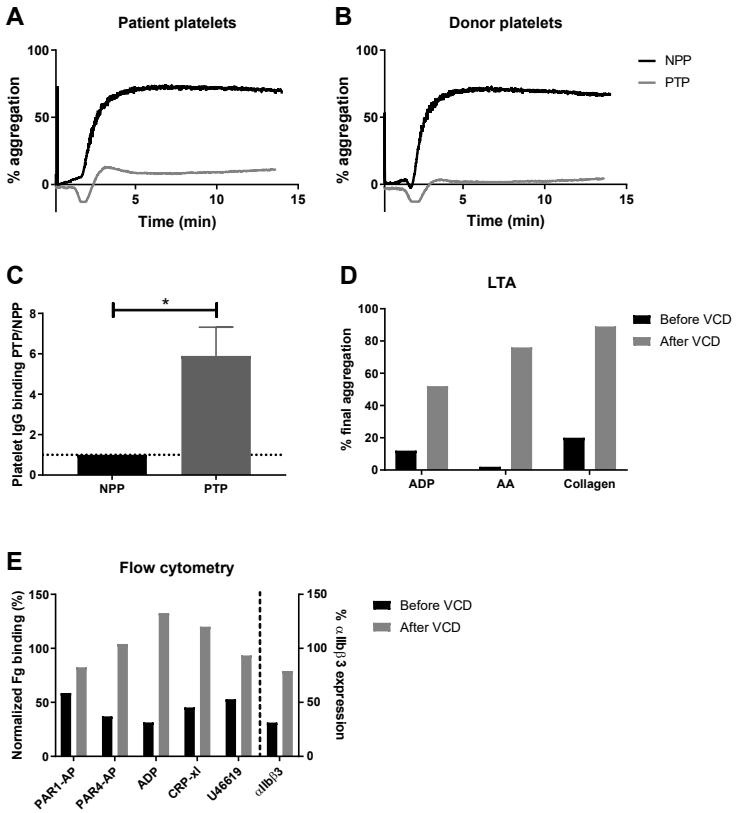

Figure 1. Acquired Glanzmann Thrombasthenia. (A) Patient platelets were incubated in PNP (black) or PTP (grey) and aggregation was initiated with 4.0 µg/mL collagen. (B) Donor platelets were incubated in PNP (black) or PTP (grey) and aggregation was initiated with 4.0 µg/mL collagen. (C) Donor platelets were incubated in PNP or PTP and IgG binding was analyzed with flow cytometry (p=0.04). (D) Platelet aggregation after stimulation with 5.0µM ADP, 1.5mg/mL arachidonic acid (AA) and 4.0µg/mL measured with light transmission aggregometry (LTA) before (black) and after VCD therapy (grey) (E) Platelets were activated with 25µM PAR-1AP, 250µM PAR4-AP, 60µM ADP, 1µg/mL CRP-xl or 5µM U46619 for 10 minutes and fibrinogen (Fg) binding was assessed with flow cytometry. Samples were taken before (grey) and after VCD therapy (black).

Table 1. Laboratory characteristics patient

	Results	Reference
Hb (mmol/L)	9.1	7.4-9.6
Platelet count (10^9/L)	513	150-450
WBC (10^9/L)	11.1	4-10
TPO (E/mL)	39	4-32
PT (s)	9.8	10-13.5
APTT (s)	33.1	<35
vWF:ag (IU/dL)	115	40-150
vWF:Rco	129	40-150
LTA (% final amplitude)		
5.0μM ADP	12	>77%
1.5mg/mL arachidonic acid	0	>65%
4.0μg/mL collagen	20	>78%
αIIbβ3 expression (%)	31%	
Blood paraprotein (subclass)	Positive, IgGk	
MAIPA	Negative	

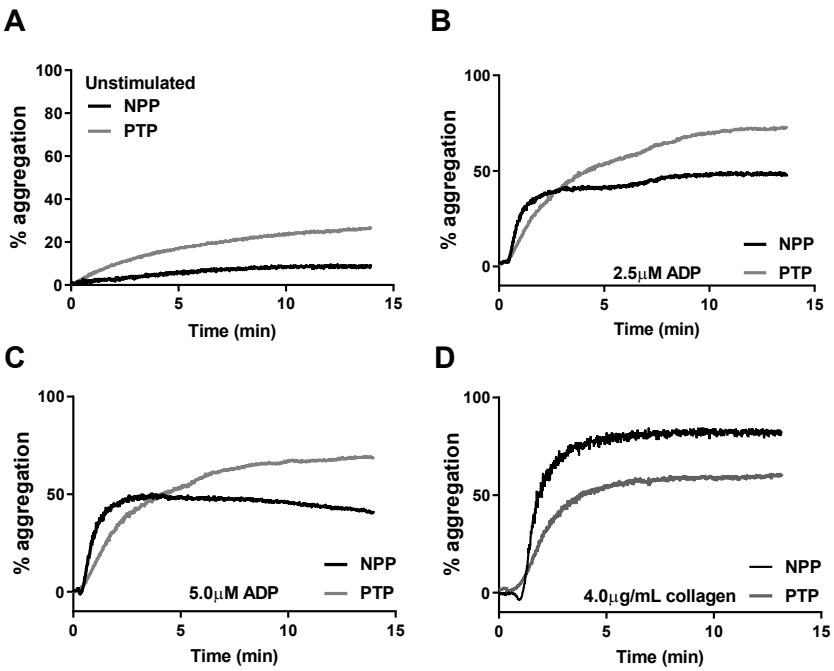

Figure 2. Acquired thrombocytopenia and spontaneous aggregation. Platelet aggregation of donor platelets in PTP (grey) and NPP (black) without stimulation (A), 2.5μM ADP (B), 5.0μM ADP (C) and 4.0μg/mL Horm collagen (D).

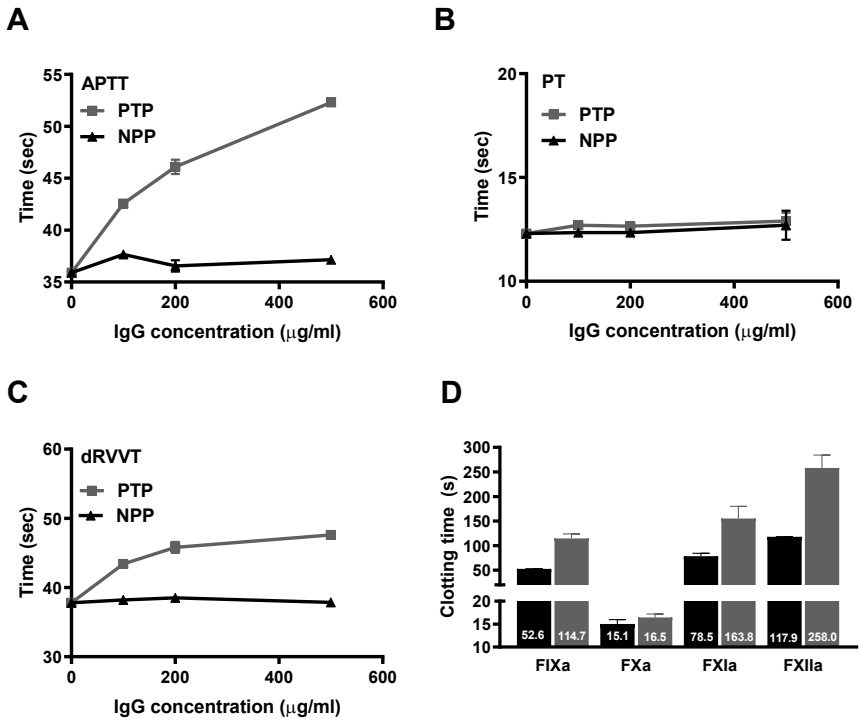

Figure 3. Acquired coagulopathy. APTT (A), PT (B) and dRVVT (C) in plasma spiked with several concentrations of isolated IgG from PTP or PNP. (D) Relative clotting time measured when clotting was initiated with FIXa, FXa, FXIa and FXIIa, in PNP (black) and PTP (grey). Original clotting times are in the graph bars.

indicate interference with the activation of FX. Because of the mild bleeding symptoms of this patient, treatment with VCD was not considered justified.

Patient 4 was a woman of 57 years who presented with compartment syndrome after intramuscular hemorrhage. Routine laboratory investigations showed a PT of 12.7s, an APTT of 47s, a prolonged APTT mixing test, FXI activity of 41% and FXII activity of 33%. The paraprotein screening was positive with an IgGk of 7 g/L. After an iliac crest puncture she had a WHO grade 3 hemorrhage, after which Hb dropped from 7.8 to 5.5 mmol/L. Bone marrow morphology showed 5% monoclonal plasma cells. Because of the suspicion of MGUS related coagulopathy and the severe limb threatening bleeding symptoms, treatment of the MGUS was initiated with 3 cycles of VCD therapy and autologous stem cell transplantation after high dose Melphalan. After therapy, the monoclonal paraprotein was not detectable and remained that way during a 5-year follow up period, the bleeding symptoms disappeared and the APTT restored to 35s. Taken together, this strongly indicates that the paraprotein was associated with the bleeding symptoms.

DISCUSSION

Here, we presented four different cases of a monoclonal gammopathy associated with a mild to severe hemorrhagic diathesis. Treatment of the monoclonal gammopathy with an anti-myeloma

chemotherapy regimen lead to remission and resolved the bleeding tendency, not only in clinical parameters but also in laboratory parameters.

There is increasing evidence that the presence of a paraprotein might cause or contribute to a wide variety of pathologies. Many different organs may be involved in MGCS and there are several ways in which a monoclonal gammopathy can become clinically significant. For instance, monoclonal gammopathy of renal significance may be caused by immunoglobulin deposits in the kidney[17], autoantibody activity targeted to collagen IV or phospholipase A2[18], or via complement pathway activation[19]. The cases presented here show direct autoantibody activity interfering on different levels of the hemostatic function. Immune thrombocytopenia was described in 3% of patients with monoclonal gammopathy as compared to 0.1% in the general population[20]. The most recognized condition is acquired von Willebrand disease (AVWD). Of all AVWD cases, 31% is related to a monoclonal gammopathy[21]. The EACH2 registry showed that a monoclonal gammopathy was found as underlying disorder in 2.6% of cases of acquired hemophilia A[22].

MGHS is difficult to diagnose, because it requires more than routine diagnostic tests to show a causal relation between the monoclonal gammopathy and the pathology. As a consequence, the incidence of MGHS might be underestimated. In patient 1, there was an antibody targeting αIIbβ3, which inhibited fibrinogen binding. It is unclear whether this paraprotein blocked our detection antibody, or whether this antibody resulted in αIIbβ3 receptor shedding. Anti-αIIbβ3 antibodies have been described, and can also result in immune thrombocytopenia (ITP)[23,24]. Our patient had a normal platelet count. We therefore hypothesize that this paraprotein had low affinity for platelets. Acquired Glanzmann Thrombasthenia is very rare, but has been described in malignant disease and can be treated in order to resolve the bleeding tendency[25].

Our second case had an extremely low platelet count (<1x10^9/L) and was non responsive to standard treatment. Functional analysis indicated that a component in patient plasma caused spontaneous platelet aggregation. Here, pre-activation of platelets could explain the severity of the observed thrombocytopenia, because platelets are cleared from the circulation after activation[26]. After VCD therapy, the platelet count normalized completely and the paraprotein disappeared.

Two of our patients developed an acquired coagulopathy. In patient 3 the monoclonal antibody attenuated the activation of FX by the intrinsic tenase complex. As the bleeding symptoms in this patient were mild, anti-myeloma therapy was not considered justified. While patient 4 presented with a similar acquired coagulopathy, the bleeding tendency was more severe, and treatment was deemed necessary. After treatment, the concentration of the paraprotein dropped and the bleeding problems resolved.

The progression from an MGUS to malignant disease is around 1% per year, and without any symptoms, immediate therapy is not necessary[27]. Spontaneous disappearance of a paraprotein is rare and occurs in up to 5% of the studied patients[28,29]. Recovery from a paraprotein might be observed in patients that are treated for auto-immune disease or infections[30]. Treatment of MGUS related disorders depends on the severity of disease, since clone directed therapy might have toxic side-effects. According the recommendations from the European Myeloma Network, treatment of MGUS related disorders should only be considered if the disease is aggressive or disabling

and is only justified when there is a clear link between the disease and the MGUS[27]. Our patients experienced (severe) bleeding symptoms that were associated with the MGUS, therefore 3 out of 4 patients were treated with VCD. In patient 3, there was a clear relation between the MGUS and bleeding symptoms, but the mild bleeding symptoms did not justify the potential side effects of treatment.

In conclusion, due to the potential severe bleeding phenotype and the potentially beneficial effect of treatment, we argue for a new entity, the Monoclonal Gammopathy of Hemorrhagic Significance (MGHS). Patients with MGHS have a MGUS, do not meet criteria for multiple myeloma, and have a clinically significant acquired bleeding phenotype. The coagulation disorder can be on the level of diminished platelet function, acquired von Willebrand disease, acquired hemophilia A or other acquired abnormalities in the plasmatic coagulation system. Treatment of the MGUS with multiple myeloma based strategies might be beneficial to control the bleeding phenotype.

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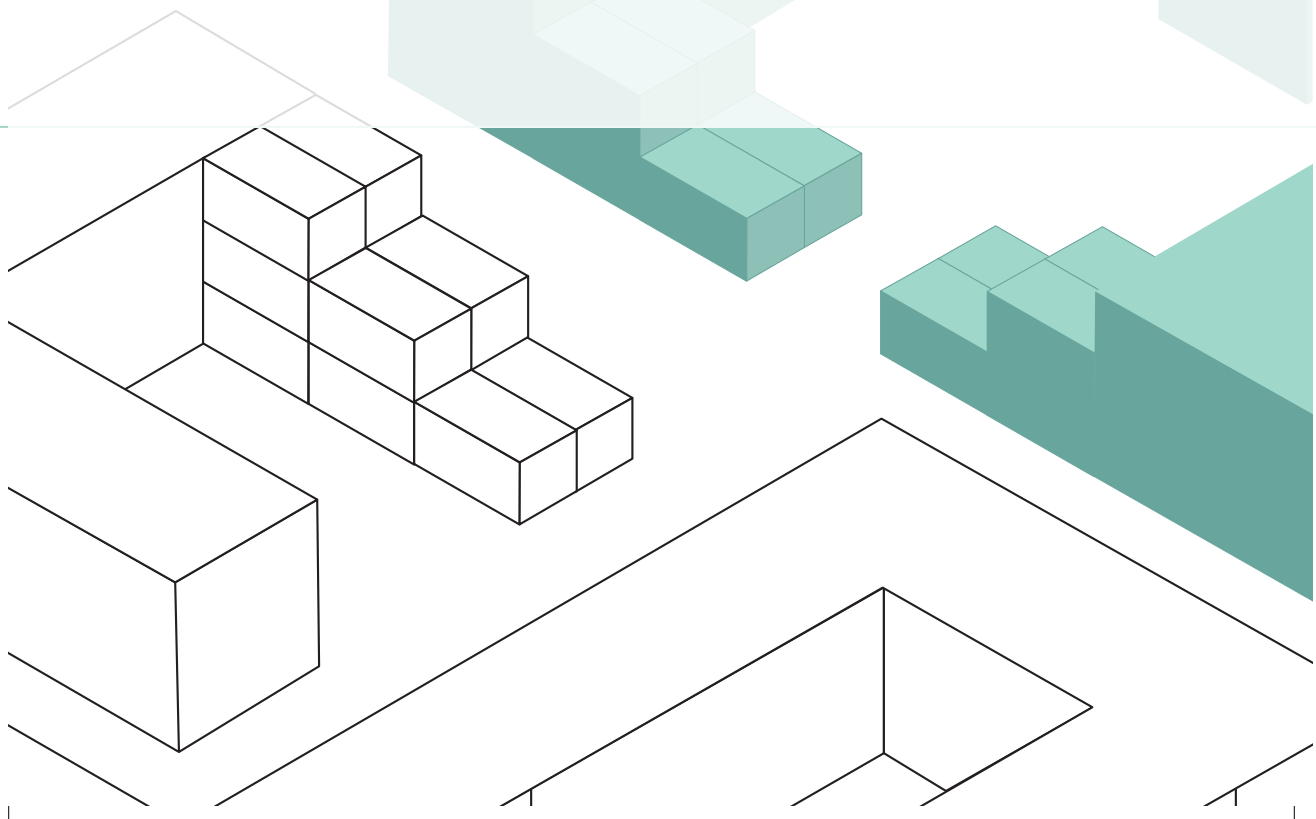
8

SUMMARY AND DISCUSSION



SUMMARY

We are in need of better diagnostic tools for platelet function disorders, because the current diagnostic tools lack sensitivity and specificity for platelet function disorders. To improve the diagnostic success rate, the International Society of Thrombosis and Haemostasis (ISTH) recommends the use of flow cytometry as diagnostic tool for platelet function disorders.

In **chapter 2** we described the different applications of flow cytometry in platelet function testing and discussed the challenge of introducing new diagnostic tools in the field of platelet function disorders. There is no gold standard test, making it difficult to validate new tools. In **chapter 3** we showed that flow cytometric platelet function testing has added value in diagnosing patients with suspected platelet function disorders compared with light transmission aggregometry alone. The diagnostic accuracy of mepacrine fluorescence measured on the flow cytometer for storage pool disease was evaluated in **chapter 4**. Although this test showed good discriminative ability, it was inferior to platelet ADP content. Nevertheless, we showed that this test can be used to exclude storage pool disease in patients suspected for a platelet function disorder. Genetic testing is an alternative for platelet function testing and its diagnostic value is described in **chapter 5**. We identified a causal genetic variant in only 17% of the patients with a platelet function disorder, indicating that genetic testing has limited value in the current diagnostic setting. To improve the genetic diagnostic yield, we need to explore the relation between newly identified genetic variants and the platelet function disorder. In **chapter 6** we identified a variant in the GNE gene in two sisters with severe macrothrombocytopenia. We showed that this variant caused platelet hyposialylation and increased hepatic platelet clearance, explaining their low platelet count. Finally, in **chapter 7,** we showed that monoclonal gammopathy can result in an acquired bleeding disorder and can be treated successfully with anti-plasma cell therapy.

Taken together, this thesis shows that flow cytometry can be used in the diagnosis of platelet function disorders. Using flow cytometry, we we have increased our understanding of certain platelet function disorders. However, we are still in need of better diagnostic tools. In the near future, we should aim to understand the pathophysiological mechanisms of platelet function disorders, in order to design more effective diagnostic tools.

DISCUSSION

Treatment of platelet function disorders

Platelets play an important role in hemostasis, a multifactorial system that prevents unnecessary blood loss during vascular injury. Bleeding from vascular injury can only be stopped when there is proper interaction between the subendothelial matrix, von Willebrand factor, platelets, coagulation factors and fibrinolytic compounds. A deficiency in one of these components can give rise to bleeding symptoms. To manage those bleeding symptoms, several treatment options are available, but diagnostic testing is essential in picking the appropriate therapy.

The treatment of patients with bleeding symptoms is usually based on the affected component of the hemostatic system. For instance, hemophilia A can be treated by the supplementation of coagulation factor VIII and von Willebrand disease can be treated by administration of von

Willebrand factor. Unfortunately, the treatment of platelet function disorders is more complicated. Platelet function disorders can be divided into quantitative (thrombocytopenia) and qualitative defects (thrombocytopathy).

The treatment options for platelet function disorders are limited. The options for treatment are platelet transfusions, desmopressin and anti-fibrinolytics[1]. These therapies are effective ways to treat bleeding symptoms, but they do not cure the underlying deficit. The severity of bleeding symptoms determines which treatment strategy is preferred. In general, desmopressin and anti-fibrinolytics are effective in treating mild platelet function disorders, but not in severe platelet function disorders[2,3]. Patients with severe platelet function disorders, like Glanzmann Thrombasthenia, are more often treated with platelet transfusions. However, platelet transfusions are associated with an increased risk of developing allo-antibodies targeted to donor platelets. The more frequent platelet transfusions are given, the higher the chance of generating allo-antibodies, for instance against glycoprotein αIIbβ3 in patients with Glanzmann Thrombasthenia. As a consequence, these allo-antibodies result in clearance and destruction of transfused platelets, reducing the efficacy of treatment[4]. Therefore, platelet transfusion are only provided when necessary.

When considering treatment options for thrombocytopenia, it is important to identify the cause of the low platelet count. Low platelet count can be either congenital or acquired and can be the result of impaired platelet production or increased platelet clearance. In case of increased platelet clearance, patients can be treated with intravenous immune globulins or splenectomy, whereas impaired platelet production can be treated by stimulation platelet production with thrombopoietin or platelet transfusions. A very illustrative example was a patient from the Thrombocytopathy in the Netherlands (TiN) study (**chapter 5**). This woman was diagnosed with immune thrombocytopenia and was "treated" with splenectomy. However, her platelet count remained low and she was referred to the UMC Utrecht for a second opinion. Here, she was diagnosed with Bernard Soulier syndrome, which is characterized by low platelet counts due to impaired production. This example emphasizes the importance of an accurate diagnosis, since a false diagnosis may lead to ineffective or unnecessary invasive treatment.

Limitations of current platelet function testing

In order to provide effective treatment for bleeding symptoms, we are in need of adequate diagnostic tools. Rare and severe platelet function disorders are easy to diagnose, but more prevalent and mild platelet function disorders are difficult to diagnose because of the lack of adequate diagnostic tools[5]. Light transmission aggregometry (LTA) has been developed in 1962 to diagnose platelet function disorders. In all the years since then, no other test was able to replace the LTA. LTA measures the light transmission through plasma during platelet activation. When platelets form aggregates, light transmission through the sample increases, which is a measure for platelet function. LTA is useful for studying several platelet activation pathways and platelet aggregation function, but cannot distinguish between platelet activation, secretion or aggregation disorders. It is sensitive for severe platelet function disorders like Glanzmann Thrombasthenia

and Bernard Soulier syndrome, but lacks sensitivity for Storage Pool disease or other mild platelet function disorders[6]. For the diagnosis of Storage Pool disease, platelet ADP content or lumiaggregometry can be used[7].

Differentiating between different platelet function disorders is easier with flow cytometry than with routine diagnostic tests and depends on the markers that are used. For instance, by using P-selectin expression or CD63 expression in response to agonist stimulation, platelet secretory function can be measured, whereas platelet fibrinogen binding can be used to measure platelet aggregation function. Mepacrine fluorescence on the flow cytometer can be used for the screening of Storage Pool disease. However, those applications are not yet able to replace LTA or platelet ADP content (**chapter 3** and **chapter 4**). Many mild or rare platelet function disorders can show decreased platelet aggregation or platelet reactivity on the flow cytometer, but the test does not allow the identification of a particular platelet function disorder. Therefore, more disorder-specific tests should be performed in addition to assessment of platelet function with flow cytometry[8]. Another advantage of flow cytometry over LTA is the detection range. Platelet aggregation is semi-quantitative because it is relative to platelet poor plasma and has a maximum of 100% aggregation. With flow cytometry, there is no maximum signal. Second, with LTA, a certain amount of αIIbβ3 activation is required to obtain maximum platelet aggregation, but it does not mean that 100% aggregation is equal to 100% αIIbβ3 activation. With flow cytometry, the entire range of αIIbβ3 activation can be assessed. Finally, analysis of platelet function with LTA is limited to samples with a platelet count above $75x10^9$/L, which precludes the use of LTA in thrombocytopenic samples. The lowest platelet count that allows analysis of platelet function with flow cytometry is expected to be much lower (<$10x10^9$/L).

In around 60% of the patients with bleeding symptoms, the underlying cause cannot be found[9]. In **chapter 3** we have shown that LTA was normal in around 50% of the patients with a suspected platelet function disorder. With flow cytometry, we found abnormal platelet reactivity in 9 out of 22 patients (41%). Based on these data, we assume that the number of false-negative results with LTA is high and, as a consequence, patients may be treated ineffectively. Moreover, there was not a good agreement between flow cytometry in LTA and flow cytometry, which makes the interpretation difficult. Therefore, we should develop more effective tools and aim at identifying as many platelet function disorders as possible to reduce the number of false-negative results. The discrimination between different forms of platelet function disorders is of less importance, but can be helpful to link a certain genotype to the laboratory findings or in genetic counseling.

Challenges for the diagnostics of platelet function disorders

An efficient diagnostic process should be able to identify as many different platelet function disorders with as few tests as possible. This is fairly difficult for platelet function disorders, because platelet behavior is complex and many different assays are required to fully evaluate platelet function. The currently used assays cover some, but not all functions of platelets in hemostasis. For instance, platelet adhesion to the vessel wall cannot be fully assessed: While platelet-von Willebrand factor binding can be measured with LTA, disturbances in platelet adhesion to

extracellular matrix proteins due to α2β1 deficiency cannot be detected with this technique. With flow cytometry, platelet surface receptors can be quantified, but it does not prove that these receptors are functional. Moreover, the reaction conditions in these assays cannot fully assess platelet function in the circulation, because shear forces and subendothelial proteins are missing. Similarly, platelet interaction with secondary hemostasis cannot be tested, but this interplay is important in hemostasis.

Although it is almost impossible to have one platelet function test that covers all aspects of platelet function in hemostasis, there is room for improvements. However, implementing new tests in the diagnostics of platelet function disorders is challenging, due to the lack of a gold standard test, definition of patient population and the limited diagnostic value of genetic testing, as described in **chapter 2** of this thesis.

Severe platelet function disorders are easy to diagnose, but the mild platelet function disorders are more difficult to identify. However, the identification of a mild platelet function disorder is equally important as the identification of a severe platelet function disorders, because the failure to identify a platelet disorder will result in the underestimation of the risk of bleeding during hemostatic challenges. In patients with mild platelet function disorders, for example, it is important to be aware of the increased bleeding risk during surgery.

The inadequate diagnostic sensitivity for mild platelet function disorders makes validating new tools for these mild disorders challenging, because we cannot rely on a reference test, as shown in **chapter 3**. In this chapter, we decided to determine the discriminative ability of both LTA and flow cytometry between healthy controls and patients with suspected platelet function disorder. The disadvantage of such an approach is that we cannot conclude which test is true positive and which is not, but we show that one test shows more abnormal results in patients compared to healthy controls. An alternative approach is to look at a particular platelet function disorder for which there is a reference test, such as is described in **chapter 4** of this thesis, where we show that mepacrine fluorescence can be used to exclude delta Storage Pool disease. In this study, only patients with suspected platelet function disorders were included. They were categorized as "positive" or "negative" for the disease based on platelet ADP content. In this patient population, we are able to determine the diagnostic accuracy of mepacrine fluorescence measured on the flow cytometer. Unfortunately, this approach is not feasible for all platelet function disorders, because some disorders are extremely rare and there is no gold standard test. Storage Pool disease was present in about 10% of our patient population, but in more rare diseases we would need bigger study populations and perhaps global collaborations to get a significant number of patients with a particular platelet function disorder.

Defining a platelet function disorder

Labeling a test result as "normal" or "abnormal" requires a definition of normal platelet function. By defining the 2.5th percentile of the response in the healthy population as a cut-off value, we accept that 2.5% of the healthy people has an abnormal test result. In our flow cytometry assay, we stimulated platelets with 5 different agonists and had 2 different read outs (e.g. fibrinogen binding

and P-selectin expression). That means that a healthy person has a 78% (97.5%^10) chance of having "normal" platelet function when measured with flow cytometry. Therefore, an abnormal platelet function assay does not necessarily mean that someone has a platelet function disorder. We can correct for multiple testing by defining that >1 variables need to be abnormal in platelet function disorders. However, we know that 1 abnormal variable, for instance in Bernard Soulier syndrome, results in bleeding symptoms. As a result, we should prioritize the variables in the assay, but this will take years of investigation. In an optimal situation we need a gold standard test to evaluate diagnostic accuracy and to define an optimal reference value.

Selecting the right patient population

For the validation of new diagnostic approaches for platelet function disorders it is important to use a clearly defined patient population. Only patients with (suspected) platelet function disorders should be included in such a design. However, it is hard to exclude all deficiencies in von Willebrand factor, coagulation and fibrinolysis, or to exclude tissue specific defects that result in bleeding. Incorrect inclusion of patients results in underestimation of the diagnostic accuracy of the test, but without a gold standard we cannot conclude whether we missed a diagnosis or whether we falsely included a patient. Moreover, including patients based solely on bleeding symptoms is difficult, because it is partially based on the patient's and their environment's perception of the symptoms. Although efforts have been made to objectify bleeding symptoms with questionnaires like the bleeding assessment tool from the International Society for Thrombosis and Hemostasis (ISTH-BAT)[10], the severity of bleeds is subjective to patient experience. For instance, there is a difference in disease perception when you suffer from mild bleeding symptoms that are common within your family, or when you are the only affected person within a family. Nevertheless, with the ISTH-BAT, 14 different items are evaluated based on the frequency, time and treatment of bleeds to objectify the bleeding symptoms. A bleeding score >3 in men and >5 in women can be used for the screening of a bleeding disorder[11]. It has recently been shown that the ISTH-BAT has high sensitivity for platelet function disorders and is therefore a useful screening tool[12]. However, it does not allow discrimination between, for instance, von Willebrand disease and a platelet function disorder.

Genetic testing of platelet function disorders

An additional approach for the diagnosis of platelet function disorders is genetic analysis. The current list of candidate genes involved in platelet function contains over 300 genes of which 56 genes are associated with a platelet function disorder[13]. It is likely that this list will become longer in the future, because there are over 5000 different proteins present in platelets[14]. Because of our increased understanding of genes involved in platelet function, it was suggested to perform genetic testing earlier in diagnostic work-up of platelet function to increase diagnostic efficiency[15,16]. In **chapter 5,** we have shown that the diagnostic yield of genetic analysis is limited in patients with a platelet function disorder. Moreover, genetic testing did not have diagnostic value when laboratory investigations were unable to identify platelet dysfunction in patients with a bleeding tendency or when laboratory results showed different abnormalities on different occasions.

Genetic testing becomes more complicated when platelet function disorders are caused by multiple genetic variants. Not only by different variants in platelet function genes, but bleeding symptoms can also occur when genetic variants in different parts of the hemostatic system occur, for instance by variants in von Willebrand factor, coagulation factors and platelet signaling. Moreover, acquired bleeding disorders, as described in **chapter 7**, lower the diagnostic success rate of genetic testing.

The low diagnostic yield of genetic testing in our study conflicts with previous reports that found a higher number of genetic variants in patients with platelet function disorders[17,18]. The primary difference between our study and those studies, is that patient inclusion criteria were different. For instance, they included patients with an established platelet function disorder. Genetic testing in patients that already have a diagnosis is not useful for their diagnosis and does not change treatment regimens, but could be performed when genetic counseling is requested. Therefore it is more fair to evaluate the diagnostic value in a more real-life patient population that contains patients with inconclusive laboratory findings and patients with a suspected platelet function disorder. In these patients, genetic testing may aid in the diagnosis. Our data show that genetic testing has limited additional value. Other studies that performed genetic testing in a more real-life patient population showed a similar success rate [13,19]. In **chapter 5** we found a genetic variant of uncertain significance (VUS) in 16 out of 170 patients (9%) and Downes et al found a VUS in 5% of patients with suspected platelet function disorders [13] . The actual number of VUSs in those patients are higher, because we selected potential VUSs if they matched the laboratory phenotype of the patients. The problem with VUSs is that they cannot be used for clinical decision making[20], although they might fit with the patients phenotype. Before using these VUSs in clinical decision making, their role in the pathology of the patient needs to be confirmed. Taken together, genetic testing has potential in the diagnosis of platelet function disorders, but it is not yet as useful as promised.

Future perspective

Genotype-phenotype relation

In order to improve the diagnostic yield for platelet function disorders, we need both improvements in functional testing and genetic testing. Platelet function testing remains challenging due to the complex and multifactorial hemostatic system. In many cases the genetic cause of a bleeding disorder remains unclear, despite a clear family anamnesis and/or the presence of bleeding symptoms from birth. Even if genetic variants are identified, we first need to prove that this variant has clinical consequences. This will take a lot of effort, but it is essential to increase the diagnostic success rate of genetic testing. For instance, in **chapter 6** we found a probably damaging genetic variant in GNE, which encodes the rate limiting enzyme of sialic acid synthesis, in two siblings with a severe congenital macrothrombocytopenia. Hitherto, GNE variants were associated with adult onset muscle atrophy and occasionally a thrombocytopenia was reported. We were able to confirm that this GNE variant resulted in platelet hyposialylation and that this hyposialylation is responsible for increased hepatic platelet clearance explaining their low platelet count. This uptake mechanism

has been described in an animal model[21], but we are the first to show this disease mechanism in human. This mechanism may also apply to other patients with unexplained thrombocytopenia, so measuring platelet sialylation status and hepatic platelet sequestration might be beneficial in the diagnosis of thrombocytopenia.

With the rise of CRISPR-CAS9 technology, introducing newly identified genetic variants in cell lines has become more facile[22]. This allows us to study the effect of genetic variants on a cellular level. In **chapter 6,** this technology was used to knock-down the GNE gene and to study its consequences. This method may already seem old-fashioned, because the possibilities for genetic editing have developed rapidly. For instance, base-editing with CRISPR technology allows the introduction of precise point mutations in living cells, without introducing double stranded DNA breaks[23]. There are many different rare platelet function disorders, so it will take a lot of time and effort to perform similar studies to elucidate the mechanism driving patients bleeding symptoms. Although it may seem as small contributions to the scientific field, those findings have big impact on the individual patient or their family. Moreover, these kind of studies will help in the interpretation of future variants in the same gene, although it remains difficult to prove that the bleeding symptoms are actually caused by such a variant.

A first approach may be to introduce genetic variants in induced pluripotent stem cells (IPSC), followed by their differentiation towards megakaryocytes and platelets in vitro[24,25]. In theory, this could be used to confirm whether the platelet function disorder of the patient is also present in the IPS cell derived platelets. While this does not prove that this platelet function defect results in bleeding symptoms, it increases the likelihood of causality between a genetic variant and a bleeding phenotype. In case of thrombocytopenia, the platelet production rate of these megakaryocytes can be evaluated to determine if platelet production is impaired. A second approach is to create an animal model that contains the same genetic trait as a patient and subsequently study the hemostatic system. However, creating an animal model is time consuming and expensive. Moreover, the hemostatic system in man is not identical to the hemostatic system in animals, which makes it difficult to draw hard conclusions.

Therapeutic options

There is a wide variety of options for treating a bleeding tendency, but before applying tailored therapy, we need to understand the exact disease mechanism. The techniques used for understanding our disease, could potentially also be used to cure our patients. The in vitro IPSC derived platelet production is a promising tool to study genetic variation, but is also a potential tool for therapeutics. Its main goal was to produce significant amounts of platelets for transfusion purpose[26], however the yield was not sufficient. Nevertheless, the IPSC models can be used to cure patients disease.

Ideally, we create IPSCs from our bleeding patient and with the genome editing tools, we selectively restore the genetic cause of bleeding. Those IPSCs can be differentiated into hematopoietic stem cells and can be transplanted into the patient to replace its previous bone marrow cells. These kind of procedures are technically available in the lab, but safety issues need

to be addressed before this kind of therapy may be applied. An alternative approach may be genome editing in vivo, but this presents an additional challenge, namely tissue specific targeting of the genome editing tools. One of the biggest obstacles is to guarantee that only the "affected" gene is restored, without unintended off-target DNA editing elsewhere in the genome.

Moreover, we cannot exclude host versus graft reactions in patients who receive autologous genetically modified stem cells. Take, for example, patients with Glanzmann Thrombasthenia, who lack the fibrinogen receptor. The fibrinogen receptor of on genetically modified autologous cells can be recognized as foreign and induce an immune response, similar as in Glanzmann Thrombasthenia patients developing immune responses against transfused platelets[27]. However, in the patient receiving our therapy, the immune response may be permanent, because those foreign platelets are continuously produced.

Mapping the diagnostic maze of platelet function disorders

Platelet function in hemostasis is complex, so diagnosing platelet function disorders is equally sophisticated. We aimed to solve this diagnostic maze, and with the validation of flow cytometric applications for the diagnosis of platelet function disorders, we made several shortcuts to navigate more efficiently through our maze. However, the bearing walls of this maze remain untouched, because the applications we investigated cannot replace the currently available tests, but should be performed alongside. The limited diagnostic value of genetic testing emphasizes that there is much more to learn. In all those patients with an evident bleeding problem, but without a genetic or laboratory diagnosis, we have to search for the mechanism that explains their bleeding symptoms to explore the size of the maze. By identifying new disease mechanisms, the diagnostic complexity increases, which is illustrated by our studies to understand the role of GNE in thrombocytopenia. In short term, it seems we are building walls in our maze, instead of breaking them down. But mapping the entire maze is crucial in order to successfully guide every single patient toward the end of their diagnostic journey. On the long term this will help us rearranging the walls of our maze to, ultimately, reduce the number of steps we need to take to find a diagnosis.

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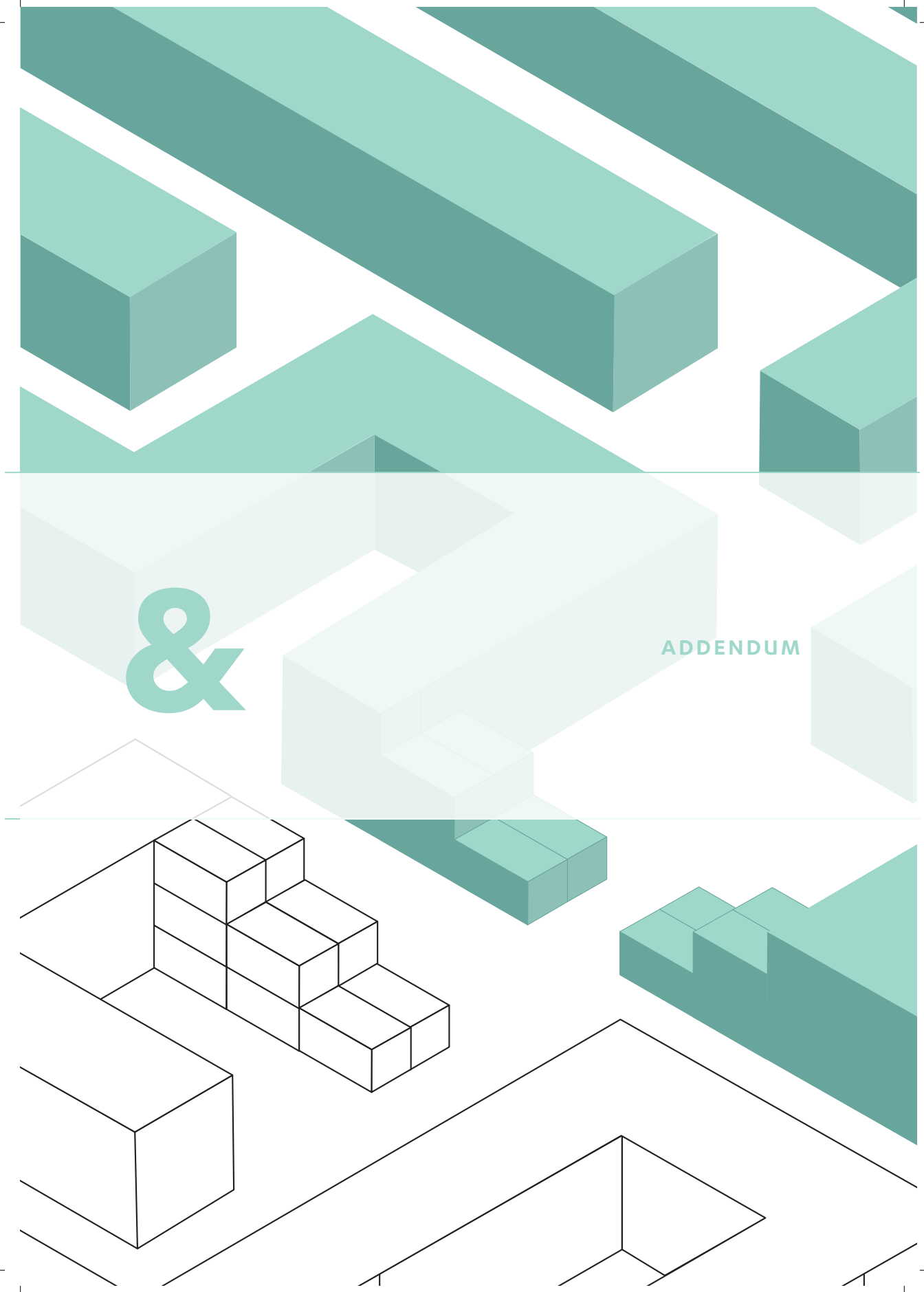
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ADDENDUM

NEDERLANDSE SAMENVATTING

In dit proefschrift staan de resultaten van het promotieonderzoek van Ivar van Asten naar de diagnostiek van bloedplaatjesfunctiestoornissen. De vertaalde titel luidt: "In kaart brengen van het diagnostische doolhof van bloedplaatjesfunctiestoornissen".

Bloedplaatjes spelen een cruciale rol in de bloedstolling en minimaliseren de hoeveelheid bloedverlies tijdens beschadigingen aan de vaatwand. Bloedplaatjes worden geactiveerd op het beschadigde bloedvat en gaan aan elkaar binden, zodat het beschadigde bloedvat wordt afgedicht. Patiënten met niet goed werkende bloedplaatjes zullen langere tijd nodig hebben om een stolsel te vormen, waardoor zij langer zullen bloeden. Typische kenmerken van bloedplaatjesfunctiestoornissen zijn regelmatige (spontane) blauwe plekken, verlengde bloedingstijd en hevige menstruaties. Ernstige bloedplaatjesfunctiestoornissen kunnen leiden tot levensbedreigende bloedingen, maar ook milde bloedplaatjesfunctiestoornissen kunnen gevaarlijk zijn. Met name wanneer er adequate bloedstolling gevraagd wordt, bijvoorbeeld tijdens chirurgische ingrepen. Het is daarom belangrijk dat zowel de ernstige, maar ook milde, bloedplaatjesfunctiestoornissen tijdig gediagnosticeerd worden, zodat patiënten hier voor behandeld kunnen worden, of er rekening mee gehouden kan worden tijdens chirurgische ingrepen.

De diagnostiek van bloedplaatjesfunctiestoornissen is complex en bovendien ontoereikend. In bijna de helft van de patiënten met bloedplaatjesfunctiestoornissen kan geen exacte oorzaak gevonden worden. Er is dus grote behoefte aan vernieuwde diagnostische testen om patiënten met een bloedplaatjesfunctiestoornis te kunnen identificeren. Een potentiële test voor de diagnostiek maakt gebruik van flow cytometry en dit wordt aangeraden door de internationale organisatie voor trombose en hemostase. Echter, deze test wordt nog maar weinig in de diagnostiek gebruikt. In **hoofdstuk 2** van dit proefschrift staat een overzichtsartikel van de mogelijke toepassingen van deze methode. Deze test kan niet alleen gebruikt worden om bloedplaatjesfunctiestoornissen te ontdekken, maar kan ook gebruikt worden om het risico van trombose in te schatten (te sterk werkende bloedplaatjes) of om de kwaliteit van bloedplaatjesconcentraten voor transfusie te kunnen meten. De grote uitdaging van het implementeren van nieuwe diagnostische testen, zoals deze test op de flow cytometer, is het gebrek aan een referentiemethode. Zonder referentiemethode is het lastig te beoordelen of de nieuwe test een terechte uitslag heeft gegenereerd. In **hoofdstuk 3** van dit proefschrift wordt de bloedplaatjesfunctie test op de flow cytometer vergeleken met de meest gebruikte diagnostische test voor bloedplaatjesfunctiestoornissen. In dit hoofdstuk laten we zien dat de test op de flow cytometer toegevoegde waarde heeft ten opzichte van de huidige test, maar dat de huidige test nog niet vervangen kan worden. **Hoofstuk 4** van dit proefschrift beschrijft een specifieke toepassing op de flow cytometer voor de diagnostiek van storage pool disease en maakt gebruikt van mepacrine. Mepacrine is een fluorescente stof die bindt aan de "dense granules" in bloedplaatjes. In patiënten met storage pool disease zijn deze dense granules 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 screening test. Echter een afwijkende uitslag betekent niet per sé dat er sprake is van storage pool disease. In **hoofdstuk 5** laten we zien dat genetisch testen nog weinig toegevoegde waarde heeft in het aantonen van bloedplaatjesfunctiestoornissen ten opzichte van laboratorium testen. Echter, het kan wel van

waarde zijn voor genetisch familie onderzoek of genetisch advies. Indien er in de toekomst meer genetische afwijkingen gevonden worden die passen bij bloedplaatjesfunctiestoornissen, dan kan genetisch onderzoek meer toegevoegde waarde krijgen voor de diagnostiek. Dit vereist wel jaren van wetenschappelijk onderzoek voordat dit in de praktijk toepasbaar wordt. Een voorbeeld van zo'n wetenschappelijke studie is beschreven in **hoofdstuk 6**. In dit hoofdstuk beschrijven we een familie waar een nieuw genetisch defect is gevonden, wat mogelijk te maken had met een extreem laag bloedplaatjes aantal in twee zusjes. Het genetisch defect werd gevonden in het GNE gen, wat verantwoordelijk is voor siaalzuur productie. In dit hoofdstuk laten wij zien dat de bloedplaatjes van deze patiënten verminderde hoeveelheid siaalzuur op de bloedplaatjes hebben en dat dit tekort aan siaalzuur leidt tot versnelde bloedplaatjes opname in de lever via een specifiek mechanisme. Als gevolg hebben deze patiënten verlaagd aantal circulerend bloedplaatjes en hebben zij een verhoogd bloedingsrisico. Dit mechanisme was alleen nog maar beschreven in diermodellen, maar wij waren de eerste die dit fenomeen in mensen hebben gevonden. Mogelijk kan men in andere patiënten met een onverklaarbaar laag aantal bloedplaatjes dezelfde defecten op sporen om zo tot een passende diagnose te komen. Naast erfelijke bloedplaatjesfunctiestoornissen, zijn er ook verworven aandoeningen die leiden tot een verhoogde bloedingsneiging, zoals beschreven in **hoofdstuk 7** van dit proefschrift. In dit hoofdstuk worden twee patiënten beschreven die door een monoklonale antistof een bloedplaatjesfunctiestoornis hebben ontwikkeld. Daarnaast worden er twee patiënten beschreven die een verworven stollingsstoornis hebben. Drie van de vier patiënten zijn behandeld met chemotherapie, om de monoklonale antistof kwijt te raken. In deze drie patiënten is de bloedingsneiging verdwenen na het behandelen van deze monoklonale antistof. Dit hoofdstuk laat zien dat het vinden van een verworven bloedingsneiging belangrijk is, omdat de klachten met succes behandeld kunnen worden. In **hoofdstuk 8** worden de hoofdstukken van dit proefschrift bediscussieerd en in het perspectief van de huidige literatuur geplaatst.

De diagnostiek van bloedplaatjesfunctiestoornissen is er erg complex en kan worden vergeleken met een doolhof. Echter, in een groot deel van de patiënten kan geen oorzaak worden gevonden en ook genetisch testen heeft nog weinig toegevoegde waarde. De exacte omvang van dit doolhof is voor ons nog onbekend, dus meer wetenschappelijk onderzoek is nodig. Door het doen van wetenschappelijk onderzoek kunnen we het diagnostische doolhof beter in kaart brengen. Op de korte termijn lijkt het doolhof er alleen maar ingewikkelder op te worden, maar zodra het hele doolhof in kaart gebracht is, kunnen we op zoek gaan naar de snelste route om tot onze diagnose te komen.

LIST OF PUBLICATIONS

Ivar van Asten, Roger E.G. Schutgens, Rolf T. Urbanus. Toward Flow Cytometry Based Platelet Function Diagnostics. *Seminars in Thrombosis and Hemostasis*. 44: 197-205 (2018).

Ivar van Asten, Roger E.G. Schutgens, Marije Baaij, Judith Zandstra, Mark Roest, Gerard Pasterkamp, Albert Huisman, Suzanne J.A. Korporaal, Rolf T. Urbanus. Validation of flow cytometric analysis of platelet function in patients with a suspected platelet function defect. *Journal of Thrombosis and Haemostasis*.16: 689-698 (2018).

Blaauwgeers MW, van Asten I, Huisman A, Urbanus RT, Schutgens REG. Congenitale trombocytopathie: huidige diagnostiek en toekomstperspectief, *Nederlands Tijdschrift voor Hematologie*. 13: 332-40 (2016).

Awards

2018 Topscoring Abstract Award from the International Society for Laboratory Hematology.

2017 Trainee Travel Award from the International Society for Laboratory Hematology

2016 Eberhard F. Mammen Young Investigator Award for best presentation in the field of thrombosis and hemostasis.

2016 Young Investigator Award from the International Society for Laboratory Hematology

Presentations

2018 Oral presentation ISLH congress in Brussel and NVTH symposium in Koudekerke:
Title: "GNE-related thrombocytopenia, a congenital thrombocytopenia due to impaired platelet sialylation"

2017 Oral presentation ISLH congress in Honolulu and NVTH symposium in Koudekerke:
Titel: "Validation of flow cytometric mepacrine staining and CD63 expression in the diagnosis of Storage Pool Disease"

2017 Poster presentations ISTH congress in Berlin:
Titles: "Validation of a flow cytometric platelet function test in a cohort of patients with a suspected platelet function disorder."
"Validation of flow cytometric mepacrine staining and CD63 expression in the diagnosis of Storage Pool Disease"

2017 Presentation NVTH symposium in Koudekerke:
Title: "Validation of a flow cytometric platelet function test in a cohort of patients with a suspected platelet function disorder."

2016 Presentation springsymposium NVKC:
Title: "Validation of a flow cytometry based platelet function test compared with light transmission aggregometry"

2016 Presentation ISLH congress in Milan
Titel: "Diagnostic value of a flow cytometry based platelet function test compared with light transmission aggregometry in patients with unknown bleeding disorders"

DANKWOORD

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Ivar

CURRICULUM VITAE

Ivar van Asten was born on the 19th of June, 1991 in Sneek, the Netherlands. In 2010 he obtained his VWO-diploma at the Rijksscholengemeenschap Magister Alvinus in Sneek and started his bachelor Biomedical Science at Utrecht University. In 2013 he obtained his Bachelor of Science degree after finishing his internship at the Laboratory of Experimental Cardiology. In 2013 he started his master programme Biology of Disease at Utrecht University in 2013 with the focus on cardiovascular disease. During his master research project he studied cardiac calcium handling at the department of Medical Physiology under the supervision of dr. T.P. de Boer. In his minor research project he validated the use of flow cytometry in the diagnosis of platelet function disorders at the department of Clinical Chemistry and Haematology of the UMC Utrecht under supervision of dr. R.T. Urbanus. He obtained his Master of Science degree after completing his masterthesis at the van Creveldkliniek under supervision of prof. R.E.G. Schutgens in 2015. Subsequently he started his PhD project at the department of Clinical Chemistry & Haematology and van Creveldkliniek in the UMC Utrecht under supervision of prof. R.E.G. Schutgens, prof. G. Pasterkamp and dr. R.T. Urbanus. In this thesis the results of his PhD project are described. In 2019 he started a four-year residency in Clinical Chemistry at Certe.