Genetic Screening in Patients Suspected of Inherited Bleeding Disorders

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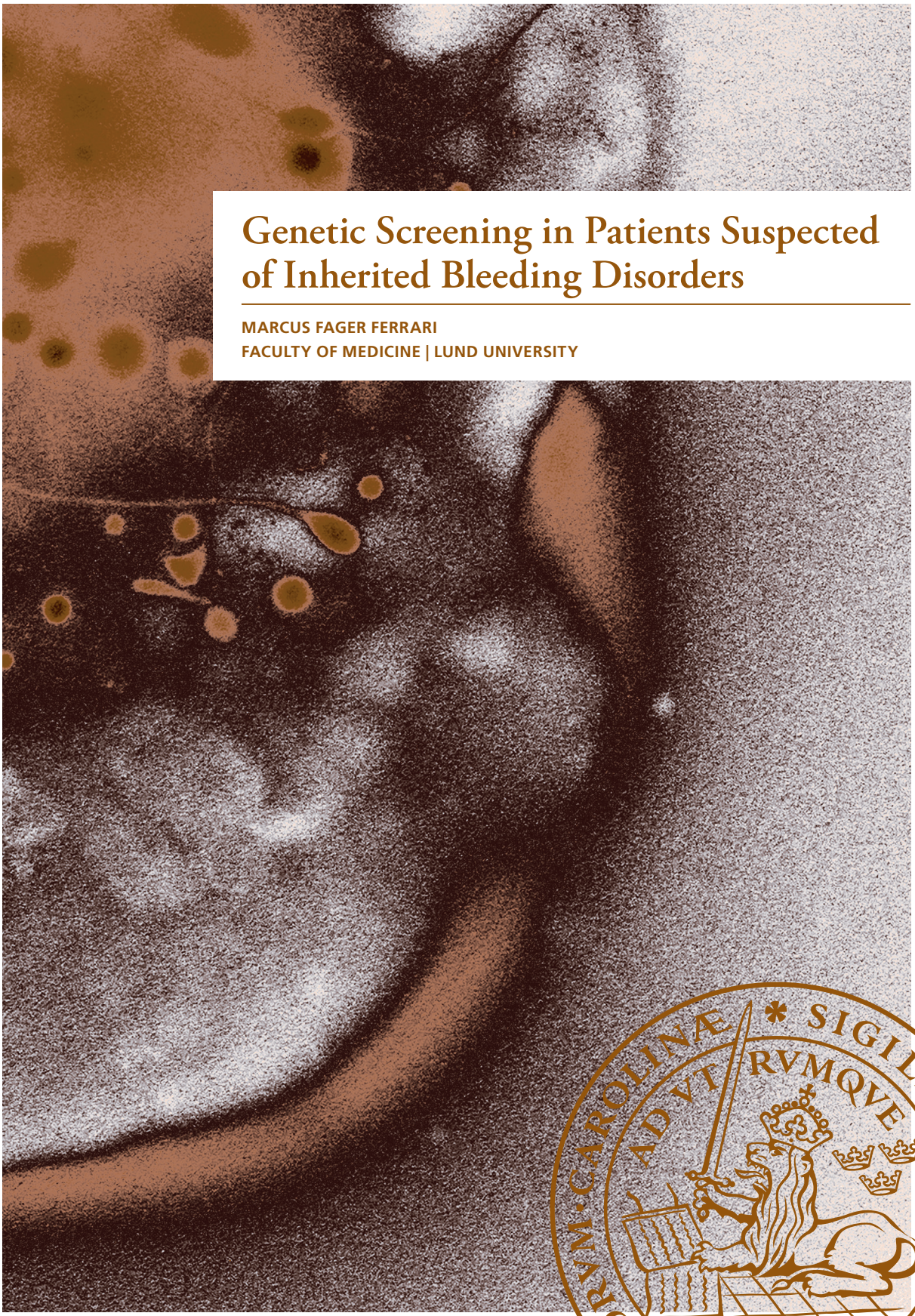

Genetic Screening in Patients Suspected of Inherited Bleeding Disorders

MARCUS FAGER FERRARI
FACULTY OF MEDICINE | LUND UNIVERSITY

Marcus Fager Ferrari, MD, is currently a resident physician at the Department of Hematology, Skåne University Hospital. This PhD thesis investigates the significance of selected genetic variants identified in patients suffering from incompletely explained bleeding tendencies. The PhD project was conducted at the Department of Translational Medicine, Faculty of Medicine, Lund University, in collaboration with the Center for Genomic Medicine and the Department of Hematology, Copenhagen University Hospital.

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Genetic Screening in Patients Suspected of Inherited Bleeding Disorders

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Marcus Fager Ferrari, MD

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Inherited bleeding disorders constitute a heterogeneous group of genetic diseases, affecting virtually all major components of the hemostatic system. The diagnostics are potentially complex, and a high proportion of patients remain without a conclusive diagnosis following work-up. In recent years, advances in high-throughput DNA sequencing technologies have facilitated the use of genetic investigations in patients suspected of inherited bleeding disorders. Even though genetic investigations have contributed appreciably to the diagnostics, a considerable proportion of the genetic variants identified are still of uncertain significance. The overall aim of this thesis was to investigate the significance of selected genetic variants identified in patients suspected of inherited bleeding disorders, presenting with incompletely explained bleeding tendencies.

In **Paper I**, the consequences of heterozygous variants in the genes *UNC13D*, *STX11* and *STXBP2* were investigated by functional methods. The results suggested an association between heterozygous variants in *UNC13D*, *STX11* or *STXBP2* and impaired platelet degranulation, possibly contributing to increased bleeding in affected patients. In **Paper II**, a rare variant in *FGB* was identified as the cause of familial hypofibrinogenemia following genetic and functional investigations. In **Paper III**, investigations using biomarkers specific for collagen formation and degradation were not able to verify any functional abnormalities hypothesized to result from heterozygous variants in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2*. However, an interesting negative correlation between plasma ascorbic acid levels and the ISTH-BAT bleeding score was observed in the patients. In **Paper IV**, two previously undescribed variants in *GNE* were found to be causative of severe congenital macrothrombocytopenia when harbored in a compound heterozygous state, as a result of markedly decreased platelet sialylation. Treatment with the sialidase inhibitor oseltamivir did not prove to be effective for increasing the platelet counts.

In summary, this thesis contributes to the continuously increasing knowledge of genetic variants associated with inherited bleeding disorders, required for adequately diagnosing a higher proportion of patients suffering from constitutionally increased bleeding.

Key words: Bleeding; Inherited Bleeding Disorders; Platelet Dysfunction; Thrombocytopenia; Genetic Screening; High-Throughput Sequencing; STX11; STXBP2; UNC13D; FGB; COL1A1; COL3A1; COL5A1; COL5A2; GNE; Sialic Acid; Oseltamivir.

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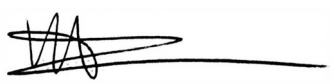
Signature  Date 2021-04-27

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A platelet whole mount transmission electron microscopy image, turned into some platelet art.

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“Now I'm a scientific expert; that means
I know nothing about absolutely everything.”

– Arthur C. Clarke

Table of contents

List of papers **11**

Abbreviations **13**

Hemostasis **17**

Primary hemostasis 17

Secondary hemostasis 19

Fibrinolysis 21

Inherited bleeding disorders **23**

Disorders of primary hemostasis 23

Disorders affecting the vascular wall 24

Inherited platelet function disorders 25

Inherited thrombocytopenias 29

von Willebrand disease 33

Disorders of secondary hemostasis 35

Hemophilia A and B 35

Congenital fibrinogen disorders 36

Prothrombin deficiency 36

Factor V deficiency 37

Combined factor V and factor VIII deficiency 37

Factor VII deficiency 37

Factor X deficiency 38

Factor XI deficiency 38

Factor XII deficiency 38

Factor XIII deficiency 38

Disorders of the fibrinolytic system 39

α2-antiplasmin deficiency 39

Plasminogen activator inhibitor-1 deficiency 39

A brief overview of hemostatic therapies 40

Diagnostic approaches **41**

Determining the clinical bleeding phenotype 41

Initial screening for hemostatic defects 41

Light transmission aggregometry 43
Flow cytometry 44
Transmission electron microscopy 45
Incompletely explained bleeding 47
Thrombocytopenia of unknown cause 47
Platelet dysfunction of unknown cause 47
Bleeding of unknown cause 48
Genetic screening 49
Rationale 49
Ethical considerations 50
Methodological principles 50
Diagnostic efficacy in inherited bleeding disorders 51
Variants of uncertain significance 52
Rationales of Papers I-IV 53
Paper I 53
Paper II 53
Paper III 54
Paper IV 54
Aims of the thesis 55
Paper I 55
Paper II 55
Paper III 55
Paper IV 55
Methods 57
Recruitment of patients 57
Genetic screening 57
Paper I 57
Paper II 59
Paper III 59
Paper IV 60
Results and discussion 63
Paper I 63
Paper II 66
Paper III 69

Paper IV 72
Conclusions and future perspectives **77**
Populärvetenskaplig sammanfattning **79**
Acknowledgements **83**
References **85**

List of papers

This thesis is based on the following papers:

Paper I

Fager Ferrari M, Leinoe E, Rossing M, Norström E, Strandberg K, Steen Sejersen T, Qvortrup K, Zetterberg E. Germline heterozygous variants in genes associated with familial hemophagocytic lymphohistiocytosis as a cause of increased bleeding. *Platelets*. 2018;29(1):56-64.

Paper II

Fager Ferrari M, Leinoe E, Rossing M, Norström E, Zetterberg E. A rare heterozygous variant in *FGB* (Fibrinogen Merivale) causing hypofibrinogenemia in a Swedish family. *Blood Coagulation and Fibrinolysis*. 2020;31(7):481-484.

Paper III

Fager Ferrari M, Zetterberg E, Rossing M, Manon-Jensen T, Pehrsson M, Karsdal MA, Lykkesfeldt J, Leinoe E. Collagen remodelling and plasma ascorbic acid levels in patients suspected of inherited bleeding disorders harbouring germline variants in collagen-related genes. *Haemophilia*. 2021;27(1):e69-e77.

Paper IV

Smolag KI[*], **Fager Ferrari M**[*], Zetterberg E, Leinoe E, Ek T, Blom AM, Rossing M[†], Martin M[†]. Severe congenital thrombocytopenia caused by compound heterozygous variants in *GNE*. *Manuscript*.

[*]Authors contributed equally as joint first authors
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Papers not included in this thesis:

Andersson NG, Rossing M, **Fager Ferrari M**, Gabrielaite M, Leinoe E, Ljung R, Mårtensson A, Norström E, Zetterberg E. Genetic screening of children with suspected inherited bleeding disorders. *Haemophilia*. 2020;26(2):314-324.

Fager Ferrari M, Lemonakis K, Förnvik Jonsson M. A rare case of IgE kappa monoclonal gammopathy of undetermined significance identified in a Swedish female. *Submitted*.

Papers I-III are reprinted according to the permissions obtained from the publishers.

Abbreviations

AA	Ascorbic acid
ACMG	The American College of Medical Genetics
AD	Autosomal dominant
ADP	Adenosine diphosphate
APTT	Activated partial thromboplastin time
AR	Autosomal recessive
ARC	Arthrogryposis, renal dysfunction and cholestasis
ATP	Adenosine triphosphate
BAT	Bleeding assessment tool
BSS	Bernard-Soulier syndrome
BUC	Bleeding of unknown cause
CAMT	Congenital amegakaryocytic thrombocytopenia
cEDS	Classical Ehlers-Danlos syndrome
CHS	Chediak-Higashi syndrome
CRP-XL	Cross-linked collagen-related peptide
ECM	Extracellular matrix
EDS	Ehlers-Danlos syndrome
ELISA	Enzyme-linked immunosorbent assay
FV(a)	(Activated) factor V
FVII(a)	(Activated) factor VII
FVIII(a)	(Activated) factor VIII
FIX(a)	(Activated) factor IX
FX(a)	(Activated) factor X
FXI(a)	(Activated) factor XI

FXII(a)	(Activated) factor XII
FXIII(a)	(Activated) factor XIII
FC	Flow cytometry
FH	Complement factor H
FHL	Familial hemophagocytic lymphohistiocytosis
gMFI	Geometric mean fluorescence intensity
GP	Glycoprotein
GPS	Gray platelet syndrome
GT	Glanzmann thrombasthenia
hEDS	Hypermobile Ehlers-Danlos syndrome
HHT	Hereditary hemorrhagic telangiectasia
HPS	Hermansky-Pudlak syndrome
HTS	High-throughput sequencing
IPD	Inherited platelet disorder
IPFD	Inherited platelet function disorder
ISTH	The International Society on Thrombosis and Haemostasis
IT	Inherited thrombocytopenia
ITP	Immune thrombocytopenia
LTA	Light transmission aggregometry
MAL II	Maackia amurensis lectin II
MYH9-RD	*MYH9*-related disease
PAI-1	Plasminogen activator inhibitor-1
PRP	Platelet rich plasma
PT	Prothrombin time
ROTEM	Rotational thromboelastometry
SD	Standard deviation
SNA	Sambucus nigra lectin
TAFI(a)	(Activated) thrombin activated fibrinolysis inhibitor
TEM	Transmission electron microscopy

TF	Tissue factor
tPA	Tissue plasminogen activator
TRAP-6	Thrombin receptor activator peptide 6
TXA_2	Thromboxane A2
uPA	Urokinase
vEDS	Vascular Ehlers-Danlos syndrome
VUS	Variant of uncertain significance
VWD	von Willebrand disease
VWF	von Willebrand factor
WAS	Wiskott-Aldrich syndrome
WES	Whole exome sequencing
WGS	Whole genome sequencing
XLR	X-linked recessive

Hemostasis

The hemostatic system is a complex physiologic machinery, regulating the fluidity of circulating blood. The process of blood clotting, also known as coagulation, protects the organism from extensive bleeding in the case of a wound. Adequate clotting is restricted to the site of injury, and the formed clot is subsequently dissolved following the restoration of vascular integrity. Consequently, the hemostatic system has an important function in maintaining the homeostatic balance between sufficient coagulation and thrombotic events. Even though the process of coagulation is highly dynamic, involving an intricate interplay between the various components of the hemostatic system, the process of coagulation can be divided into three phases: primary hemostasis, secondary hemostasis and fibrinolysis.

Primary hemostasis

In primary hemostasis, platelets play a crucial role for the initiation of coagulation. Platelets are small discoid cell fragments derived from megakaryocytes, with an average diameter 2-5 μm, normally present at a concentration of 150 to 350 x 10^9/L [1]. In the event of a vascular injury, subendothelial extracellular matrix (ECM) structures such as fibrillar types I and III collagen are exposed, leading to the adhesion of platelets to the site of injury [2]. Platelets express the surface receptor glycoprotein Ib-IX-V complex (GPIb-IX-V complex), which binds to the exposed subendothelial collagen via the von Willebrand factor (VWF), a large multimeric glycoprotein synthesized in endothelial cells and megakaryocytes [3, 4]. Platelets also bind directly to exposed subendothelial collagen via the platelet receptors glycoprotein VI (GPVI) and integrin α2β1 [2, 5]. Although regarded as the main aggregating receptor in platelets, the fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa) also mediates platelet adhesion by its ability to bind VWF [6]. Adhered platelets are subsequently activated via various signaling pathways, resulting in increased levels of cytoplasmic Ca^{2+} (Figure 1). When GPIb-IX-V is engaged by VWF, intracellular signaling ultimately result in a conformational change of GPIIb/IIIa to its activated state, exhibiting high affinity for the major ligand fibrinogen [7, 8]. Moreover, signaling via GPVI also contributes to platelet activation [9]. Activated platelets undergo shape changes by reorganization of the actin cytoskeleton, increasing the platelet surface area [10]. Increased levels of

cytoplasmic Ca^{2+} leads to the exposure of the negatively charged phospholipid phosphatidylserine at the platelet surface, providing a procoagulant Ca^{2+} -binding surface for the activation of plasma coagulation factors such as factor X (FX) and prothrombin [11, 12]. The increased concentration of cytoplasmic Ca^{2+} further result in the secretion of autocrine and paracrine mediators of activation, amplifying the activation of adjacent platelets in the manner of a positive feedback loop (**Figure 1**).

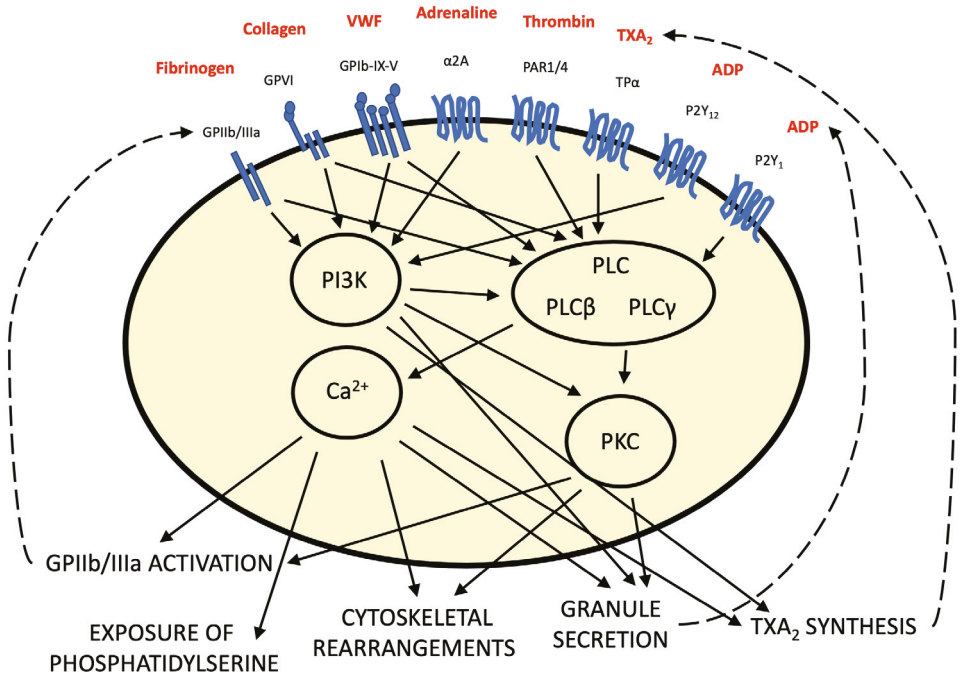

Figure 1. Overview of platelet surface receptors involved in the process of platelet activation
Activation of platelets results in the secretion of platelet agoniststs activating adjacent platelets, generating an amplified platelet response. Ligands for the surface receptors are shown in red. PLC, phopholipase C; PKC, protein kinase C; PI3K, phosphatidylinositide-3-kinase. Adapted from *Journal of Thrombosis and Haemostasis* with permission from John Wiley & Sons Ltd (Bye AP, Unsworth AJ, Gibbins JM. Platelet signaling: a complex interplay between inhibitory and activatory networks. *J Thromb Haemost*. 2016;14(5):918-930). © 2020 The Authors.

Activating platelet agonists such as thromboxane A2 (TXA_2) acting on the platelet thromboxane receptor (TPα) [13] are released, as well as procoagulant factors stored in platelet alpha granules and dense granules, that are secreted upon activation. Alpha granules contain hemostatic factors such as VWF, factor V (FV) and fibrinogen [14], while dense granules contain the major platelet activator adenosine diphosphate (ADP) together with adenosine triphosphate (ATP), serotonin and Ca^{2+} [15]. The nucleotide ADP binds to the platelet ADP receptors $P2Y_1$ and $P2Y_{12}$, resulting in further activation and aggregation of platelets [16]. In addition, thrombin generated by the parallel process of plasma coagulation activates platelets

by acting directly on platelet thrombin receptors PAR1 and PAR4 [17]. Following adhesion and activation, adjacent platelets finally aggregate by binding fibrinogen via their activated GPIIb/IIIa receptors, forming a fibrinogen-rich platelet plug at the site of vessel injury [8].

Secondary hemostasis

The process of secondary hemostasis involves the sequential activation of plasma coagulation factors, ultimately leading to the generation of thrombin. Thrombin is a central component of hemostasis, amplifying various stages of the coagulation system, while finally mediating the conversion of fibrinogen to fibrin for the formation of a stable hemostatic clot. Traditionally, coagulation have been presented as a model resembling a cascade since the 1960s [18]. In this "coagulation cascade" model, each coagulation factor is only presented in the form of an inactive proenzyme, which is activated by the preceding activated coagulation factor upstream. The current model of coagulation, however, integrates the significant contributions of active cell surfaces to hemostasis *in vivo*. Accordingly, the current cell-based model of coagulation can be divided into three different phases: the initiation phase, the amplification phase and the propagation phase (**Figure 2**).

In vivo, secondary hemostasis is initiated by tissue factor (TF), exposed by subendothelial cells in the event of a vessel injury [19]. In the initiation phase, exposed TF binds to factor VII (FVII), promoting activation of FVII to FVIIa. The TF/FVIIa complex activates small amounts of factor IX (FIX) and FX to activated factor IX (FIXa) and activated factor X (FXa) through proteolytic cleavage. Activated factor X subsequently forms a prothrombinase complex with the cofactor activated factor V (FVa) on TF-bearing cells, that converts prothrombin (also known as factor II) into active thrombin (also known as factor IIa) (**Figure 2 A**) [20]. In the amplification phase, the small amounts of thrombin generated in the initiation phase activates FV and FXI on platelet surfaces, also activating FVIII dissociating from VWF [21, 22]. In addition, thrombin contributes to further activation of platelets adhering at the site of vessel injury (**Figure 2 B**) [19]. The propagation phase takes place on activated platelets, exposing procoagulant phospholipids [12]. Activated factor IX generated in the initiation phase binds to FVIIIa on the platelet surfaces, forming a FIXa/FVIIIa tenase complex that activates more FX to FXa. Activated factor XI (FXIa) generated in the amplification phase also contributes by activating additional FIX. Activated factor X subsequently associates with FVa on the platelet surfaces, establishing a FXa/FVa prothrombinase complex for additional thrombin generation (**Figure 2 C**) [19]. Consequently, the large number of platelets recruited to the site of vessel injury provides an optimal setting for extensive thrombin generation. As a final step, the generated thrombin converts the fibrinogen in the fibrinogen-rich platelet plug to

fibrin, providing a stable fibrin clot for definitive hemostasis [23]. Thrombin also activates factor XIII (FXIII) to activated factor XIII (FXIIIa), further stabilizing the fibrin clot by crosslinking the fibrin strands [24].

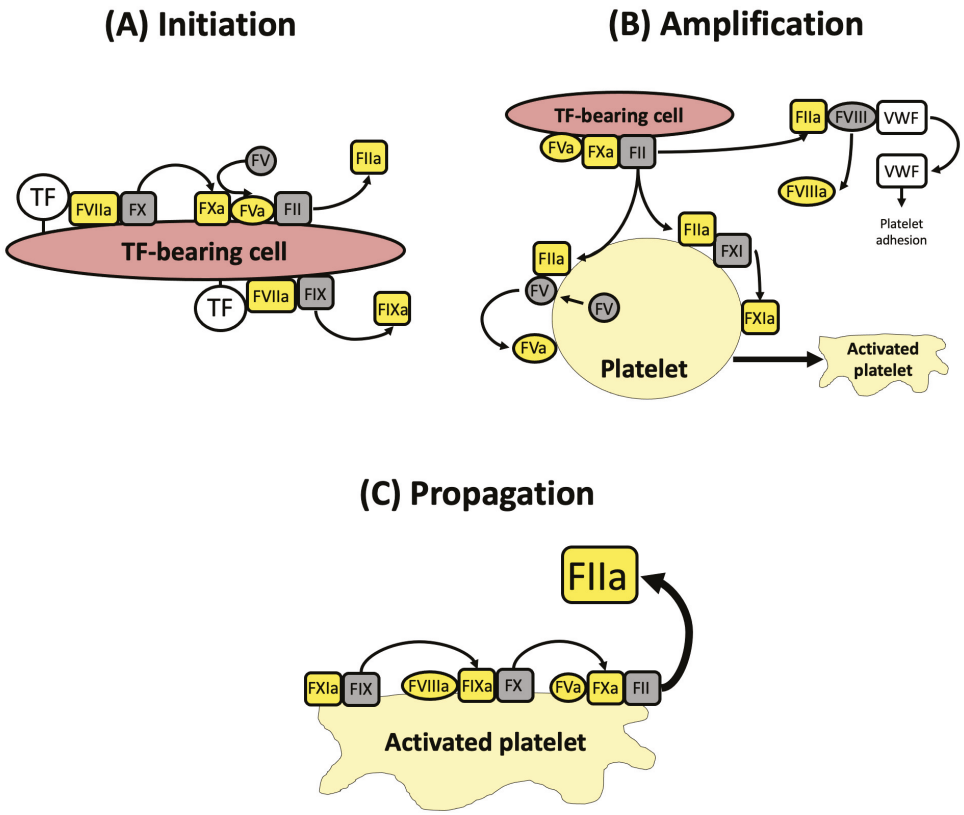

Figure 2. The cell-based model of coagulation
(A-C) The plasma coagulation occurs on the surfaces of TF-bearing cells and platelets. The generation of thrombin (FIIa) ultimately leads to the formation of a stable fibrin clot. Adapted from *Journal of Veterinary Emergency and Critical Care* with permission from John Wiley & Sons Ltd (Smith SA. The cell-based model of coagulation. *J Vet Emerg Crit Care (San Antonio)*. 2009;19(1):3-10). © Veterinary Emergency and Critical Care Society 2009.

In order to avoid uncontrolled coagulation, the hemostatic system also include factors with anticoagulant properties, tightly regulating the process of coagulation. Central anticoagulant factors include antithrombin which binds to endothelial heparan sulfated proteoglycans, subsequently inactivating coagulation factors including mainly thrombin and FXa [25]. Moreover, endothelial cells express thrombomodulin. In the presence of thrombin, thrombomodulin activates protein C, that together with its cofactor protein S inhibits further thrombin generation by inactivating FVa and FVIIIa [26]. In addition, tissue factor pathway inhibitor

present on endothelial cells acts both by the direct inhibition of FXa and the inhibition of formed TF/FVIIa/FXa complexes [23].

Fibrinolysis

Upon healing of the vessel injury, the formed fibrin clot is dissolved by the process of fibrinolysis. Endothelial tissue plasminogen activator (tPA) and urokinase (uPA) from monocytes and macrophages converts plasminogen to the major fibrinolytic protease plasmin [27]. Plasmin subsequently cleaves the fibrin polymers of the hemostatic clot. Fibrinolysis is tightly regulated by specific fibrinolytic inhibitors such as plasminogen activator inhibitor-1 (PAI-1), inhibiting tPA and uPA, and α2-antiplasmin acting by directly inhibiting plasmin [28]. Thrombin activated fibrinolysis inhibitor (TAFI) activated by thrombomodulin-bound thrombin also contributes to the regulation of fibrinolysis by reducing the generation of plasmin (**Figure 3**) [29].

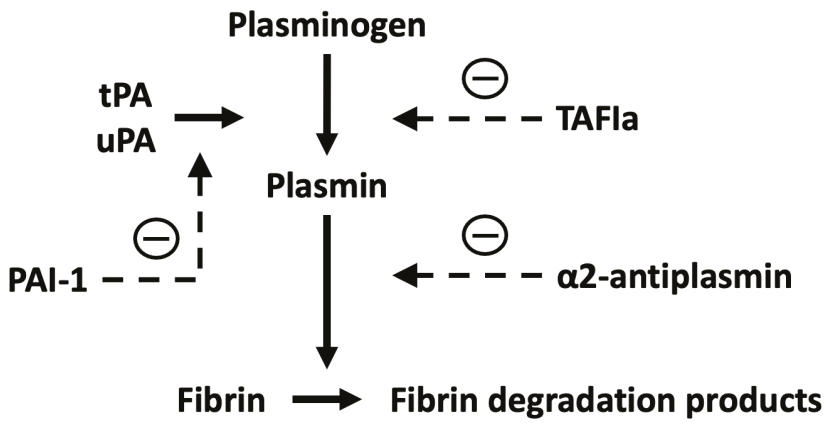

Figure 3. Simplified model of fibrinolysis
The process of fibrinolysis is regulated by fibrinolytic inhibitors such as PAI-1, α2-antiplasmin and activated TAFI (TAFIa).

Inherited bleeding disorders

Inherited bleeding disorders constitute a heterogeneous group of genetic diseases, affecting virtually all major components of the hemostatic system [30]. Hence, inherited bleeding disorders range from deficiencies in the primary hemostasis (e.g., impaired megakaryopoiesis resulting in thrombocytopenia) to deficiencies of coagulation factors involved in secondary hemostasis (e.g., reduced levels of FVIII; Hemophilia A). Accordingly, affected patients suffer from increased bleeding of varying character and severity, depending on the specific underlying disorder (**Table 1**). The symptoms range from spontaneous bleeds to increased bleeding following hemostatic challenges such as surgery or childbirth. The prevalences of the inherited bleeding disorders are highly variable. von Willebrand disease (VWD) is regarded as the most prevalent, with an estimated prevalence of 1:100 to 1:10 000 [31]. In contrast, some of the inherited bleeding disorders, such as α2-antiplasmin deficiency, have only been described in a few patients worldwide [32].

Table 1. Examples of phenotypical differences observed in patients with inherited bleeding disorders
Defects of primary hemostasis are commonly associated with a mucocutaneous bleeding pattern. Bleeds affecting muscles and joints are primarily seen in deficiencies of coagulations factors such as FVIII and FIX. Adapted from *Indian Journal of Pediatrics* with permission from Springer Nature (Bansal D, Oberoi S, Marwaha RK, Singhi SC. Approach to a child with bleeding in the emergency room. *Indian J Pediatr*. 2013;80(5):411-420). © Dr. K C Chaudhuri Foundation 2012.

Findings	Defects of primary hemostasis (e.g., VWD and platelet disorders)	Deficiencies of coagulation factors (e.g., hemophilia A and B)*
Major sites of bleeding	Mucous membranes, skin	Soft tissues, muscles, joints
Excessive bleeding following minor cuts	Yes	Not usual
Petechiae	Common	Rare
Echymoses	Small, superficial	Large, palpable
Hemarthrosis	Rare	Common
Soft tissue hematomas	Rare	Characteristic
Onset of bleeding following trauma/surgery	Immediate	Delayed

* The phenotype is variable depending on the specific underlying coagulation factor deficiency.

Disorders of primary hemostasis

The inherited disorders of primary hemostasis commonly manifest as easy bruising and bleeding from mucous membranes such as gums or the gastrointestinal tract (**Table 1**). Hence, the bleeding phenotype is commonly referred to as a

"mucocutaneous bleeding pattern" [33]. The inherited disorders of primary hemostasis can be divided into disorders affecting the vascular wall, platelet disorders and VWD. The platelet disorders can be further divided into inherited platelet function disorders (IPFDs) and inherited thrombocytopenias (ITs).

Disorders affecting the vascular wall

The structural integrity of the vessel wall is crucial for maintaining circulating blood in the vascular system. In the event of vascular rupture, bleeding occurs, and the process of hemostasis is initiated. The principal organization of the vessel wall includes an inner layer of endothelial cells (tunica intima), surrounded by a middle layer of smooth muscle cells (tunica media) and an outer layer (tunica adventitia) containing ECM [34]. The vascular ECM consists of a basement membrane and an interstitial matrix, providing tensile strength mediated by collagen fibers. Types I, III, IV, VI, XV and XVIII collagen are present in the vessel wall [35]. Of the vascular collagens, fibrillar type I collagen constitute 60%, while fibrillar type III constitute 30%. Both types are mainly located to the interstitial matrix. Type VI collagen acts as a link between the interstitial matrix and the basement membrane, while types IV, XV and XVIII collagen are found in the basement membrane. Fibrillar type V collagen has also been located to the vessel wall [36, 37], and is critical for the fibrillogenesis of types I and III collagen [38]. Several inherited disorders affecting vascular integrity are known to be associated with increased bleeding [39]. Important examples include Ehlers-Danlos syndrome (EDS) and hereditary hemorrhagic telangiectasia (HHT).

Ehlers-Danlos syndrome

Ehlers-Danlos syndrome is a disease of the connective tissues, comprising 13 different subtypes according to the current classification [40]. Although the symptomatology differ somewhat between the subtypes, EDS commonly presents with joint hypermobility and hyperextensibility of the skin [41]. Interestingly, increased bleeding is a common symptom, listed as a diagnostic criterion in the majority of the EDS subtypes [39, 40]. The most common subtypes of EDS associated with bleeding diathesis are classical EDS (cEDS) and vascular EDS (vEDS), with an estimated prevalence of 1:20 000 and 1:90 000, respectively [42]. Classical EDS is caused by heterozygous variants in the genes *COL5A1* and *COL5A2* encoding the procollagen chains forming type V collagen [43], while vEDS is caused by heterozygous variants in the gene *COL3A1*, responsible for the synthesis of the procollagen chains forming type III collagen [44]. Rarely, variants in the gene *COL1A1* are also causative of cEDS and vEDS [45]. Interestingly, types I, III, IV and VI collagen are known to interact with hemostatic components such as platelets, VWF and FIX [35, 46]. Recently, platelet function abnormalities have been described in patients with EDS, including various EDS subtypes [47, 48].

However, the traditional pathophysiological model attributing the bleeding symptoms to the mechanical weakness of the vessel wall still remains valid [39, 49]. The synthesis of mature collagen molecules is dependent on sufficient levels of ascorbic acid (AA; also known as vitamin C), acting as a cofactor in the process of procollagen hydroxylation [50]. A severe deficiency of AA results in scurvy, associated with symptoms such as impaired wound healing and increased bleeding attributed to an inadequate collagen synthesis [51]. In fact, AA supplements have been recommended based on expert opinion for reducing bleeding symptoms in cEDS [43, 52].

Hereditary hemorrhagic telangiectasia

Although not a collagenopathy, but rather a vascular dysplasia, HHT is characterized by the development of abnormal vascular structures, resulting in vascular fragility and arteriovenous malformations [53]. Common symptoms include frequent nosebleeds, gastrointestinal bleeds and telangiectasias of the buccal mucosa and the skin. Hereditary hemorrhagic telangiectasia (estimated prevalence: 1:6 000) is an autosomal dominant (AD) disease, caused by deleterious variants in the genes *ENG*, *ACVRL1* and *SMAD4* [54].

Inherited platelet function disorders

The inherited platelet disorders (IPDs) represent a diverse group of disorders affecting the function and/or numbers of platelets [55]. The defined IPDs are very rare, with reports often being limited to a small number of patients. Consequently, the true prevalences of the disorders are many times unknown. The International Society on Thrombosis and Haemostasis (ISTH) recently published a curated list of diagnostic-grade genes with significant evidence of causing bleeding, thrombotic and platelet disorders [56]. The list is updated annually, and currently includes 63 different genes known to cause IPDs. The IPDs can be divided into IPFDs characterized by normal platelet counts [57] and ITs [58]. However, it should be noted that some of the ITs show concomitant defects of platelet function.

The IPFDs affect a wide variety of structures critical for normal platelet function (**Figure 3**). In this section, examples of genes known to be involved in IPFDs associated with normal platelet counts (with the exception of Bernard-Soulier syndrome; BSS) will be presented according to the mechanisms involved.

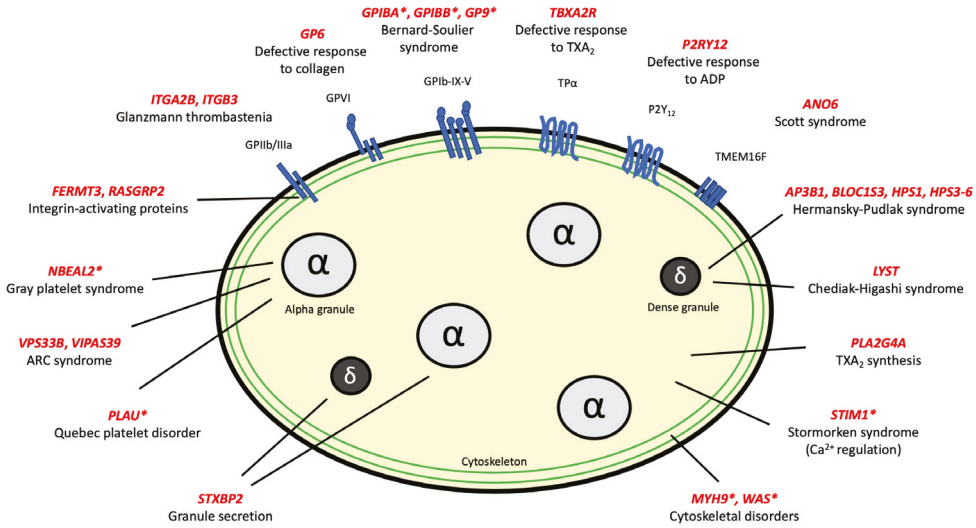

Figure 3. Examples of genes involved in the pathogenesis of inherited platelet disorders
Examples of genes associated with defects of surface proteins as well as intracellular proteins and organelles are presented. The affected genes are depicted in red. Genes associated with thrombocytopenia are denoted by an asterisk.

Platelet glycoprotein receptors

Variants in *GP1BA*, *GP1BB* and *GP9* cause defects in the platelet GPIb-IX-V receptor, essential for VWF-binding. Variants in these genes give rise to BSS (estimated prevalence: 1:1 000 000), characterized by an inability of platelets to adhere to the vascular subendothelium [59]. Although being a defect of platelet surface receptors, BSS is further characterized by a mild to moderate macrothrombocytopenia, with an average platelet count of 60 x 10^9/L [60]. Due to the deficient VWF-binding, the disorder is associated with severe bleeding. The disorder is commonly inherited in an autosomal recessive (AR) fashion, although milder AD forms caused by monoallelic variants in *GP1BA* or *GP1BB* are also described [59, 61, 62].

The two integrins αIIb and β3, forming the platelet fibrinogen receptor GPIIb/IIIa (also known as integrin αIIbβ3), are encoded by *ITGA2B* and *ITGB3,* respectively. Deleterious variants causing quantitative or qualitative defects of the encoded integrins result in Glanzmann thrombasthenia (GT), an AR disorder characterized by normal platelet counts and an inability of platelets to aggregate by the cross-linking normally mediated via fibrinogen-binding [63]. Even though GT has been described as the most common IPFD [63], it is a very rare disorder, with an estimated prevalence of 1:1 000 000 [64]. An AD form of GT, associated with platelet dysfunction and mild macrothrombocytopenia has been described, attributed to gain-of-function variants in *ITGA2B* and *ITGB3* [65-68].

The platelet collagen receptor GPVI is encoded by the gene *GP6*. Homozygous or compound heterozygous variants in *GP6* result in an absent platelet response to collagen, accompanied by a mild clinical bleeding phenotype [69, 70].

Variants in *FERMT3* affect the signaling of integrins β1, β2 and β3, causing a GT-like bleeding phenotype in an AR mode of inheritance [71]. The biallelic variants give rise to a leukocyte adhesion deficiency syndrome (LAD-III), that in addition to bleeding is associated with leukocyte adhesion defects resulting in recurrent bacterial infections [72]. Like in GT, platelets do not aggregate when stimulated with all physiological agonists. However, GPIIb/IIIa is present, but do not bind to the PAC-1 monoclonal antibody (directed to active GPIIb/IIIa) upon platelet activation [71].

Another gene affected in the signaling pathway of GPIIb/IIIa is *RASGRP2*, encoding the calcium and diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) [73]. Biallelic variants in *RASGRP2* impair normal GPIIb/IIIa signaling via Rap1, resulting in a GT-like phenotype [74]. Notably, the agonist phorbol 12-myristate 13-acetate activates Rap1 independently of upstream receptor signaling and constitutes a diagnostic test for *RASGRP2*-associated platelet dysfunction [71].

Platelet G-protein-coupled receptors and associated ligands

Biallelic variants in *P2RY12*, encoding the platelet ADP receptor $P2Y_{12}$, result in moderate to severe bleeding [75, 76]. Monoallelic variants have also been reported to give rise to bleeding diathesis [76, 77]. Consequently, platelets fail to aggregate normally when stimulated with ADP, and stimulation with other agonists may only induce partially reversible aggregation due to a limited effect of ADP released from activated platelets [78].

The gene *TBXA2R* encodes TPα, the major platelet TXA_2 receptor. Monoallelic and biallelic variants result in mild bleeding diathesis; platelets show decreased aggregation in response to arachidonic acid or TXA_2 analogs such as U46619 [79-81].

The gene *PLA2G4A* encodes cytosolic phospholipase A2, catalyzing the release of arachidonic acid from cell membranes during the synthesis of TXA_2. Biallelic variants result in bleeding diathesis, and laboratory investigations show impaired aggregation in response to ADP and collagen [82, 83].

Platelet granules

Defects of platelet granule biogenesis or secretion are observed both in pure IPFDs and ITs. Here, some examples of genes involved in IPFDs characterized by granule defects will be presented. Hermansky-Pudlak syndrome (HPS; subtypes HPS1-10) is an AR multisystem disorder caused by variants in 10 different genes: *HPS1* (HPS1), *AP3B1* (HPS2), *HPS3* (HPS3), *HPS4* (HPS4), *HPS5* (HPS5), *HPS6*

(HPS6), *DTNBP1* (HPS7), *BLOC1S3* (HPS8), *BLOC1S6* (HPS9) and *AP3D1* (HPS10) [84]. The prevalence has been estimated to 1:1 000 000 in the general population, even though a higher prevalence has been described in certain populations, such as in Puerto Ricans (estimated prevalence of HPS1: 1:1 800) [85]. The genes affected in HPS encode proteins crucial for the biogenesis of platelet dense granules. Accordingly, platelets in patients with HPS are devoid of dense granules, resulting in increased bleeding. The absence of dense granules can be confirmed by platelet whole mount transmission electron microscopy (TEM), and aggregation studies show absent secondary aggregation due to the non-existent release of dense granule content [86]. The genes involved in HPS are also essential for the synthesis of melanosomes in melanocytes, normally contributing with pigmentation of organs such as the skin and the eyes. Consequently, in addition to bleeding diathesis, patients with HPS exhibit oculocutaneous albinism, manifesting as hypopigmentation of skin, hair and eyes. Depending on the HPS subtype, additional features such as progressive pulmonary fibrosis (HPS1, HPS4), neutropenia (HPS2, HPS10), granulomatous colitis (HPS1, HPS4) are described [71, 84].

Chediak-Higashi syndrome (CHS) is caused by biallelic variants in *LYST* [87]. The syndrome is characterized by bleeding diathesis due to a reduction of platelet dense granules, resulting from impaired dense granule biogenesis [88]. Hence, CHS has a platelet phenotype similar to the one observed in HPS, and patients with CHS also show hypopigmentation of the skin, hair and eyes [89]. Notably, CHS is associated with immunodeficiency, related to neutropenia and profound functional defects in cytotoxic T lymphocytes and natural killer cells [90]. Moreover, approximately 85% of patients are reported to develop an “accelerated phase” of CHS resulting in hemophagocytic lymphohistiocytosis due to the immune dysregulation [91].

Biallelic variants in *VPS33B* and *VIPAS39* are associated with the arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome, a multisystem disorder of the newborn with a dismal prognosis [92, 93]. The encoded proteins VPS33B and VPS16B are known to be required for the biogenesis of platelet alpha granules [94, 95]. Consequently, platelets from affected patients lack alpha granules, resulting in increased bleeding. Platelet TEM show absence of alpha granules, while the dense granule content is unaffected [92].

In addition to quantitative deficiencies of platelet granules, defects in the secretion of existing granules have the potential of resulting in a functional deficiency of platelet granules. Familial hemophagocytic lymphohistiocytosis (FHL) is a rare genetic hyperinflammatory disorder, characterized by an uncontrolled immune response resulting from impaired granule secretion in cytotoxic T lymphocytes and natural killer cells [96]. The subtypes FHL3, FHL4 and FHL5 are caused by biallelic variants in the genes *UNC13D*, *STX11* and *STXBP2*, respectively [97]. The genes encode the proteins Munc13-4 (*UNC13D*), Syntaxin-11 (*STX11*) and Munc18-2 (*STXBP2*), essential for the exocytosis of cytotoxic granules by cytotoxic T

lymphocytes and natural killer cells [98]. The encoded proteins are also crucial for the secretory machinery mediating granule release in platelets [99-103]. Accordingly, severely impaired release of platelet alpha granules and dense granules has been demonstrated in FHL-patients harboring biallelic variants in either *UNC13D*, *STX11* or *STXBP2* [103-106].

Platelet procoagulant activity

Scott syndrome is a defect of platelet procoagulant activity caused by biallelic variants in *ANO6* [107]. Scott syndrome is characterized by deficient phosphatidylserine exposure on platelets, which is normally required for the Ca^{2+}-dependent activation of FX and prothrombin on platelet surfaces [108]. Flow cytometry (FC) can be used to identify the reduced phosphatidylserine exposure on activated platelets by assessing annexin V-binding [109].

Inherited thrombocytopenias

In the ITs, the low platelet counts results from two principal mechanisms: reduced platelet production or increased platelet removal [58]. Platelets are produced from megakaryocytes, which differentiates from hematopoietic stem cells during an intricate maturation process regulated by multiple transcription factors in an interplay with cytokines and the stromal niche of the bone marrow [110-112]. Consequently, many of the genes affected in ITs are known to be essential for various aspects of platelet production, such as megakaryocyte maturation and platelet release (**Figure 4**) [113]. Variants affecting transcription factors involved in early megakaryopoiesis are associated with a risk of developing hematological malignancies. Furthermore, some of the ITs are syndromic, being associated with additional extrahematological manifestations [114]. Thrombocytopenia, commonly defined as a platelet count <150 x 10^9/L, can be graded as mild (>100 x 10^9/L), moderate (50 to 100 x 10^9/L) or severe (<50 x 10^9/L) [1, 115]. Moreover, ITs affecting the size of platelets are usually classified as either macrothrombocytopenias (large platelets) or microthrombocytopenias (small platelets) [116]. In this section, examples of genes known to be affected in ITs will be presented. The ITs will be categorized according to their pathophysiological mechanisms or associated phenotypical characteristics.

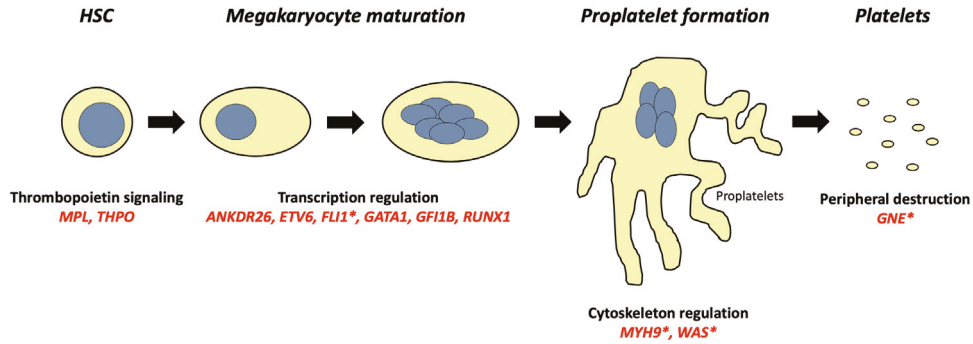

Figure 4. Examples of genes involved in the pathogenesis of inherited thrombocytopenias
Affected genes are shown in red. Genes associated with extrahematological manifestations are denoted by an asterisk. HSC; hematopoietic stem cell.

Thrombopoietin signaling

Biallelic deleterious variants in *MPL*, encoding the thrombopoietin receptor, cause congenital amegakaryocytic thrombocytopenia (CAMT) [117]. Thrombopoietin-mediated signaling via the thrombopoietin receptor is central for the production of megakaryocytes and platelets [118]. Accordingly, the disorder is associated with a severe congenital thrombocytopenia, showing platelets with normal size and a markedly reduced number of megakaryocytes in the bone marrow [119]. Affected patients commonly progress to multi-lineage aplasia during childhood, further resulting in anemia and leukopenia, and the condition is fatal unless patients undergo allogeneic hematopoietic stem cell transplantation [119].

Biallelic variants in *THPO*, encoding thrombopoietin, have been described to cause a CAMT phenotype in a number of families; accordingly, treatment with thrombopoietin receptor agonists has been reported as successful [120-122]. Moreover, monoallelic variants in *THPO* was recently shown to cause a mild thrombocytopenic phenotype, only affecting the platelet lineage [123].

Transcription regulation

Monoallelic variants in *RUNX1* give rise to moderate thrombocytopenia with generally normal platelet size [124]. Platelets show a reduced secretion and content of dense granules; bleeding is mostly mild [125, 126]. *RUNX1* encodes the master hematopoietic transcription factor RUNX1, and haploinsufficiency of RUNX1 is further associated with a predisposition to myeloid malignancies [127].

Variants in *ANKRD26* cause an AD moderate normothrombocytopenia associated with mild bleeding diathesis and a reduced number of alpha granules [128]. Affected patients are at risk of developing myeloid malignancies [129]. The ANKRD26 protein is a target for the transcription factor RUNX1, explaining the phenotypic similarities [130].

Monoallelic variants in the transcription factor-encoding gene *ETV6* leads to moderate normothrombocytopenia, accompanied by a predisposition to mainly lymphoid malignancies [131, 132].

Paris-Trousseau syndrome is the designation of the severe macrothrombocytopenia associated with the Jacobsen syndrome, a developmental disorder caused by an AD deletion at chromosome 11q [133]. The transcription factor-encoding gene *FLI1* have been shown to be causative of the thrombocytopenia [134, 135]. Isolated variants in *FLI1* have been described to cause non-syndromic thrombocytopenia with large, fused alpha granules in a subset of circulating platelets [125, 136].

Biogenesis of platelet granules

Gray platelet syndrome (GPS), associated with an AR inheritance pattern, manifests as moderate macrothrombocytopenia, characterized by platelets devoid of alpha granules [137]. The lack of alpha granules results in platelets appearing as gray-colored on a stained peripheral blood smear, hence the name of the syndrome. The absence of alpha granules can clearly be visualized by platelet TEM [138]. The severity of the resulting bleeding diathesis is variable; bleeding symptoms have been described to range from mild to severe [139]. Variants in the gene *NBEAL2* was identified as causative of GPS in 2011 [140-142]. In addition of being imperative for the biogenesis of alpha granules, the encoded protein NBEAL2 also seems to be involved in proplatelet formation [143, 144]. GPS is further associated with myelofibrosis and splenomegaly, which has the potential of exacerbating the thrombocytopenia [145]. Increased serum levels of vitamin B12 is another characteristic feature of the syndrome, and an overrepresentation of autoimmune features has recently been described in GPS patients [146].

Mainly monoallelic variants in the transcription factor-encoding gene *GFI1B* have been described to cause mild to moderate macrothrombocytopenia associated with erythrocyte defects [147-149]. Furthermore, variants in *GFI1B* also result in a deficiency of platelet alpha granules [150].

Another gene associated with macrothrombocytopenia (mainly severe) and a paucity of platelet alpha granules is *GATA1* [151]. Variants affecting the encoded hematopoietic transcription factor GATA1 commonly result in dysmegakaryopoiesis and abnormal erythropoiesis [152]. Myelofibrosis is also described as an associated feature [153]. The reduced alpha granule content of platelets is explained by GATA1 normally regulating the expression of NBEAL2 [154]. The condition has a X-linked recessive (XLR) inheritance pattern. Consequently, males are predominantly affected.

Cytoskeletal abnormalities

Heterozygous variants in *MYH9* causes moderate to severe macrothrombocytopenia, associated with mild to moderate bleeding diathesis [155].

A characteristic hematological finding is the presence of Döhle-like inclusion bodies in neutrophils [156]. The gene *MYH9* encodes the heavy chain of non-muscle myosin IIA, an actin binding protein essential for various processes involving the chemomechanical actions of the cytoskeleton, such as cell migration and cell adhesion [157]. Variants in *MYH9* cause impaired proplatelet formation and platelet release from megakaryocytes, resulting in thrombocytopenia [158, 159]. Non-muscle myosin IIA is widely expressed in the cells of the human body. Consequently, variants in *MYH9* are associated with a syndromic disorder, commonly referred to as *MYH9*-related disease (MYH9-RD). The prevalence has been estimated to 3:1 000 000 [159]. In addition to the congenital macrothrombocytopenia, late-onset features include sensorineural hearing loss, glomerulonephritis associated with end-stage renal disease, early cataracts and elevated liver enzymes [159]. Regrettably, most patients develop at least one of the described late-onset manifestations [159, 160].

Wiskott-Aldrich syndrome (WAS) is a XLR disorder, classically characterized by moderate to severe microthrombocytopenia, severe immunodeficiency and eczema [161]. The syndrome is caused by hemizygous variants in *WAS* affecting the encoded WAS protein, that has a central role in actin polymerization and cytoskeletal remodeling of hematopoietic cells [162]. The mechanism of the microthrombocytopenia is likely multifactorial, involving abnormal proplatelet formation and peripheral destruction of platelets [162, 163]. The incidence has been estimated to 1:100 000 live births [164].

Platelet sialylation

Platelet surface sialic acid (5-*N*-acetylneuraminic acid; Neu5Ac) is known to be essential for the lifespan of platelets. Senescent desialylated platelets are cleared from the circulation via the hepatic Ashwell-Morell receptor [165, 166]. The rate limiting enzyme of sialic acid biosynthesis, UDP-*N*-acetyl-glucosamine-2-epimerase/*N*-acetylmannosamine kinase (also known as the GNE enzyme), is encoded by the gene *GNE* [167]. Deleterious variants in GNE are associated with the rare AR neuromuscular disorder GNE myopathy, where thrombocytopenia have been described as an additional manifestation in a few affected families [168-170]. Recently, biallelic variants in *GNE* have been described in patients presenting with isolated severe macrothrombocytopenia, attributed to a reduced expression of platelet surface sialic acid [171-174].

Platelet channelopathies

Stormorken syndrome is caused by heterozygous gain-of-function variants in *STIM1* [175]. Patients affected by the syndrome show moderate to severe macrothrombocytopenia, associated with reduced platelet lifespan and spontaneous expression of platelet phosphatidylserine [176]. Other manifestations of the syndrome include immune dysfunction, anemia and myopathy. The encoded protein

STIM1 acts as a Ca^{2+}-sensor that regulates the influx of Ca^{2+} into cells via the cell surface channel ORAI1 [177]. Gain-of-function variants in *STIM1* renders the ORAI1 channel constitutively open, resulting in elevated Ca^{2+}-levels and platelet preactivation [175]. Gain-of-function variants in *STIM1* have also been identified as the cause of York platelet syndrome, sharing several of the phenotypical features observed in Stormorken syndrome [178]. Consequently, the two disorders have been suggested to represent the same clinical condition [179].

Platelet-dependent hyperfibrinolysis

Quebec platelet disorder is an AD disorder caused by a tandem duplication involving the *PLAU* gene, encoding uPA [180]. This tandem duplication results in increased levels of uPA in megakaryocytes and platelets, leading to the degradation of alpha granule proteins by plasmin [181]. Furthermore, platelets release increased amounts of uPA resulting in an accelerated platelet-dependent fibrinolysis [182]. Quebec platelet disorder is associated with abnormal platelet aggregation, and thrombocytopenia is present in some, but not all, of the affected patients [183, 184]. The mechanism underlying the thrombocytopenia is unknown. The prevalence has been estimated to 1:655 000 in Canadians [181].

von Willebrand disease

von Willebrand disease is regarded as the most common inherited bleeding disorder (estimated prevalence: 1:100 to 1:10 000) [31]. The disorder results from qualitative or quantitative deficiencies of VWF, a multimeric glycoprotein with a central role in both primary and secondary hemostasis. The VWF protein is crucial for the adhesion and aggregation of platelets, while also acting by stabilizing FVIII [185, 186]. Hence, low functional levels of VWF result in decreased levels of circulating FVIII. Mainly being a disorder of primary hemostasis, VWD is primarily associated with a mucocutaneous bleeding pattern. However, in severe forms of the disorder, musculoskeletal bleeding, including joint bleeds, also occurs [187]. von Willebrand disease is commonly subclassified according to the severity or the mechanisms underlying the disorder [31, 188]. The subtypes include type 1 VWD (partial quantitative deficiency of VWF), type 2 VWD (qualitative deficiency of VWF) and type 3 VWD (virtually complete absence of VWF). Type 2 VWD is further divided into type 2A VWD (reduced/absent high-molecular-weight VWF multimers), type 2B VWD (gain-of-function variants in *VWF* resulting in increased binding of VWF to platelets), type 2M VWD (reduced binding of VWF to platelets or collagen) and type 2N VWD (reduced binding of VWF to FVIII). Notably, type 2B VWD sometimes presents with macrothrombocytopenia due to the increased platelet-binding properties of VWF [189]. Moreover, type 1C VWD has also been recognized as an important subtype, resulting from increased VWF clearance. von Willebrand disease is generally caused by variants in the *VWF* gene, encoding the

VWF protein. The disorder is commonly associated with an AD inheritance pattern, with the exception of type 3 VWD and type 2N VWD, being associated with biallelic variants [188, 190]. However, the genetics of the disorder are complex, with a high degree of genetic variability in *VWF* in healthy subjects, and a poor genotype-phenotype correlation in type 1 VWD [191, 192]. Genetic modifiers outside the *VWF* gene, such as ABO blood group (25% lower VWF levels in individuals with blood group O) and other identified genetic loci are thought to contribute to low VWF levels in a subset of patients lacking disease-causing variants in *VWF* [193, 194]. Consequently, the diagnostics of VWD are largely based on bleeding history and functional testing [31]. Genetic testing is however regarded as useful in establishing the diagnosis of type 2 VWD (type 2B VWD in particular), as disease-causing variants are well-characterized [31, 192]. Genetic investigations can also be valuable in the diagnostics of type 3 VWD [195]. A special form of VWD that deserves to be mentioned is the platelet-type VWD. This rare AD disorder is caused by specific gain-of-function variants in *GP1BA*, resulting in an increased affinity of platelets to VWF [196]. Similar to type 2B VWD, platelet-type VWD is associated with low platelet counts and increased platelet volumes. Consequently, genetic investigations can be useful for discriminating between the two disorders [197].

Disorders of secondary hemostasis

The inherited disorders of secondary hemostasis include deficiencies of plasma coagulation factors essential for the process of thrombin generation and subsequent fibrin formation (**Figure 5**).

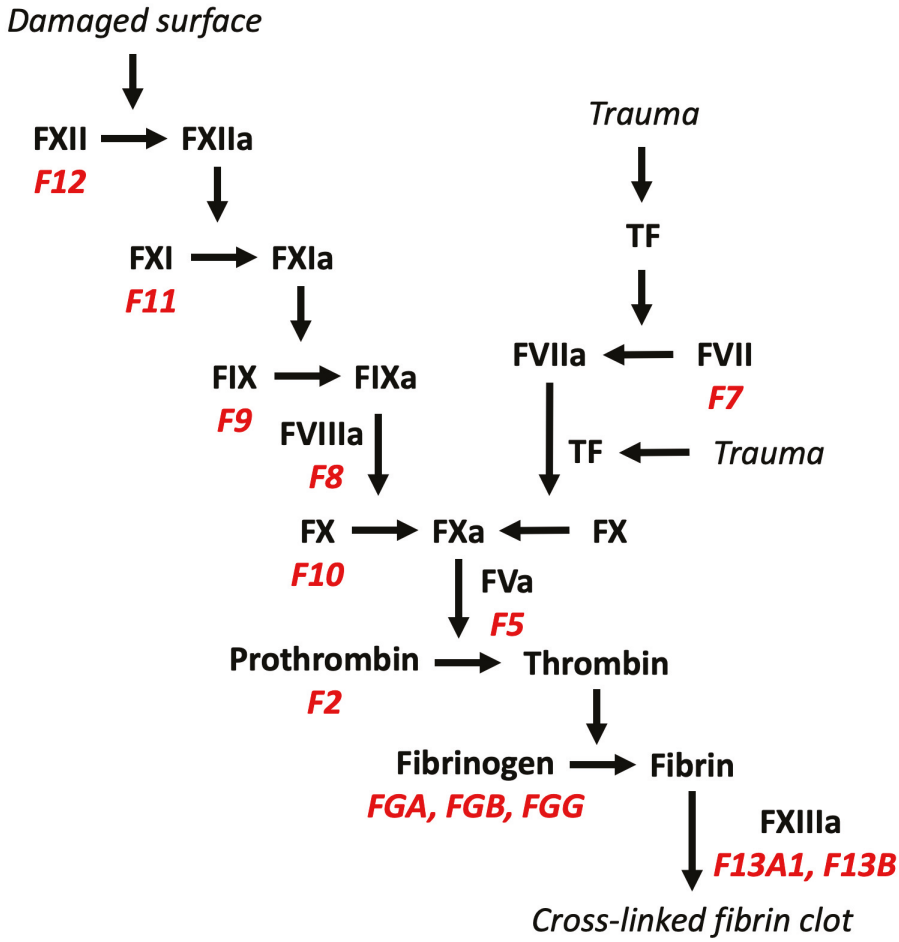

Figure 5. Examples of genes affected in the inherited disorders of secondary hemostasis
Simplified model of secondary hemostasis including examples of genes involved in the inherited disorders of secondary hemostasis. Affected genes are shown in red.

Hemophilia A and B

The most prevalent disorders of secondary hemostasis are hemophilia A and B, characterized by a deficiency of FVIII and FIX, respectively [198]. The deficiencies

result from deleterious variants in the genes *F8* (encoding FVIII) and *F9* (encoding FIX), which are both located to the X-chromosome. Accordingly, the disorders are inherited in an XLR manner, predominantly affecting males. Hemophilia A has an estimated prevalence of 1:5 000 male births, while hemophilia B is less common, with an prevalence of 1:30 000 male births [199]. The bleeding tendency is related to the plasma concentration of the affected factor, and the disorders are classified according to the remaining functional plasma levels. Hemophilia A and B are hence divided into mild (>5-40% of normal functional plasma levels), moderate (1-5%) and severe (<1%) forms [198]. In contrast to the bleeding pattern observed in the disorders of primary hemostasis, the bleeding phenotype in hemophilia A and B is characterized by joint and muscle bleeds [200].

Congenital fibrinogen disorders

The congenital fibrinogen disorders are divided into quantitative (afibrinogenemia and hypofibrinogenemia) and/or qualitative (dysfibrinogenemia and hypodysfibrinogenemia) deficiencies of fibrinogen [201, 202]. The disorders are caused by variants in the genes *FGA*, *FGB* and *FGG*, encoding the three polypeptide chains forming the fibrinogen molecule [203]. Afibrinogenemia is inherited in an AR fashion and is characterized by the complete absence of fibrinogen [201]. The bleeding phenotype is mainly, but not exclusively, mucocutaneous [204]. Patients affected by the mainly AD disorders hypofibrinogenemia, dysfibrinogenemia and hypodysfibrinogenemia show highly variable degrees of bleeding tendencies. In hypofibrinogenemia, however, the plasma levels of fibrinogen correlate strongly with the severity of bleeding symptoms [201]. Hypofibrinogenemia is categorized, according to the functional plasma fibrinogen levels, as mild (1-2 g/L), moderate (0.5-0.9 g/L) and severe (<0.5 g/L) [202]. Interestingly, the congenital fibrinogen disorders are associated with a paradoxically increased risk of thromboembolic events [201, 205]. The congenital fibrinogen disorders are rare; the prevalence of afibrinogenemia has been estimated to 1:1 000 000 [201]. Although being regarded as more common than afibrinogenemia, the considerable proportion of asymptomatic individuals have made it difficult to establish the prevalences of hypofibrinogenemia and dysfibrinogenemia [206]. Hypodysfibrinogenemia seems to be the least prevalent subtype [205].

Prothrombin deficiency

Prothrombin deficiency is one of the rarest inherited disorder of secondary hemostasis, with an estimated prevalence of 1:2 000 000 [207]. The disorder is caused by variants in the prothrombin-encoding gene *F2* and is inherited in AR manner. Prothrombin deficiency is further subclassified into hypoprothrombinemia and dysprothrombinemia. Severe hypoprothrombinemia (<5% of normal

prothrombin activity in plasma) is characterized by significant bleeding tendencies [208]. Dysprothrombinemia is associated with reduced plasma prothrombin activity, usually between 5-10%, and commonly normal/near normal quantitative plasma levels of prothrombin; the bleeding phenotype is variable but less pronounced compared with severe hypoprothrombinemia [209]. Heterozygous individuals are usually asymptomatic; increased bleeding has, however, been observed following surgical procedures [210].

Factor V deficiency

Symptomatic FV deficiency is caused by biallelic variants in *F5*. Increased bleeding is generally only seen in the severe form of FV deficiency, defined as functional FV levels <10-15% [211]. Severe deficiency of FV is a clinically heterogeneous condition, showing a relatively weak correlation between the plasma levels of FV and the bleeding severity. The prevalence of FV deficiency in its homozygous form has been estimated to 1:1 000 000 [208]. Mild to moderate FV deficiency is usually seen in asymptomatic heterozygous carriers, showing functional FV levels >20-30% [212].

Combined factor V and factor VIII deficiency

Combined FV and FVIII deficiency (F5F8D) is a rare AR disorder with an estimated prevalence of 1:1 000 000 [208]. The condition is caused by variants in *LMAN1* and *MCFD2*, encoding proteins involved in the intracellular transport required for normal secretion of FV and FVIII [213]. Combined FV and FVIII deficiency is characterized by reduced functional levels of both FV and FVIII. Affected patients commonly show FV and FVIII levels of 5-20% [214]. The bleeding phenotype is usually mild [208].

Factor VII deficiency

Factor VII deficiency is caused by variants in *F7*. Increased bleeding is generally associated with functional plasma FVII levels <30%, resulting from biallelic deleterious variants [215]. However, the disorder is phenotypically variable with patients showing a bleeding phenotype ranging from asymptomatic to life-threatening, that is further poorly correlated to the plasma levels of functional FVII [215, 216] The global prevalence has been estimated to 1:500 000 [208].

Factor X deficiency

Factor X deficiency is mainly an AR disorder with an estimated prevalence of 1:1 000 000 [208]. The disorder is caused by variants in *F10* and is generally characterized by mucocutaneous bleeding. However, the sometimes life-threatening phenotype of severe FX deficiency (functional plasma levels <1%) is also associated with joint bleeds [217]. Joint bleeds have also been reported in patients with moderate FX deficiency (functional plasma levels 1-5%), while patients with mild FX deficiency (functional plasma levels 5-10%) mainly suffer from mucocutaneous bleeds; factor X levels >20% are rarely associated with bleeding [217].

Factor XI deficiency

Factor XI deficiency is caused both by monoallelic and biallelic variants in *F11*. While spontaneous bleeds are uncommon, the bleeding phenotype is characterized by excessive bleeding following surgery or trauma [218]. Generally, there is a weak correlation between bleeding risk and functional plasma levels of FXI [219]. However, FXI levels >40% are usually not associated with severe bleeding [220]. The global prevalence of AR FXI deficiency has been estimated to 1:1 000 000 [221].

Factor XII deficiency

Factor XII (FXII) deficiency is a rare condition not associated with increased bleeding [222]. Nevertheless, a significant FXII deficiency prolongs the activated partial thromboplastin time (APTT) commonly used in routine coagulation screens.

Factor XIII deficiency

Factor XIII deficiency is an AR disorder caused by variants in *F13A1* and *F13B*, encoding the A and B subunits of the FXIII molecule, respectively. Variants affecting the A subunit are the more common cause of symptomatic FXIII deficiency and are usually associated with severe bleeding tendencies with heterogeneous presentations [208, 219]. Functional FXIII levels between 2% and 5% are in general sufficient for avoiding severe bleeding [208]. Moreover, patients with FXIII levels >30% have been reported to primarily remain asymptomatic [223]. Variants affecting the B subunit of FXIII have mostly been associated with surgical and/or mild mucocutaneous bleeding [224]. The estimated prevalence of FXIII deficiency is 1:2 000 000 [208].

Disorders of the fibrinolytic system

The inherited bleeding disorders of the fibrinolytic system include rare deficiencies of the fibrinolytic inhibitors α2-antiplasmin and PAI-1 (**Figure 6**). Both conditions result in increased bleeding by an accelerated fibrinolysis. The prevalences of the disorders are not known, but they are likely very rare [225].

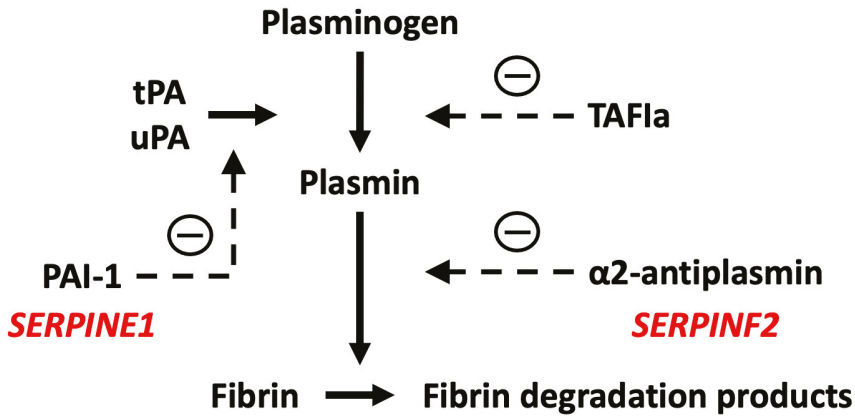

Figure 6. Examples of genes affected in the inherited disorders of the fibrinolytic system
Deficiecies of the fibrinolytic inhibitors α2-antiplasmin and PAI-1 result in increased fibrinolysis. Affected genes are shown in red.

α2-antiplasmin deficiency

α2-antiplasmin deficiency is mainly an AR disorder, caused by deleterious variants in *SERPINF2* [32]. The deficiency results in moderate to severe bleeding, commonly described following surgical procedures or trauma [225].

Plasminogen activator inhibitor-1 deficiency

Biallelic variants in *SERPINE1* underlie PAI-1 deficiency, associated with mild mucocutaneous bleeding and post-operative bleeds of a more severe character [32]. Heterozygous patients are usually asymptomatic [225].

A brief overview of hemostatic therapies

In patients with inherited bleeding disorders, the treatment or prevention of bleeding generally depends on the severity of the specific disorder and the prevailing situation. Some patients do not suffer from frequent spontaneous bleeds but may require prophylactic treatments prior to hemostatic challenges such as surgical interventions (e.g., mild IPFDs or type 1 VWD). On the other hand, patients with disorders such as severe deficiencies of coagulation factors may need regular prophylactic treatment in order to avoid spontaneous bleeds (e.g., severe hemophilia A or type 3 VWD). In addition to general measures such as avoiding medications associated with increased bleeding, using local hemostatic agents and considering hormonal therapies to reduce menorrhagia, several systemic hemostatic therapies exists [71]. In general, fibrinolytic inhibitors such as tranexamic acid are widely used, as sole treatment of mild bleeding symptoms or in combination with other hemostatic agents in the treatment of most inherited bleeding disorders. Furthermore, tranexamic acid constitutes the cornerstone treatment in the fibrinolytic disorders [32]. In IPFDs, tranexamic acid is commonly combined with desmopressin, a synthetic vasopressin analogue increasing the endogenous levels and FVIII and VWF [226]. Platelet transfusions can also be used in conjunction with severe bleeding or surgical procedures to replenish the circulation with normal-functioning platelets [227]. In ITs, platelet transfusions are also used in critical situations to increase the platelet counts, together with tranexamic acid and possibly desmopressin. In disorders with a high risk of developing platelet alloantibodies following platelet transfusions, such as GT, the use recombinant FVIIa is commonly advocated [228]. Recently, the use of the thrombopoietin receptor agonist eltrombopag was reported to successfully raise platelet counts in patients with ITs of varying etiologies [229]. For coagulation factor deficiencies, including severe deficiencies of VWF, replacement of the affected coagulation factor can be achieved by recombinant or plasma-derived factor concentrates (or fresh frozen plasma if concentrates are not available) [230, 231]. Since desmopressin increase the circulating levels of FVIII and VWF, it is commonly used in milder forms of hemophilia A and VWD.

Diagnostic approaches

The most common causes of abnormal bleeding are acquired, including a wide variety of factors such as concomitant diseases (e.g., renal dysfunction or liver disease) or medications (e.g., platelet-inhibiting drugs or directs oral anticoagulants) [232]. Consequently, acquired causes need to be ruled out by obtaining a careful medical history and performing an appropriate general laboratory screening. The same principle is true for incidentally identified thrombocytopenias, that may not always be associated with increased bleeding if the thrombocytopenia is mild [233, 234]. If the suspicion of an inherited bleeding disorder persists, a variety of methods are available for performing further investigations. Some methods are highly specialized and usually limited to research settings. In this section, a brief overview of common approaches and methods used to investigate patients suspected of inherited bleeding disorder will be presented.

Determining the clinical bleeding phenotype

The present and historical bleeding phenotype should be carefully assessed, and the severity of the bleeding symptoms should be quantified using a standardized bleeding score derived from bleeding questionnaires, such as the ISTH-BAT [235-237]. Moreover, a detailed family history is of importance for unveiling a possible familial bleeding disorder. In the context of a suspected IT, the clinician should be observant of additional symptoms that could be associated with a syndromic IT [238]. Due to the association between increased bleeding and connective tissue-disorders such as EDS, a clinical assessment for identifying generalized joint hypermobility can also be considered [39].

Initial screening for hemostatic defects

A full blood count should always be performed, including a peripheral blood smear, potentially revealing an altered morphology of platelets in the setting of thrombocytopenia (e.g., GPS or MYH9-RD). An automated assessment of the mean platelet volume is further useful for identifying thrombocytopenias with an altered

platelet size (e.g., macrothrombocytopenias). If an IT is suspected, further investigations can be guided by the clinical phenotype and/or the initial laboratory findings (**Figure 7**) [239].

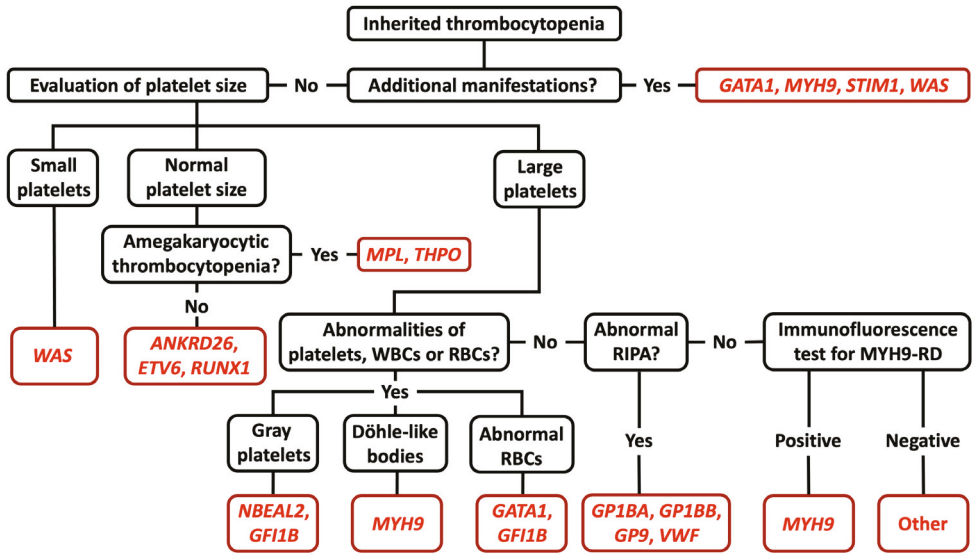

Figure 7. Flowchart for investigating a suspected inherited thrombocytopenia
Examples of findings guiding further investigations in a number of ITs presented in this thesis. Potentially affected genes are shown in red. Some genes are associated with ITs characterized by variable clinical presentations and are hence presented in multiple pathways. RIPA, ristocetin-induced platelet aggregation; RBCs, red blood cells (erythrocytes); WBCs, white blood cells (leukocytes). Adapted from *Clinical Genetics* with permission from John Wiley & Sons Ltd (Pecci A. Diagnosis and treatment of inherited thrombocytopenias. *Clin Genet*. 2016;89(2):141-153). © 2015 John Wiley & Sons A/S.

In the absence of thrombocytopenia explaining the bleeding diathesis, widely used tests for assessing the functional levels of plasma coagulation factors include a combination of prothrombin time (PT; Quick method), APTT and functional fibrinogen levels (Clauss method) [240]. In Sweden, the PT is commonly analysed by the Owren method, and is referred to as PK(INR) [241]. PK(INR) assess the activity of prothrombin, FVII and FX, while the PT according to Quick also includes the activity of fibrinogen and FV. APTT measures the activity of fibrinogen, prothrombin, FV, FVIII, FIX, FX, FXI and contact activation factors not involved in bleeding disorders such as FXII [242]. Functional assays for all the individual coagulation factors are available. Factor VIII and FIX are commonly included in the coagulation screen due to the higher prevalences of hemophilia A and B compared to other factor deficiencies. Screening tests such as APTT do not identify mildly reduced levels of an isolated coagulation factor, and is hence not always sufficient for identifying, e.g., a mild hemophilia [243]. In addition, none of the common screening tests include the activity of FXIII. Consequently, assessment of additional coagulation factors may be considered for excluding rare deficiencies of factors

such as FXI and FXIII. To screen for VWD, the absolute levels of the VWF protein as well as the functional activity of VWF are assessed [30, 244]. Factor VIII is commonly included in a VWD screen due to its interactions with VWF [186, 240]. Tests for assessing platelet function are commonly recommended if the observed bleeding diathesis cannot be explained by deficiencies of coagulation factors or VWF [30, 240, 245]. Examples of such assays include light transmission aggregometry (LTA) and FC, which are the recommended functional methods for investigating suspected IPFDs according to the guidelines issued by the ISTH [245].

Light transmission aggregometry

Since its introduction in the 1960s, LTA has been regarded as the gold standard method for assessing platelet function *in vitro* [246, 247]. The assay is based on a photometric measurement of platelet aggregation in platelet rich plasma (PRP), following the addition of various platelet-activating agonists. In a healthy subject, activated platelets aggregate, which reduce the number of light-scattering particles and decreases the optical density of the PRP (**Figure 8 A**) [248]. The generated light transmission curve allows for the interpretation of aggregation parameters such as maximum aggregation amplitude, presence of secondary wave (release of platelet granules) and reversibility (stability of platelet aggregates) (**Figure 8 B**) [249].

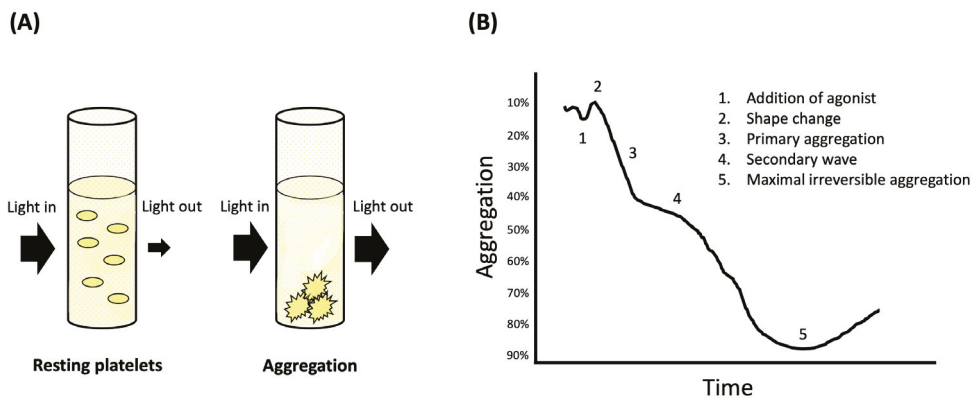

Figure 8. Principles of light transmission aggregometry
(A) Principle of light transmission aggregometry. Platelet aggregation in respone to added agonists result in increased light transmission through the sample. (B) Aggregation profile generated from the light transmission over time.

Examples of agonists used in LTA include ADP, adrenaline, collagen, trombin receptor activator peptides, arachidonic acid and ristocetin (an antibiotic inducing platelet aggregation by strongly enhancing the interaction between VWF and GPIb-IX-V), acting on platelet surface receptors (**Figure 1**) [250]. The aggregation profile

is interpretated in relation to the different agonists, which are used in separate experiments. Some well-defined aggregation patterns include the absent aggregation to all agonists except ristocetin in GT, while BSS (when VWD is excluded) is characterized by reduced/absent aggregation in response to ristocetin only [248]. Other examples include absent response to collagen in platelet GPVI deficiency and impaired and/or reversible aggregation in response to ADP in the setting of platelet $P2Y_{12}$ deficiency [249]. The methodological principle for LTA can also be used for assessing platelet release of dense granules by lumiaggregometry. In lumiaggregometry, released ATP stored in dense granules react with an added luciferin-luciferase reagent, generating a light emission proportional to the concentration of ATP [251].

Flow cytometry

In brief, platelets are exposed for antibodies targeting a specific platelet surface component of interest for investigation. Each type of antibody is labelled with a specific fluorochrome. The antibodies bind to the platelet structures of interest and when platelets pass through the flow cytometer, the fluorochromes are excited by laser beams and emits light at a specific wavelength. The amount of light emitted from each platelet is registered and is proportional to the number of antibodies bound to the platelet, which corresponds to the expression of the investigated platelet surface component. Multiple types of antibodies targeting different platelet structures are commonly used in the same sample, made possible by the use of different fluorochromes specific for each type of antibody [252]. For example, FC can be used to diagnose GT and BSS by analysing the expression of platelet surface glycoproteins such as GPIb-IX-V and GPIIb/IIIa, using antibodies against GPIb (CD42b) and GPIIb (CD41), respectively [253]. Flow cytometry can also be used for investigating suspected platelet function defects, by measuring the expression of markers for activations in response to stimulation with agonists such as ADP and trombin receptor activating peptides. Commonly used activation markers on platelet surfaces include CD62P (release of alpha granules) and CD63 (release of dense granules and lysosomes), as well as activated GPIIb/IIIa targeted by the antibody PAC-1 (**Figure 9**) [254].

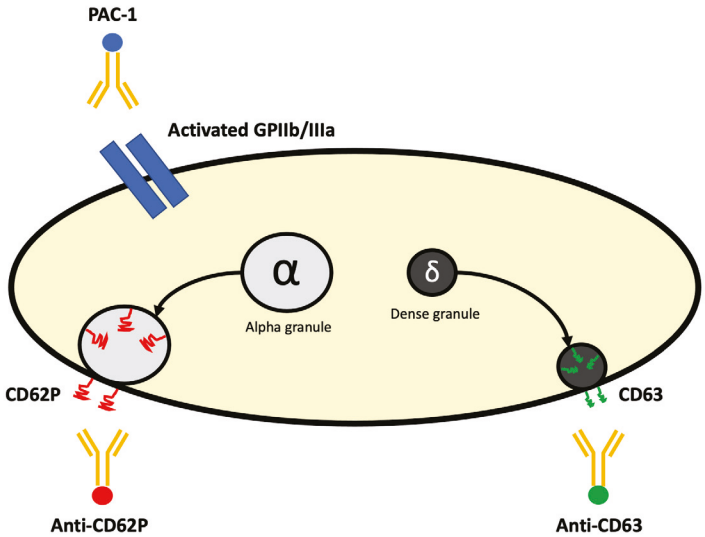

Figure 9. Examples of markers used for assessing platelet function by flow cytometry
Fluorochrome-labeled antibodies are used for detecting proteins of interest on platelet surfaces. The proteins CD62P and CD63 are exposed on the platelet surface following granule-release.

Transmission electron microscopy

Transmission electron microscopy allows for investigating platelets at a very high magnification and is regarded as the gold standard for investigating ultrastructural abnormalities in IPDs [255]. The thin section TEM method is used for assessing the platelet content of alpha granules and various other structures such as microtubules and glycogen (**Figure 10 A**). In thin section TEM, the platelets are stained, and the specimen is prepared into thin sections. Accordingly, each platelet section does not represent the total platelet content of a given structure, but the method is still useful for identifying structural abnormalities. For example, the method can be used to confirm deficiencies of alpha granules in GPS [138]. The whole mount TEM method investigates whole platelets without any staining (**Figure 10 B**). The method is the gold standard for investigating the total dense granule content of platelets, since dense granules are electron dense due to their high Ca^{2+} and phosphate content [256]. Hence, whole mount TEM is useful in diagnosing disorders such as HPS and CHS, characterized by dense granule deficiencies [86].

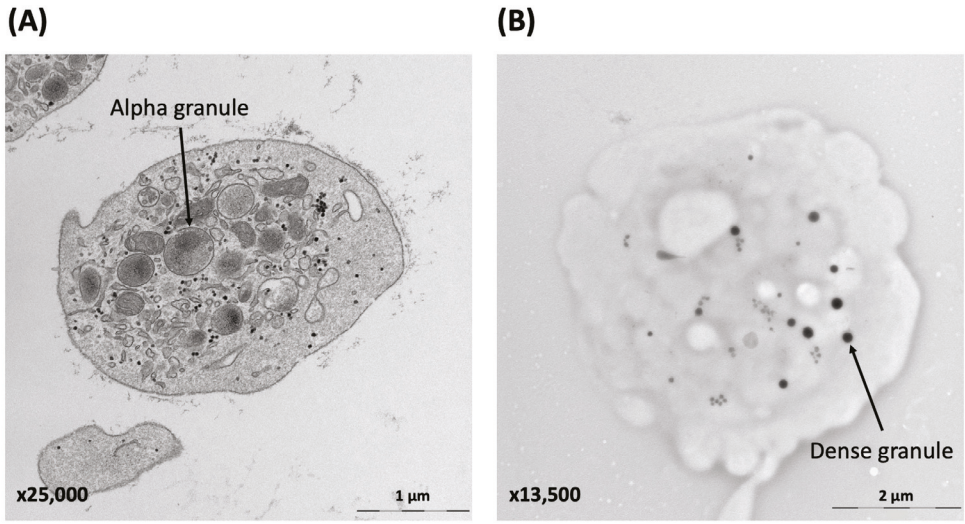

Figure 10. Platelet transmission electron microscopy
(A) A thin section TEM image displaying the ultrastructure of a platelet. Original magnification: x25.000. (B) A whole mount TEM image showing the total dense granule content of a platelet. Original magnification: x13.500.

Incompletely explained bleeding

Far from all patients suspected of inherited bleeding disorders receive a defined diagnosis following work-up. In this section, some examples of the clinical challenges associated with diagnosing inherited bleeding disorders will be presented.

Thrombocytopenia of unknown cause

The diagnostic difficulties in patients with constitutional thrombocytopenias are not to be underestimated. In clinical practice, an important "diagnostic" step consists of actually considering the possibility of an IT. Accordingly, a recent report on a cohort consisting of 181 patients with ITs showed that 31% of the patients were previously misdiagnosed with immune thrombocytopenia (ITP) [257]. Hence, the work-up of constitutional thrombocytopenias needs to be carefully guided by a synthesis of medical history, family history, potential associated features, the finding of morphologically abnormal platelets and other possible hematological manifestations. In addition, a concomitant platelet dysfunction can sometimes be indicative of an IT (e.g., *RUNX1*-associated thrombocytopenia or BSS). However, even if the clinical phenotype is indicative of an IT, the process of reaching a specific diagnosis can still be difficult. Although recent advances in DNA sequencing technologies have significantly improved the knowledge of genes affected in ITs, approximately 50% of patients highly suspected of ITs never receive an explanation to their low platelet counts [113].

Platelet dysfunction of unknown cause

A proportion of patients with normal platelet counts suffering from increased bleeding have historically been diagnosed with platelet dysfunction following work-up including various functional platelet assays [258-260]. Commonly, these patients are distinguished from patients with bleeding of unknown cause (BUC), even if the underlying cause of the platelet dysfunction is not established [258]. Many of the major IPDs, such as GT or BSS, are well characterized and are readily diagnosed

by LTA or FC. However, milder defects of platelet function are usually harder to define using functional methods. The overall prevalence of constitutional platelet dysfunction is regarded as unknown [249, 258]. However, incompletely characterized platelet dysfunction has sometimes been suggested to be as prevalent as VWD in patients with a mucocutaneous bleeding phenotype [260-262]. These conclusions are uncertain, and even though LTA has been used in many of the studies, the assay is widely known to suffer from poor standardization regarding execution and interpretation of results. Accordingly, several efforts have been made in recent years to improve the consistency and comparability of LTA findings [245, 250, 263]. Today, patients are still diagnosed with platelet dysfunction of unknown cause following functional investigations if the bleeding phenotype is supportive of the diagnosis.

Bleeding of unknown cause

Bleeding of unknown cause is commonly defined as a significant bleeding history accompanied by normal hemostatic tests, after excluding acquired factors interfering with hemostasis [264, 265]. In patients referred because of a suspected mild bleeding disorder, it has been estimated that 49%-75% remain without a diagnosis following laboratory work-up [258]. Accordingly, another recent review of previous studies estimated that 40%-70% of patients with mild bleeding tendencies show normal results following laboratory investigations, and that 30%-40% of patients report a family history of bleeding [265]. However, comparisons between different studies are limited by the poor standardization of patient selection and the included laboratory testing. Currently, several established structured bleeding assessment tools (BATs) for identifying significantly increased bleeding exist, such as ISTH-BAT [235, 236], Vicenza BAT [266] and MCMDM-1 VWD BAT [267]. The use of standardized bleeding scores has not proven useful in distinguishing patients with mild to moderate bleeding and laboratory-confirmed hemostatic defects from patients with BUC [268, 269]. Still, BATs are useful for grading bleeding symptoms, and a high ISTH-BAT score is predictive of future bleeding episodes in patients with BUC [270]. Nonetheless, a significant proportion of patients with increased bleeding never receive an explanation to their symptoms. For some reported patients with BUC, the increased bleeding may be caused by hemostatic defects only possible to diagnose by highly specialized functional assays [30]. An additional potential cause not assessed by laboratory testing is the presence of connective tissue-disorders. Interestingly, a high prevalence of symptomatic generalized joint hypermobility (24%) have been observed in patients with mucocutaneous bleeding disorders including BUC, suggesting a possible involvement of collagen-related defects [271]. Taken together, the cause of bleeding in this group of patients is enigmatic.

Genetic screening

As may be evident from previous sections, the defined inherited bleeding disorders are highly heterogeneous, and the diagnostic methods are labor-intensive and commonly limited to centers specialized in bleeding disorders and/or research settings. Moreover, functional testing is sometimes inconclusive, especially in the case of functional platelet investigations [57]. In addition, a considerable proportion of patients suffering from increased bleeding never receive an explanation to their symptoms following rather extensive laboratory investigations [258, 265]. Consequently, the idea of using genetic screening to reach a molecular diagnosis in patients suspected of inherited bleeding disorders is appealing.

Rationale

In addition to offering patients an explanation to their bleeding symptoms, an adequate molecular diagnosis can also be of importance for correct treatment, prognostication and genetic counselling, which is especially evident in the context of ITs [272]. The importance can be exemplified by patients with ITs being misdiagnosed with ITP, which expose patients for unnecessary treatments and their associated side-effects (e.g., corticosteroids or splenectomy) [257, 273, 274]. Moreover, syndromic ITs can be associated with additional manifestations that needs to be addressed, as in the case of MYH9-RD, where renal involvement can be pharmacologically targeted [275]. Other important examples include ITs caused by germline variants in *RUNX1*, *ANKRD26* and *ETV6.* The lifetime risk of developing a hematological malignancy has been estimated to 40% for *RUNX1*-associated thrombocytopenia [276], while ~10% of patients with thrombocytopenia caused by variants in *ANKRD26* have been reported to develop hematological malignancies [129, 277]. In addition, ~50% of patients with variants in *ETV6* are diagnosed with predominantly lymphoid, but also myeloid malignancies [278]. The identification of such variants has potential implications for regular monitoring and genetic counseling [279].

Ethical considerations

Prior to performing genetic investigations, consenting patients (or parents/legal guardians) need to be well-informed regarding the potential diagnostic benefits and possible clinical implications of the results [280]. Importantly, patients need to be informed about the risk of identifying variants associated with a risk of developing hematological malignancies which could potentially cause psychological stress. There is also the possibility of incidental findings not primarily related to the specific phenotype of the patient, even if only genes associated with inherited bleeding disorders are investigated. Hence, it is commonly recommended that patients should have the option of deciding whether they want to receive information of incidental findings or not [281].

Methodological principles

Since the 1970s, Sanger sequencing has been applied for sequencing separate genes suspected to harbor variants causative of a specific disease-phenotype [282]. The introduction of high-throughput DNA sequencing (HTS) techniques in the 2000s has allowed for the parallel sequencing of a large number of genes, at a significantly improved speed and at lower costs [283]. Using HTS, two principally different approaches can be applied: whole exome sequencing (WES) and whole genome sequencing (WGS). In WES, the protein-coding regions of the genome (the exome) are sequenced. The exome constitutes approximately 1% of the genome but has been estimated to harbor ~85% of known genetic variants associated with disease [284]. In WGS, the whole genome is sequenced, including the non-coding regions. For example, WGS allows for the detection of larger copy number variations and variants in promotor sites or enhancers regulating gene expression. Even though there is a significant difference in the amount of data acquired by WGS compared to WES, both methods generate extensive datasets. Consequently, approaches targeting only a specific set of genes of interest are widely used in both WGS and WES [285]. In brief, germline DNA extracted from peripheral blood is fragmented, and the DNA fragments are cloned into a high number of copies. In the case of WES, fragments from exonic regions are isolated (target enrichment). The nucleotide sequences of the fragments are then registered (sequencing). The registered sequences (reads) are then aligned to the reference genome for the identification of variants in genes or possibly other regions of interest (variant calling) (**Figure 11**) [286]. In addition, *in silico* gene panels can be used for filtering the findings to only include variants in a predefined set of genes. Guidelines for assessing the significance of detected variants have been published by the American College of Medical Genetics (ACMG) [287]. The framework includes parameters such as the allele frequency in population databases, *in silico* models predicting the

effect of the variant, results of functional studies, segregation data and mode of inheritance for the associated disorder. According to the ACMG guidelines, variants are graded as either benign (class 1), likely benign (class 2), variant of uncertain significance (VUS) (class 3), likely pathogenic (class 4) or pathogenic (class 5).

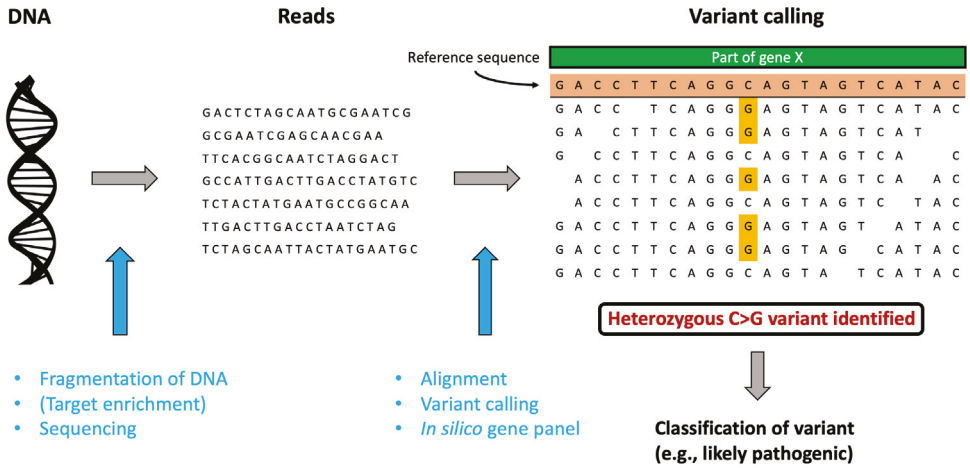

Figure 11. Workflow for genetic screening using whole exome and whole genome sequencing
In WES, target enrichment is performed to isolate exonic regions. A prediefined *in silico* gene panel is commonly used for only investigating variants in specific genes of interest.

Diagnostic efficacy in inherited bleeding disorders

Over the last years, HTS using predefined gene panels have been implemented the diagnostics of inherited bleeding disorders [288]. Accordingly, several studies have been published, investigating the diagnostic yield in patients suspected of different types of inherited bleeding disorders. The number of genes included in the panels have ranged from 23 to 358, depending on the design of the studies [289]. One study reported class 4-5 variants in 68.3% of the patients, suffering from lifelong thrombocytopenia or laboratory abnormalities consistent with IPFDs [290]. Another study including patients with functionally suspected IPDs following laboratory work-up, encompassing patients with both normal and reduced platelet counts, showed class 4-5 variants in 26% of all included patients [291]. In patients with abnormal coagulation factor parameters or confirmed coagulation deficiencies, the proportion of patients with a corresponding class 4-5 variant have been reported as ~60% [292] and 100% [293], respectively. In the context of a suspected IT, the percentages of patients found to have a class 4-5 variant have been reported as 25.8% [294], 30% [295], 34.9% [296], ~35% [292], 46% [297], 46% [298] and 55.6% [299]. In patients with normal platelet counts suspected of inherited bleeding

disorders based on the bleeding phenotype, the diagnostic yield have been significantly lower: in four different studies, class 4-5 variants were only found in 0% [299], 0% [300], 4% [301] and 11% [295] of the patients. To date, the largest study evaluating the use of HTS in inherited bleeding disorders was conducted by the ThromboGenomics group in the UK [292]. In 430 patients suspected of IPFDs, showing platelet function abnormalities, class 3-5 variants were found in 26.1% of the patients, with class 4-5 corresponding to ~20%. However, in 619 patients with unexplained bleeding following relatively extensive laboratory testing (e.g., coagulation factors, VWF and platelet function testing), class 4-5 variants were only identified in 2.5% of the patients [289, 292]. Hence, with the present knowledge regarding the genetic causes of inherited bleeding disorders, HTS seems like a promising clinical tool for establishing a molecular diagnosis in patients presenting with laboratory signs of factor deficiencies and patients suspected of ITs. However, the diagnostic yield is lower for patients suspected of IPFDs and, in particular, patients with BUC following extensive work-up.

Variants of uncertain significance

One of the main challenges of using HTS is the interpretation of variants designated as VUSs. Accordingly, many VUSs have been identified in the studies previously published on utilizing HTS in the setting of suspected inherited bleeding disorders [292, 295, 299, 300]. For example, in one of the studies by Johnson *et al*., VUSs were found in 51% of the patients [294]. In the future, efforts for increasing international data sharing, such as the ISTH GoldVariants database, may increase the knowledge regarding variants currently classified as VUSs [289]. Until then, one approach consists of performing specific functional studies for investigating the significance of such variants [302].

Rationales of Papers I-IV

The rationales behind Papers I-IV will be presented in the present section. The genes specifically investigated in this thesis have already been introduced in the context of inherited bleeding disorders. However, for the convenience of the reader, a brief resume of these genes and their associated conditions will also be included.

Paper I

Biallelic variants in *UNC13D*, *STX11* and *STXBP2* are causative of FHL, resulting from impaired exocytosis of cytotoxic granules in cytotoxic T lymphocytes and natural killer cells [97, 98]. The genes are also essential for the secretory machinery mediating granule release in platelets [99-103]. Accordingly, severely impaired release of platelet granules has been demonstrated in FHL-patients harboring biallelic variants in either *UNC13D*, *STX11* or *STXBP2* [103-106].

Of the initial 156 patients in the Öresund Region undergoing genetic screening because of incompletely explained bleeding tendencies [295], 7.7% of the patients were found to harbor heterozygous variants in *UNC13D*, *STX11* or *STXBP2.* To the best of our knowledge, heterozygous variants in these genes had previously not been functionally investigated in the context of suspected inherited bleeding disorders.

Paper II

The congenital fibrinogen disorders are divided into quantitative and/or qualitative deficiencies of fibrinogen [201, 202]. The disorders are caused by variants in the genes *FGA*, *FGB* and *FGG*, encoding the three polypeptide chains forming the fibrinogen molecule [203].

A woman was found to suffer from increased bleeding and low fibrinogen levels. The phenotype was also identified in a number of her first-degree relatives. The combination of genetic screening and functional testing offers the possibility of specifically characterizing a suspected familial fibrinogen disorder, potentially

contributing with new knowledge regarding specific disease-causing genetic variants.

Paper III

Ehlers-Danlos syndrome is a disease of the connective tissues, commonly presenting with joint hypermobility and hyperextensibility of the skin [41]. Increased bleeding is a common symptom, listed as a diagnostic criterion in the majority of the EDS subtypes [39, 40]. Heterozygous variants in the collagen-encoding genes *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* are known to cause subtypes of EDS that are associated with increased bleeding [42]. Moreover, the synthesis of mature collagen molecules is dependent on sufficient levels of AA [50]. Accordingly, a severe deficiency of AA results in scurvy, associated with increased bleeding [51].

In the initial cohort of 156 patients with incompletely explained bleeding diathesis [295], 9% of the patients were found to harbor heterozygous variants in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2* following genetic screening, suggesting a possible association between the variants and increased bleeding in this patient population.

Paper IV

Biallelic variants in *GNE* have recently been described as a very rare cause of severe macrothrombocytopenia, attributed to a reduced expression of platelet surface sialic acid [171-174]. Platelet surface sialic acid is essential for the lifespan of platelets: senescent desialylated platelets are normally cleared from the circulation via the hepatic Ashwell-Morell receptor [165, 166]. The GNE enzyme, encoded by *GNE*, is the rate limiting enzyme of sialic acid biosynthesis [167].

A patient with severe congenital macrothrombocytopenia was shown to harbor two novel compound heterozygous variants in *GNE*. Even though the variants were suspected to be causative, functional investigations are required for verifying an association between the genotype and the phenotype. Moreover, in the case of an established diagnosis of *GNE*-associated thrombocytopenia, it was hypothesized that treatment with an approved sialidase inhibitor possibly could increase the platelet counts by an inhibition of endogenous sialidases.

Aims of the thesis

The overall aim of this thesis was to investigate the significance of selected genetic variants identified in patients suspected of inherited bleeding disorders but with incompletely explained bleeding tendencies. In each paper, variants in a specific category of genes have been investigated:

Paper I

The aim was to investigate the functional consequences of heterozygous variants in *UNC13D*, *STX11* and *STXBP2* for platelet granule secretion in patients with incompletely explained bleeding.

Paper II

The aim was to molecularly characterize a suspected familial fibrinogen disorder in a Swedish family.

Paper III

The aim was to assess the functional consequences of heterozygous variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* in patients with incompletely explained bleeding.

Paper IV

The aims were to functionally assess the consequences of two compound heterozygous variants in *GNE* identified in a patient with severe congenital macrothrombocytopenia, and to evaluate the use of the sialidase inhibitor oseltamivir for increasing the platelet counts.

Methods

Recruitment of patients

All included patients were referred to the Department of Coagulation, Skåne University Hospital, Malmö or the Department of Hematology, Rigshospitalet, Copenhagen between 2013 and 2018 because of a suspected inherited bleeding disorder. Due to an incompletely explained bleeding phenotype following work-up, the patients underwent genetic screening for potentially improving the diagnostic yield. Signed informed consent was obtained from all included patients and healthy controls participating in the studies. The studies were approved by the regional ethical committees in Lund (Dnr 2014/409) and Copenhagen (H-15011677).

Genetic screening

Whole exome sequencing was performed using an *in silico* gene panel targeting genes associated with bleeding disorders. The method is described in detail in Paper I. The *in silico* gene panel was continuously updated, and consisted of 87 genes in Paper I (Paper I, Table S1), 94/95 genes in Paper II (Paper II, Table S1) and 94 genes in Paper III (Paper III, Table S1). In Paper IV, WES was initially performed using the gene panel described in Paper I. However, the genetic investigation in Paper IV was later expanded by using WGS.

Paper I

Study population

In total, 12 patients with incompletely explained bleeding diathesis were found to harbor heterozygous or compound heterozygous variants in *UNC13D*, *STX11* or *STXBP2* following genetic screening.

Methods for assessing platelet granule secretion

Whole mount TEM was used to investigate the release of platelet dense granules in response to stimulation with thrombin [105, 255]. Platelet rich plasma was carefully prepared from each subject. One aliquot of PRP was kept resting, while one aliquot was stimulated with thrombin. The separate samples consisting of unstimulated and stimulated PRP was subsequently prepared for investigation by whole mount TEM. Images of 50 platelets from each of the two samples were randomly recorded using a transmission electron microscope. All images were blinded, and the platelets were assessed for the presence of platelet dense granules in a separate session. The mean number of dense granules per platelet was calculated in 50 thrombin-stimulated platelets (DG_T) and in 50 unstimulated platelets (DG_U). The secretion of dense granules was assessed by a secretory ratio (R_S), where a value <1 was regarded as suggestive of granular secretion (**Equation 1**).

$$R_S = \frac{DG_T}{DG_U} \qquad \text{(Equation 1)}$$

The result of each patient was compared to a reference range derived from 19 healthy controls.

Secretion of platelet alpha granules and dense granules was investigated by FC: the platelet expression of CD62P and CD63 was assessed following stimulation with ADP, cross-linked collagen-related peptide (CRP-XL) and thrombin receptor activator peptide 6 (TRAP-6). The percentages of platelets positive for each of the markers were compared to the local clinical reference ranges.

Light transmission aggregometry, including lumiaggregometry for investigating the release of dense granules, was performed using the agonists ADP, adrenaline, collagen, ristocetin and TRAP-6. Traces for both aggregation and ATP release were registered and interpreted in relation to results of healthy controls.

Statistical methods

For the dense granule secretion analysis using whole mount TEM, the R_S values obtained in the control group were normal distributed (visual inspection of histogram and boxplot, Shapiro-Wilk test, Q-Q plot). Consequently, a 95% reference range for the assay was derived from the controls by calculating the mean ± 1.96 standard deviations (SD).

Paper II

Study population

A woman (the proband) and her first-degree relatives (mother, father, sister and brother) were investigated because of a suspected familial fibrinogen disorder.

Whole exome sequencing

Whole exome sequencing was performed using an *in silico* gene panel targeting 95 (proband) or 94 (first-degree relatives) genes associated with bleeding disorders, including *FGA*, *FGB* and *FGG* (Paper II, Table S1).

Fibrinogen assays

The functional fibrinogen levels and the fibrinogen antigen levels were determined. FIBTEM analysis (rotational thromboelastometry; ROTEM) of fibrinogen-depended clot formation was also performed [303, 304], as well as analyses of fibrin clot structure [305].

Assessment of platelet function

The expression of activated GPIIb/IIIa (PAC-1 binding), CD62P and CD63 in response to stimulation with ADP, CRP-XL and TRAP-6 was assessed by FC. Light transmission aggregometry was also performed.

Paper III

Study population

Following genetic screening, a total of 31 patients with incompletely explained bleeding diathesis were found to harbor at least one heterozygous variant in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2*.

Clinical assessment of EDS criteria

All patients underwent a specific clinical examination for the assessment of potential symptoms compatible with EDS, according to the current diagnostic criteria [40]. All listed diagnostic criteria for the EDS subtypes associated with variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* were addressed, and the presence of generalized joint hypermobility was investigated using the Beighton score [306] as well as the five-point questionnaire [40, 307].

Collagen remodeling

The genes *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* encode the procollagen chains forming types I, III and V collagen, respectively. In order to investigate the functional consequences of the identified variants, the formation and degradation of types I, III and V collagen were examined by enzyme-linked immunosorbent assays (ELISAs), using monoclonal antibodies directed against highly specific neo-epitopes exposed during the synthesis and degradation of the collagens. Collagen formation was assessed by the serum levels of PRO-C1 (type I collagen), PRO-C3 (type III collagen) and PRO-C5 (type V collagen) [308-311]. Collagen degradation was assessed by the serum levels of C1M (type I collagen), C3M (type III collagen) and C5M (type V collagen) [312-314]. The tissue balances of types I, III and V collagen were expressed as PRO-C1/C1M, PRO-C3/C3M and PRO-C5/C5M, respectively [315, 316]. The remodeling of types I, III and V collagen was assessed in all patients and was compared with the results of 20 healthy controls included in the study.

Ascorbic acid

It was hypothesized that the plasma levels of AA possibly could reflect a potentially altered collagen remodeling. Hence, the plasma levels of AA were investigated in all patients by high-performance liquid chromatography [317]. Patients with reduced plasma levels of AA were classified as having either suboptimal status (23-50 μM), marginal deficiency (11-23 μM) or severe deficiency (<11 μM) [318].

Statistical methods

The distributions of the data were determined visually and by using the D'Agostino-Pearson omnibus K2 normality test. Outliers were defined using the Tukey's fences method. The results of the patients were compared with the controls using Mann-Whitney tests, due to a consistently non-normal distribution of the compared parameters. Potential correlations between clinical and laboratory parameters were investigated by creating scatterplots, including correlation coefficients and *p*-values. The Pearson (normally distributed data) or the Spearman (non-normally distributed data) correlation coefficient was used. A p-value <0.05 was regarded as significant for all analyses.

Paper IV

Study population

A young boy (the proband) was investigated because of a severe congenital macrothrombocytopenia of unknown cause. His non-thrombocytopenic first-degree

relatives (mother, father and brother) were also included for investigation, in order to identify possible heritable factors explaining the phenotype of the proband.

Whole genome sequencing

In 2015, WES was performed using the 87-gene panel described in Paper I (Paper I, Table S1) without any plausible explanations for the thrombocytopenia. Consequently, WGS was undertaken in the proband and his first-degree relatives. Initially, the WGS data were analyzed without any clear causative findings. In 2019, data had emerged on variants in *GNE* causing a severe isolated macrothrombocytopenia, resembling the phenotype observed in the proband. Consequently, the WGS data were specifically reinvestigated for variants in *GNE*.

Platelet and leukocyte sialic acid

The GNE enzyme is known to be required for the normal sialylation of platelets and other hematopoietic cells [319]. Consequently, the levels of sialic acid were assessed on platelets, granulocytes, lymphocytes and monocytes by FC, using fluorochrome-labeled Sambucus nigra lectin (SNA) and Maackia amurensis lectin II (MAL II) for detecting α-2,6- and α-2,3-linked sialic acid, respectively [320]. Results were presented as the delta geometric mean fluorescence intensity (Δ gMFI), adjusting for potential autofluorescent properties of investigated cells. The results of five healthy controls were used as reference.

Complement factor H binding in platelet and leukocytes

Sialic acid is required for the binding of complement factor H (FH) to hematopoietic cells [321]. The binding of FH to platelets, granulocytes, lymphocytes and monocytes was investigated by FC, using fluorochrome-labeled purified FH. Results were presented as Δ gMFI. The results of five or six healthy controls were used as reference.

Complement-mediated hemolysis

Surface-bound FH is essential for protecting erythrocytes from lysis mediated by the complement system [322]. The binding of FH to erythrocytes was indirectly evaluated by hemolytic assays, comparing erythrocytes from the proband with erythrocytes from five healthy controls. Free hemoglobin was measured following the incubation of erythrocytes with freshly frozen normal human serum in increasing concentrations.

Treatment with oseltamivir

The sialidase inhibitor oseltamivir is commonly used for treating influenza, and acts by inhibiting viral sialidases [323]. Oseltamivir has been shown to increase platelet counts in patients with ITP, were platelet desialylation recently has been proposed as a relevant pathophysiological mechanism [320, 324]. Increased platelet counts

have also been demonstrated in influenza patients treated with oseltamivir [325]. Therefore, it was hypothesized that oseltamivir possibly could increase the platelet counts in *GNE*-associated thrombocytopenia by increasing the levels of platelet sialic acid through an inhibition of endogenous sialidases. Consequently, the proband was treated with a regular dosage of oseltamivir (75 mg twice daily). The platelet counts were monitored during the treatment period and the platelet sialylation was reassessed by FC after 18 days of treatment.

Statistical methods

For each of the FC experiments investigating the levels of sialic acid and FH binding, the Δ gMFI values were expressed as the percentage of the median Δ gMFI value of the healthy controls.

Results and discussion

Paper I

In the 12 patients, a total of eight different genetic variants were found in *UNC13D*, *STX11* or *STXBP2*. One of the patients (P5) was compound heterozygous for two variants in *UNC13D*. All other patients were heterozygous for one variant in either *UNC13D*, *STX11* or *STXBP2*. In addition, a total of 27 heterozygous variants were found in the 12 patients, whereof only one variant in *RUNX1* was assessed as significant (**Table 2**; Paper I, Table S2). The *RUNX1* variant was found in the related patients P4 (mother) and P5 (son).

The 11 heterozygous patients were all available for investigation by the functional whole mount TEM assay (**Figure 12**).

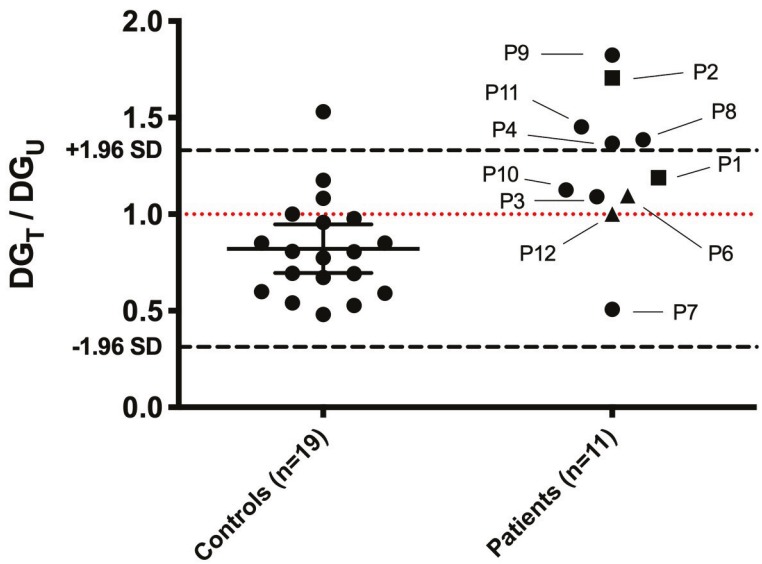

Figure 12. Secretion of platelet dense granules assessed by whole mount transmission electron microscopy
A possibly impaired secretion of dense granules was shown in five out of the 11 patients available for the investigation. The mean with the 95% confidence interval is shown for the 19 controls. Grid lines indicate mean ± 1.96 standard deviations (black, dashed) and $DG_T = DG_U$ (red, dotted). The gene potentially affected in each patient is depicted as follows: ●, *UNC13D*; ■, *STXBP2*; ▲, *STX11*. SD, standard deviation.

The individual R_S values of the patients were compared with the reference range calculated from the results of the controls: a R_S value >1.33 was interpreted as impaired secretion of dense granules. Five patients presented with R_S values exceeding the cutoff (**Figure 12**). Nine patients were available for examination by FC. Abnormal results, including reduced expression CD63, was found in three of the patients (**Table 2**). Assessment by LTA with lumiaggregometry was possible in seven patients. Reduced aggregation with the absence of a secondary wave following stimulation with at least one agonist was observed in three patients, whereof one patient also showed impaired ATP secretion in response to ADP (**Table 2**; Paper I, Figure 4).

Table 2. Results of the platelet granule secretion assays
An identical variant in *UNC13D* was found in six patients; the variant is highlighted using bold letters. All additional genetic variants are heterozygous. The class (1-5) of each variant is presented in brackets. Abnormal results are shown in red. Asterisks denote related individuals. The agonists associated with abnormal results in LTA and FC are shown in brackets. ATPR, ATP release by lumiaggregometry; A, ATP; C, CRP-XL; COL, collagen; T, TRAP-6; N/A, not applicable.

Patient	Variants in FHL-associated genes	TEM (R_S)	LTA	FC	Additional variants
P12	*STX11:* c.499A>T, p.Ile167Phe	1.00	N/A	N/A	*F13B* (1)
P6	*STX11:* c.799G>A, p.Val267Met	1.10	↓ (A, COL) ↓ ATPR (A)	Normal	*GP9* (1), *ITGA2* (1), *ANKRD26* (1), *PTGS1* (2), *MASTL* (1)
P2	*STXBP2:* c.911C>T, p.Thr304Met	1.71	Normal	Normal	*COL5A1* (2)
P1	*STXBP2:* c.1001C>T, p.Pro334Leu	1.19	Normal	Normal	*LYST* (2), *F2RL3* (2), *TBXAS1* (2)
P4*	***UNC13D:* c.2896C>T, p.Arg966Trp**	1.37	N/A	↓ CD63 (C, T)	*RUNX1* (4)
P5*	***UNC13D:* c.2896C>T, p.Arg966Trp** *UNC13D*: c.283_285del, p.Glu95del	N/A	N/A	↓ CD63 (C, T) ↓ CD62P (A) ↓ PAC-1 (A)	*RUNX1* (4), *LYST* (2), *COL3A1* (2)
P3	***UNC13D:* c.2896C>T, p.Arg966Trp**	1.09	Normal	Normal	*P2RY1* (2), *FGG* (1), *HPS5* (1)
P7	***UNC13D:* c.2896C>T, p.Arg966Trp**	0.51	N/A	N/A	*P4HB* (1)
P8	***UNC13D:* c.2896C>T, p.Arg966Trp**	1.39	Normal	Normal	*ITGB3* (1), *TUBB1* (1)
P10	***UNC13D:* c.2896C>T, p.Arg966Trp**	1.13	↓ (A)	↓ CD63 (C, T)	*HOXA11* (1), *F7* (N/A), *F8* (1)
P9	*UNC13D:* c.466C>T, p.Arg156Trp	1.82	↓ (A)	Normal	*BLOC1S3* (1), *VWF* (1), *F2R* (1)
P11	*UNC13D:* c.1759C>T, p.Arg587Cys	1.45	N/A	N/A	*TBXAS1* (1)

In total, 7/12 patients (58%) were available for investigation by all three of the assays for platelet granule secretion. Using whole mount TEM, FC and LTA, a total of eight 8/12 patients (67%) presented with abnormal findings by at least one of the assays; out of these eight patients, three patients showed abnormal results by two different methods (**Table 2**).

Regarding the additional variants detected in the patients, only the variant in *RUNX1* identified in P4 and P5 was considered to have a potential impact on the performed functional investigations. Both patients suffered from thrombocytopenia, consistent with the variant having deleterious effects (Paper I, Table 1). In addition to

thrombocytopenia, haploinsufficiency of *RUNX1* is known to be associated with a reduced number of platelet dense granules, which is reflected as a reduced secretion of dense granules when investigated by FC and LTA [125, 126]. Indeed, patient P4 was shown to have a low number of platelet dense granules compared with the healthy controls (Paper I, Figure 5). Unfortunately, P5 was not available for investigation by whole mount TEM.

Even though it is not possible to draw any definitive conclusions about the significance of the specific variants in *UNC13D*, *STX11* and *STXBP2* identified in the study, some degree of impaired platelet degranulation was associated with 6/8 variants identified (75%). If excluding the two patients with the *RUNX1* variant possibly affecting the results of the functional testing, 5/7 identified variants (71%) were linked to abnormal results. Heterozygous deleterious variants in two different FHL-associated genes have been described as causative of fulminant FHL, consistent with a synergistic effect of variants in different genes affecting the secretory machinery of hematopoietic cells [326]. These results indirectly support the possibility of single heterozygous variants having the potential of affecting the granule secretion machinery in platelets to some extent. Platelet granule secretion was previously investigated in one patient heterozygous for a variant in *STXBP2*, showing partially impaired secretion [105]. However, to our knowledge, the platelet phenotype of patients heterozygous for variants in FHL-associated genes have not been extensively investigated prior to the present study. Taken together, our findings are interesting and suggests an association between heterozygous variants in FHL-associated genes and increased bleeding due to affected platelet granule secretion, that warrants further investigations.

To the best of our knowledge, this is the first study to use whole mount TEM for assessing the secretion of platelet dense granules in patients with variants in FHL-associated genes. Thin section TEM combined with thrombin stimulation was previously used by Al Hawas *et al*. for identifying impaired platelet granule secretion when investigating biallelic variants in *STXBP2* [105]. A platelet-specimen investigated by thin section TEM commonly has a thickness of ~50 nM and, hence, do not investigate the full granule content of platelets. Moreover, to our experience, dense granules can be hard to identify using thin section TEM and the whole mount TEM method is commonly preferred for this purpose [327]. Consequently, the whole mount TEM method was chosen for developing an assay for platelet dense granule secretion due to the possibility of investigating the total dense granule content of platelets. Another rationale for developing the method was to possibly correct for a constitutionally reduced number of dense granules by using each subject as its own control, hence assessing the net secretion of dense granules. Methods such as LTA with lumiaggregometry and CD63-exposure by FC are not able distinguish a pure secretion defect from a reduced net secretion caused by a reduced platelet dense granule content [328]. Limitations of the whole mount TEM method used in the study obviously include that it is novel and has not been

validated. The accuracy of the assay could possibly have been improved by including a higher number of healthy controls. The examination of 50 platelets (or even less) for calculating the mean number of dense granules per platelet have previously been described in the literature for whole mount TEM [327]. However, it could be argued that investigating a higher number of platelets could have increased the performance of the assays.

For future functional studies, comprising a larger cohort of patients harboring heterozygous variants in *UNC13D*, *STX11* or *STXBP2*, it could be of interest to use additional methods for more accurately assessing platelet granule secretion. Examples for investigating secretion of dense granules in a research setting could include an initial assessment by whole mount TEM for the exclusion of a reduced number of dense granules, followed by the investigation of the release of radio-labeled serotonin incorporated in dense granules [327]. A mepacrine-based FC assay could potentially also be used for examining the net secretion of dense granules [329]. Since variants potentially affecting the platelet secretory machinery are also expected to affect leukocytes, as in fully developed FHL, complementary investigations of degranulation in cytotoxic T lymphocytes and natural killer cells could possibly be used to identify a partial secretory defect [90, 330]. Investigating the expression of the encoded proteins in platelets could also be an interesting approach [330-332].

Paper II

A 27-year-old woman (the proband) was referred because of a history of spontaneous bleeds and increased bleeding following childbirth. She showed a significant ISTH-BAT score of 12 (reference range: 0-5). The initial work-up showed a normal APTT and PK(INR), but a prolonged PT using the Quick method; the functional fibrinogen levels were shown to be reduced to 0.8 g/L (reference range: 2.0-4.0 g/L). FIBTEM analysis by ROTEM showed absent clot formation. Analysis of the fibrin structure showed normal results. The fibrinogen antigen levels were proportionally reduced in relation to the functional fibrinogen levels, resulting in a normal ratio of 0.96 (normal range: 0.92-1.19). Consequently, the results were consistent with the diagnosis of moderate hypofibrinogenemia [202].

It became evident that the sister, brother and mother of the proband also had a history of bleeding diathesis of varying degrees. The sister, brother and father of the proband were shown to suffer from a mild to moderate hypofibrinogenemia (**Table 3**). Interestingly, the mother of the proband showed the most severe bleeding phenotype of the first-degree relatives but presented with normal fibrinogen levels (**Table 3**).

Table 3. Genetic variants and additional characteristics of the family members
The variant in *FGB* underlying the familial hypofibrinogenemia is highlighted using bold letters. All identified genetic variants are heterozygous; only the *FGB* variant was assessed as significant. Abnormal results are shown in red. An abnormal ISTH-BAT score is defined as >5 in adult females and >3 in adult males. Reference range for fibrinogen: 2.0-4.0 g/L. The agonists associated with abnormal results in FC are shown in brackets. A, ATP; C, CRP-XL; T, TRAP-6; F, female; M, male; N/A, not applicable.

Patient	Age/Sex	ISTH-BAT	Fibrinogen (g/L)	Genetic variants	LTA	FC
Proband	27/F	12	0.8	***FGB*: c.854G>A, p.Arg285His** *FGG*: c.323C>G, p.Ala108Gly *MPL*: c.305G>C, p.Arg102Pro *BLOC1S3*: c.298C>A, p.Pro100Thr	Normal	↓ PAC-1 (A, T)
Sister	25/F	5	0.9	***FGB*: c.854G>A, p.Arg285His** *FGG*: c.323C>G, p.Ala108Gly *MPL*: c.305G>C, p.Arg102Pro *F2RL3*: c.412C>T, p.Arg138Cys *GP6*: c.1550T>G, p.Met517Arg	Normal	Normal
Brother	29/M	4	1.0	***FGB*: c.854G>A, p.Arg285His** *FGG*: c.323C>G, p.Ala108Gly *WAS*: c.1203_1208delACCGCC, p.Pro403_Pro404del	N/A	N/A
Mother	61/F	11	3.0	*ITGA2*: c.2678T>C, p.Ile893Thr *BLOC1S3*: c.298C>A, p.Pro100Thr *GP6*: c.1550T>G, p.Met517Arg *WAS*: c.1203_1208delACCGCC, p.Pro403_Pro404del	Normal	↓ CD62P (C, T) ↓ CD63 (T)
Father	63/M	2	1.2	***FGB*: c.854G>A, p.Arg285His** *FGG*: c.323C>G, p.Ala108Gly *MPL*: c.305G>C, p.Arg102Pro *F2RL3*: c.412C>T, p.Arg138Cys	Normal	Normal

Subsequent genetic screening undertaken in the proband and her first-degree relatives revealed the presence of two heterozygous variants segregating with the hypofibrinogenemia: *FGB*: c.854G>A, p.Arg285His and *FGG*: c.323C>G, p.Ala108Gly (**Table 3**). The *FGG* variant was found to be previously reported in several heterozygous carriers, showing normal fibrinogen levels [333, 334]. The *FGB* variant, however, was previously described in a New Zealand European family, giving rise to symptomatic hypofibrinogenemia in the heterozygous individuals; the resulting variant Bβ chain was absent in the plasma of the patients, functionally confirming the significance of the variant [335]. Moreover, no information on the allele frequency in the general population could be retrieved, indicating that the variant is likely to be very rare. Consequently, the *FGB* variant was regarded as the cause of the hypofibrinogenemia observed in the Swedish family investigated in the present study (**Figure 13**).

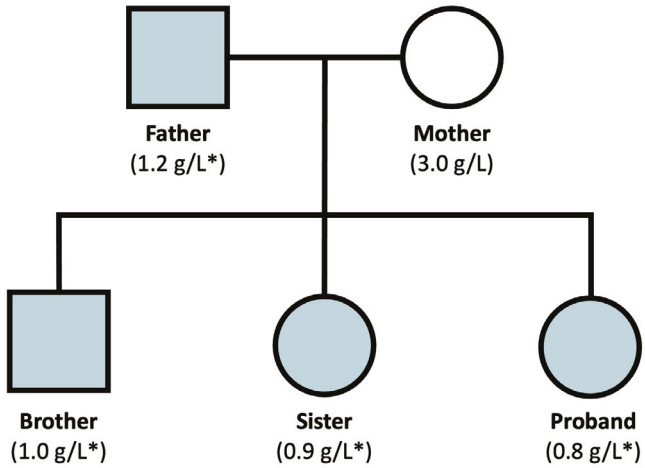

Figure 13. Pedigree chart
The family members shown to be heterozygous for the variant in *FGB* (c.854G>A, p.Arg285His) are indicated by blue pedigree symbols. The functional fibrinogen levels are shown in brackets. The asterisks denote abnormal results.

In hypofibrinogenemia, the severity of the bleeding symptoms is known to correlate well with the levels of fibrinogen [202]. However, there was a notable discrepancy between the bleeding phenotypes presented by the proband and her sister, showing similar fibrinogen levels (**Table 3**). Furthermore, the proband and her normofibrinogenemic mother both presented with a bleeding history clearly distinguishing them from the other family members. Accordingly, the possibility of a concomitant inheritable cause of increased bleeding was considered. Following investigations of platelet function, the proband and the mother showed abnormal results by FC, indicating a platelet dysfunction (**Table 3**). Consequently, the additional heterozygous variants found by genetic screening were reviewed for a possible explanation of the clinical phenotypes. However, except for the *FGB* variant, none of the variants identified in the family were found to be of significance (**Table 3**; Paper II, Table 1). Nevertheless, the unexpectedly severe bleeding phenotype observed in the proband, as well as the phenotype of her mother, were attributed to the platelet dysfunction identified by FC.

The current knowledge of genes associated with inherited bleeding disorder is often not sufficient for fully explaining a clinically significant bleeding diathesis, as illustrated in the proband and her mother. A proportion of the cases presenting with incompletely defined bleeding likely result from variants in genes or non-coding regions currently not known to be associated with bleeding disorders, or from incompletely defined polygenic mechanisms [289]. However, genetic investigations are still of value for identifying causative variants, as in the case of the familial hypofibrinogenemia. Indeed, genetic screening in the presence of coagulation factors deficiencies have been shown to have a relatively good chance of identifying

disease-causing variants [292, 293]. Furthermore, the present study highlights the potential discrepancies between assays of platelet function such as LTA and FC, which is also observed in Paper I. A weak correlation between LTA and FC has previously been shown in patients investigated because of increased bleeding [336]. Even though LTA is regarded as the gold standard method for investigating platelet function, the method lacks sensitivity for mild IPFDs, and FC has been showed to offer an additional value when using the tests complementary [337]. Taken together, the study shows how a combination of genetic screening and functional testing have the potential of contributing to the knowledge of specific variants and their significance, as illustrated by the identified *FGB* variant.

Paper III

In the 31 patients, a total of 28 different variants in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2* were identified; all variants were heterozygous except for a homozygous *COL3A1* variant found in patient P2 (Paper III, Table 1). A total of 51 additional unique heterozygous variants were identified, whereof four were assessed as significant (*VWF*, *MYH9*, *GP1BB* and *FLI1*) (Paper III, Table S2); these variants were not expected to affect the investigations of collagen remodeling and plasma AA levels.

The clinical criteria for EDS were met in 3/31 patients (9.7%): patient P20 was previously diagnosed with hypermobile EDS (hEDS; an EDS subtype of unknown molecular cause), while patients P14 and P28 were found to fulfill the criteria for cEDS when investigated in the present study.

On a group level, the patients were shown to have significantly lower levels of C5M compared with the healthy controls ($p = 0.033$) (**Figure 14 H**). No other significant differences could be demonstrated for the remaining biomarkers PRO-C1, PRO-C3, PRO-C5, C1M, C3M, PRO-C1/C1M, PRO-C3/C3M and PRO-C5/C5M (**Figure 14**). Subgroup analyses investigating patients with variants in specific collagen-related genes and their associated biomarkers (e.g., PRO-C3, C3M and PRO-C3/C3M in patients harboring *COL3A1* variants) was performed. Significantly decreased levels of C5M were found in patients harboring *at least* one *COL5A1* variant ($p = 0.017$); no other significant differences could be observed compared with the healthy controls. The group of 31 patients was investigated for outlier values for each of the biomarkers: it was more common for patients to present as an outlier for a biomarker *not* potentially associated with a harbored variant. Importantly, none of the three patients showing a phenotype consistent with EDS (P14, P20 and P28) were found to be outliers for the investigated biomarkers.

The values of the nine biomarkers were separately investigated for possible correlations with AA levels and ISTH-BAT scores in the patients, without any significant findings.

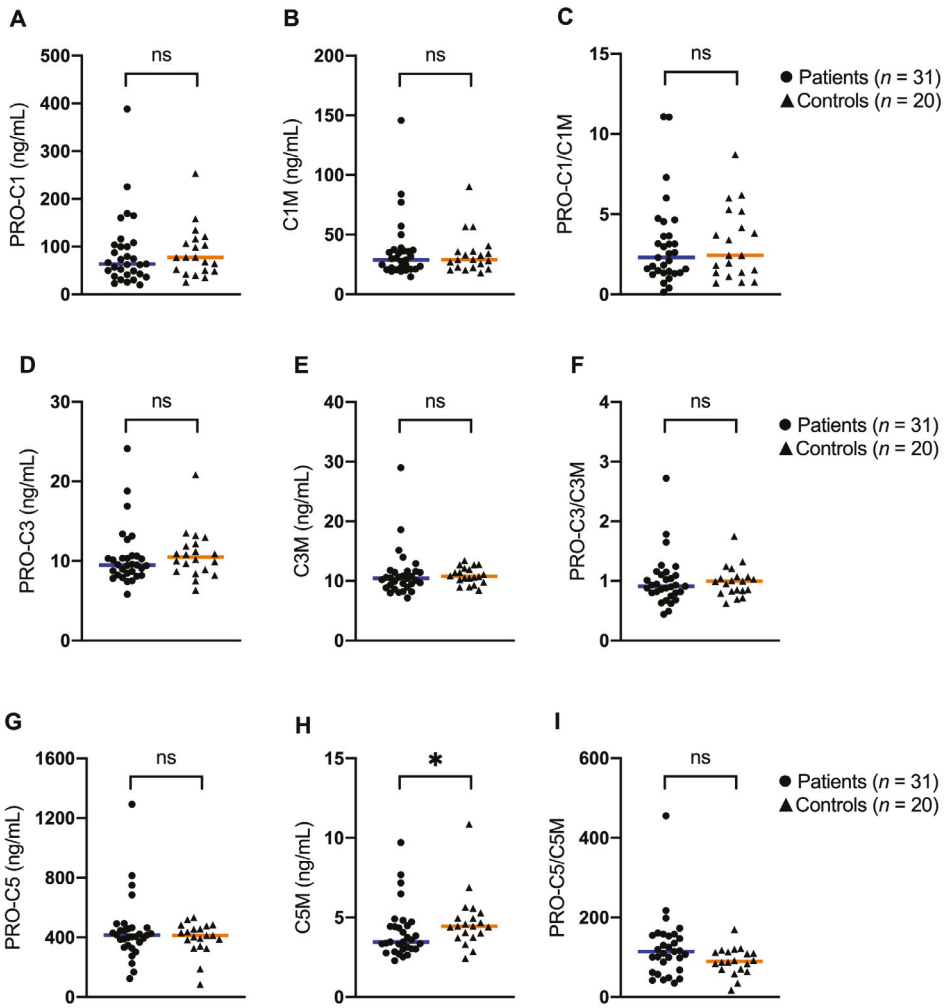

Figure 14. Remodeling of types I, III and V collagen

(A-I) Levels of PRO-C1, PRO-C3, PRO-C5, C1M, C3M and C5M were analysed in patients and healthy controls. Collagen tissue balance was assessed using the following ratios: PRO-C1/C1M, PRO-C3/C3M and PRO-C5/C5M. (H) The patients showed significantly lower levels of C5M ($p = 0.033$). No additional significant differences were found. Median values are shown (blue line, patients; orange line, controls). ns, not significant; *, significant ($p < 0.05$). Reprinted from *Haemophilia* with permission from John Wiley & Sons Ltd (Fager Ferrari M, Zetterberg E, Rossing M, Manon-Jensen T, Pehrsson M, Karsdal MA, Lykkesfeldt J, Leinoe E. Collagen remodelling and plasma ascorbic acid levels in patients suspected of inherited bleeding disorders harbouring germline variants in collagen-related genes. *Haemophilia*. 2021;27(1):e69-e77). © 2020 The Authors.

The plasma levels of AA were found to be decreased in 8/31 patients (26%): 6/31 patients (19%) showed suboptimal AA status, and 2/31 patients (6.5%) had a marginal AA deficiency (**Figure 15**). Of note, both patients with a phenotype consistent with cEDS (P14 and P28) showed suboptimal AA status. A negative correlation between plasma AA levels and ISTH-BAT score was found, that was assessed as significant ($r = -0.42$; $r^2 = 0.17$; $p = 0.020$) (**Figure 15**).

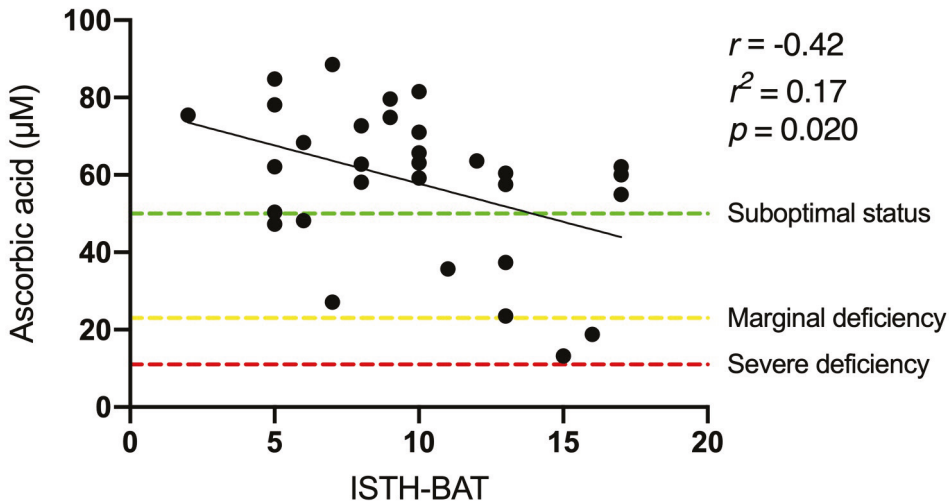

Figure 15. Correlation between plasma levels of ascorbic acid and ISTH-BAT score in patients
A significant negative correlation between plasma levels of AA and ISTH-BAT score was found ($r = -0.42$; $r^2 = 0.17$; $p = 0.020$). In addition, 8/31 patients (26%) showed at least suboptimal AA status. The levels of AA were interpreted as follows: <11 µM: severe deficiency; 11-23 µM: marginal deficiency; 23-50 µM: suboptimal status. AA, ascorbic acid; ISTH-BAT, International Society on Thrombosis and Haemostasis bleeding assessment tool. Reprinted from *Haemophilia* with permission from John Wiley & Sons Ltd (Fager Ferrari M, Zetterberg E, Rossing M, Manon-Jensen T, Pehrsson M, Karsdal MA, Lykkesfeldt J, Leinoe E. Collagen remodelling and plasma ascorbic acid levels in patients suspected of inherited bleeding disorders harbouring germline variants in collagen-related genes. *Haemophilia*. 2021;27(1):e69-e77). © 2020 The Authors.

Regarding the significantly decreased levels of C5M observed in the group of 31 patients, it was not possible to establish a correlation between low levels of C5M and variants in a specific gene. Reduced levels of C5M would theoretically suggest a lower rate of degradation of type V collagen. The implications of isolated reduced C5M levels on a group level are unknown in the present context. Taken together, it was not possible to verify any functional abnormalities resulting from the heterozygous variants in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2*.

The presence of reduced plasma AA levels in 26% of the patients was an interesting finding. Even though no conclusions can be drawn regarding causality, the plasma AA levels were shown to negatively correlate with the ISTH-BAT score, suggesting a more severe of bleeding phenotype in patients with low plasma AA levels.

To our knowledge, this is the first study investigating the significance of variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2* in patients suspected of inherited bleeding disorders by using biomarkers for collagen remodeling. The approach is interesting, since there are no established rapid functional tests for investigating these patients. This type of biomarkers has previously been investigated in conditions such as systemic sclerosis and axial spondyloarthritis and have been suggested to be of value for disease monitoring and diagnostics [338, 339]. However, the biomarkers have not been systematically investigated in EDS patients, harboring disease-causing variants in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2*. Interestingly, the patients presenting with a clinical phenotype consistent with EDS could not be distinguished from the other patients using the biomarkers for collagen remodeling. Thus, the usefulness of the biomarkers used in this study remains unknown in patients with variants in *COL1A1*, *COL3A1*, *COL5A1* and *COL5A2*. Limitations of the study include the relatively low number of included patients. Moreover, the assessment of variants in specific collagen-encoding genes was hampered by the fact that many of the patients harbored multiple variants in different collagen-encoding genes (Paper III, Table 1). Another limitation was that that the plasma AA levels were never assessed in the healthy controls, which theoretically could have been a factor affecting the comparison of biomarker levels in patients and healthy controls. However, the effects of the plasma AA levels on the biomarkers for collagen remodeling used in the present study are unknown.

For future studies, it would be of interest to evaluate the biomarkers for collagen remodeling in a cohort of patients with confirmed cEDS or vEDS, in order to assess their performance when clearly deleterious variants in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2* are present. The same is true for the plasma AA levels, which also would be interesting to investigate in a larger cohort of patients with incompletely explained bleeding diathesis.

Paper IV

A now 17-year-old boy (the proband) was referred because of a severe congenital macrothrombocytopenia. Since birth, he had consistently been showing platelet counts <5 to 10 x 10^9/L, resulting in multiple spontaneous and periprocedural bleeding episodes. Despite extensive investigations over the years, no cause of the thrombocytopenia had been identified. In 2015, WES was performed, showing two VUSs (*FLNA* and *SLFN14*) that were assessed as nonsignificant following functional investigations. Whole genome sequencing was subsequently performed, and in 2019, two previously undescribed heterozygous variants in *GNE* were identified in the proband (c.416_426del, p.Ile139Argfs*4 and c.1352G>A, p.Arg451Gln) (**Figure 16**). The mother was heterozygous for the p.Ile139Argfs*4 variant, while the father was heterozygous for the p.Arg451Gln variant. The brother

of the proband was heterozygous for the paternal p.Arg451Gln variant. The first-degree relatives of the proband were all healthy and showed normal platelet counts.

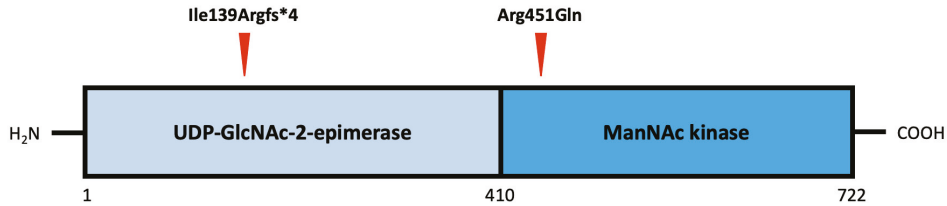

Figure 16. *GNE* variants identified in the proband
Linear domain organization of UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (also known as the GNE enzyme) encoded by *GNE*. The approximate positions of the variants identified in the proband are shown.

In the compound heterozygous proband, the levels of sialic acid on platelets and leukocytes were markedly reduced compared with healthy controls when assessed by both SNA and MAL II binding (**Figure 17 A-D**; Paper IV, Figure 3). Moreover, the binding of FH to platelets and leukocytes was clearly reduced, consistent with the reduced sialic acid levels (**Figure 17 E-H**). The heterozygous parents did not show any evidence of sialylation defects or systematically reduced FH binding (**Figure 17**). The erythrocytes of the proband showed a high rate of lysis compared with erythrocytes from healthy controls, suggesting an increased complement-mediated lysis due to decreased FH binding (Paper IV, Figure 5). Accordingly, the results were highly indicative of the variants in *GNE* causing insufficient sialylation of platelets, leukocytes and erythrocytes, when present in a compound heterozygous state. Furthermore, the immature platelet fraction was observed to be significantly elevated, varying between 45% to 72% (reference range: 1.1-6.1%), supporting the hypothesized mechanism of increased platelet clearance secondary to low platelet sialic acid levels. Indeed, a high immature platelet fraction has previously been reported in patients with *GNE*-associated thrombocytopenia [171, 172].

In an attempt to increase the platelet sialic acid levels and hence the platelet counts in the proband, treatment with oseltamivir was initiated. After 17 days of treatment, no clinically significant increase in platelet counts could be observed (**Figure 18**). After 18 days of treatment, the levels of platelet sialic acid were reassessed by FC: the platelet sialic acid levels were, however, persistently decreased (**Figure 19**). The treatment was terminated following the reassessment by FC.

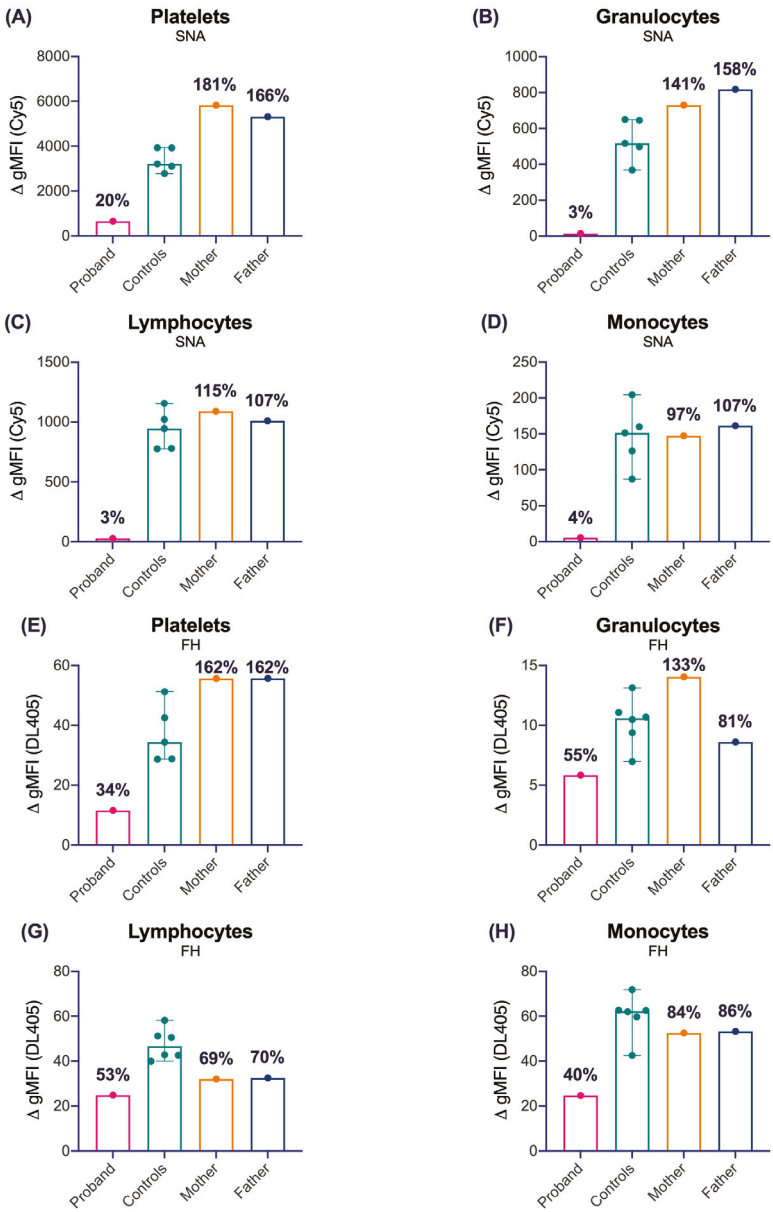

Figure 17. Sialylation and complement factor H binding in platelets and leukocytes

(A-D) A markedly decreased sialylation of platelets, granulocytes, lymphocytes and monocytes was demonstrated in the proband compared with healthy controls. The heterozygous parents of the proband did not show reduced levels of sialic acid on the investigated cell types. (E-H) A decreased binding of FH to platelets, granulocytes, lymphocytes and monocytes was demonstrated in the proband compared with healthy controls. The heterozygous parents showed normal binding of FH to platelets, granulocytes and monocytes; FH binding to lymphocytes was however slightly reduced compared with healthy controls. The results of the healthy controls (n = 5-6) are shown as medians with min-max. The results in the proband and the parents are presented as percentages of the median Δ gMFI in the control group. SNA, Sambucus nigra lectin; FH, complement factor H; gMFI, geometric mean fluorescence intensity.

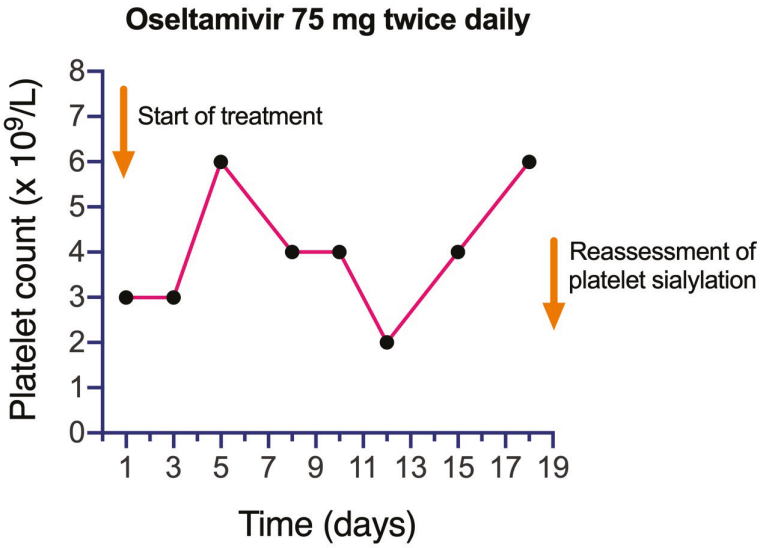

Figure 18. Platelet counts in the proband during treatment with oseltamivir
The platelet counts in the proband were monitored during treatment with oseltamivir 75 mg twice daily. No clinically significant increase in platelet counts was observed after 17 days of treatment. Platelet sialylation was reassessed by flow cytometry after 18 days of treatment.

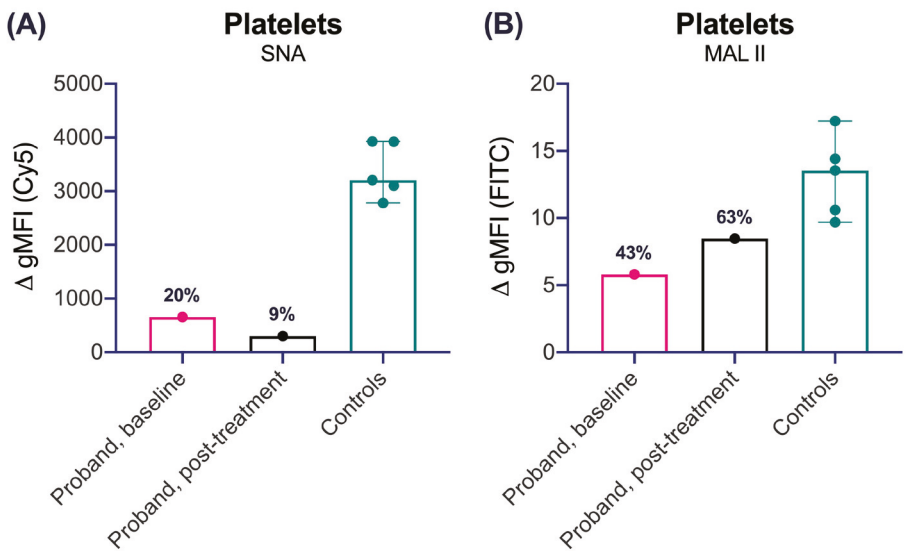

Figure 19. Sialylation of platelets after treatment with oseltamivir
(A, B) A persistently decreased sialylation of platelets was observed in the proband following 18 days of treatment with oseltamivir. The results of the healthy controls (n = 5) are shown as medians with min-max. The results in the proband are presented as percentages of the median Δ gMFI in the control group. SNA, Sambucus nigra lectin; MAL II, Maackia amurensis lectin II; gMFI, geometric mean fluorescence intensity.

Taken together, the study shows evidence of the identified *GNE* variants being causative of the congenital macrothrombocytopenia in the proband. Accordingly, decreased sialylation was observed in platelets and leukocytes, while hemolytic assays showed indirect signs of decreased sialylation. The variants seem to be associated with a preserved sialylation in the heterozygous parents, which is in line with the AR inheritance pattern previously described for *GNE*-associated thrombocytopenia [171, 172]. As previously mentioned, biallelic variants in *GNE* are further associated with GNE myopathy. The proband did only present with an isolated thrombocytopenia, showing no signs of myopathy. However, the disorder is known to commonly manifest at an age between 20 and 40 years [340]. Hence, in theory, the patient could be at risk for developing GNE myopathy in the future.

To our knowledge, the present study is the first to evaluate the use of the sialidase inhibitor oseltamivir in *GNE*-associated thrombocytopenia. It was hypothesized that the platelet sialic acid levels could be increased by the inhibition of endogenous sialidases, based on previous findings (discussed in the methods section). Regrettably, the proband did not show a clinically significant response regarding the platelet counts, and the effects of oseltamivir were insufficient for increasing the already low levels of platelet sialic acid. This could possibly be due to a severely impaired sialic acid biosynthesis at baseline, where endogenous sialidases play a minor role in the contribution to the low levels of platelet sialic acid. Moreover, at therapeutic concentrations, oseltamivir has previously been shown to have a rather scarce effect on endogenous sialidases *in vitro* [341]. Therefore, it could also be hypothesized that a limited effect of oseltamivir *in vivo* contributed to the failure of the treatment. Isolated thrombocytopenia caused by variants in *GNE* is a very rare condition, to our knowledge only described in four previous publications [171-174]. Currently, no established specific treatment exists for *GNE*-associated thrombocytopenia. However, therapies for GNE myopathy targeting the underlying defect in sialic acid biosynthesis are under development [340, 342]. Hence, an approved therapy for GNE myopathy could theoretically be an option for treating isolated *GNE*-associated thrombocytopenia in the future.

Conclusions and future perspectives

In this thesis, a subset of genetic variants identified by genetic screening in patients with incompletely explained bleeding diathesis have been investigated. Various functional methods have been applied in order to assess the significance of the variants; in the context of establishing a suspected diagnosis or for examining potentially novel explanations for familial bleeding diathesis. The main conclusions from the papers included in this thesis are as follows:

- Heterozygous variants in *UNC13D*, *STX11* and *STXBP2* seem to be associated with an impaired platelet degranulation, that could possibly contribute to increased bleeding in affected patients (Paper I).
- The variant *FGB*: c.854G>A, p.Arg285His causes familial hypofibrinogenemia in a heterozygous state (Paper II).
- Biomarkers specific for collagen formation and degradation were not able to identify any abnormalities attributable to heterozygous VUSs in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2*, harbored by patients suffering from incompletely explained bleeding. Consequently, no association between the variants and increased bleeding could be established by functional methods (Paper III).
- A negative correlation between plasma AA levels and ISTH-BAT score was observed in patients with incompletely explained bleeding tendencies harboring heterozygous VUSs in *COL1A1*, *COL3A1*, *COL5A1* or *COL5A2*, suggesting a more severe bleeding phenotype in the patients with low plasma AA levels (Paper III).
- The previously undescribed variants *GNE*: c.416_426del, p.Ile139Argfs*4 and *GNE*: c.1352G>A, p.Arg451Gln are causative of a severe congenital macrothrombocytopenia when harbored in a compound heterozygous state, resulting from decreased levels of platelet sialic acid (Paper IV).
- Treatment with the sialidase inhibitor oseltamivir do not seem to increase the platelet counts or the levels of platelet sialic acid in *GNE*-associated thrombocytopenia (Paper IV).

Although genetic screening currently seems quite useful for providing a molecular diagnosis in a subset of patients (e.g., patients suspected of ITs or heritable coagulation factor deficiencies), a high proportion of patients suspected of inherited bleeding disorders still never receive a conclusive diagnosis following genetic investigations. There are probably many genes and non-coding regions left to be identified as affected in inherited bleeding disorders [289, 302, 343]. There is also the theoretical possibility of underlying polygenic mechanisms that remains to be unveiled [265]. Furthermore, even when performing genetic screening targeting genes known to be affected in inherited bleeding disorders, the significance of the identified variants is not always obvious [289, 294, 295], as evident by the studies conducted in the context of the present PhD project. Initiatives for increasing the possibilities of variant annotation and international data sharing are important strategies for facilitating the interpretation of VUSs, including the reclassification of variants over time [289, 302, 343]. Moreover, functional studies remain an essential approach for further examining the consequences of VUSs [302]. In this thesis, the significance of specific variants in genes associated with inherited bleeding disorders have been investigated in an attempt to increase the knowledge regarding their potential importance. The results of this thesis contribute to the continuously increasing knowledge of genetic variants associated with inherited bleeding disorders, required for adequately diagnosing a higher proportion of patients suffering from constitutionally increased bleeding.

Populärvetenskaplig sammanfattning

Medfödda blödningsrubbningar utgör en heterogen grupp av ärftliga sjukdomar som påverkar kroppens förmåga att åstadkomma en normal blodkoagulering (levring av blodet). För en normal blodkoagulering måste blodet bl.a. innehålla ett normalt antal blodplättar med normal funktion, samt normala koncentrationer av ett flertal olika proteiner (koagulationsfaktorer) som samverkar för att stilla en uppkommen blödning. Därutöver är normala och mekaniskt stabila blodkärl viktiga för att hålla kvar blodet i cirkulationen vid ett visst mått av yttre påfrestningar. Medfödda blödningsrubbningar kan översiktligt indelas i tillstånd som ger upphov till brist på blodplättar, försämrad blodplättsfunktion, brist på en specifik koagulationsfaktor eller försvagade blodkärl. Varje enskild medfödd blödningsrubbning som man idag känner till orsakas vanligen av en eller flera skadliga mutationer i en särskild gen. Vissa av dessa sjukdomar kan relativt enkelt diagnostiseras med hjälp av analysmetoder som direkt kan påvisa t.ex. en koagulationsfaktorbrist. Sjukdomarna kan ofta i ett senare skede bekräftas med en riktad genetisk analys, men detta krävs inte alltid för att en diagnos ska kunna ställas. Det är dock relativt vanligt att man inte kan finna någon specifik förklaring till blödningssymtomen hos patienter där det föreligger en stark misstanke om en ärftlig blödningssjukdom. Den snabba tekniska utvecklingen gällande genetiska analysmetoder har förbättrat möjligheterna till att utföra genetiska undersökningar vid misstanke om medfödda blödningsrubbningar som ter sig svårdiagnosticerade. Ett vanligt tillvägagångssätt är att man letar efter medfödda sjukdomsorsakande mutationer i ett antal utvalda gener. Vanligen undersöks gener som man med stor säkerhet vet är påverkade vid olika blödningssjukdomar, men man kan också undersöka gener som har en något mer osäker koppling till ökade blödningssymtom för att hitta nya förklaringar. Om en mutation påvisas i en relevant gen så måste även betydelsen av denna specifika mutation värderas: vissa specifika mutationer orsakar sjukdom medan andra inte har någon sjukdomsorsakande effekt. En annan viktig faktor att ta hänsyn till gällande genetiska sjukdomar är arvsmönstret. De flesta generna hos en individ finns i dubbel uppsättning, där den ena uppsättningen har nedärvts från mamman och den andra från pappan. En person kan alltså ha mutationer i antingen en eller båda kopiorna av en specifik gen. Vissa sjukdomar förutsätter att båda genkopiorna är skadade av mutationer (förekomst av en homozygot mutation eller sammansatt heterozygota mutationer), medan det räcker att enbart en genkopia är skadad (förekomst av en heterozygot mutation) vid andra typer av sjukdomstillstånd. Genetiska analysmetoder kan i flera fall påvisa välkända specifika mutationer som man med

stor säkerhet vet är sjukdomsorsakande. Dock påvisas ofta en mängd mutationer som man inte riktigt vet betydelsen av.

I denna avhandling undersöks betydelsen av mutationer i ett antal utvalda grupper av gener, som påvisats hos patienter som misstänks lida av medfödda blödningsrubbningar men inte kunnat få någon fullständig förklaring till sina besvär.

I **Artikel I** undersöktes betydelsen av heterozygota mutationer i generna *UNC13D*, *STX11* och *STXBP2*. Sjukdomen familjär hemofagocyterande lymfohistiocytos orsakas vanligen av mutationer som skadar båda kopiorna av någon av dessa gener, alltså t.ex. en homozygot mutation. Denna sjukdom debuterar vanligen under de första levnadsåren och karaktäriseras av ett okontrollerat, livshotande inflammatoriskt tillstånd. Sjukdom uppstår då mutationerna hindrar vissa typer av vita blodkroppar från att släppa ut viktiga ämnen som finns lagrade i strukturer som visuellt liknar små korn (granulae). Hos patienter med familjär hemofagocyterande lymfohistiocytos har man tidigare visat att mutationerna även påverkar blodplättarnas förmåga att släppa ut sina granulae, innehållande ämnen som krävs för en normal blodkoagulering. Det rör sig således om en defekt i maskineriet som ser till att både vissa typer av vita blodkroppar och blodplättar kan släppa ut sina granulae på ett normalt sätt. Mot bakgrunden av detta ville vi undersöka om en heterozygot mutation i någon av dessa gener är tillräckligt för att orsaka blodplättsdefekter som leder till blödningsbenägenhet. Tre olika analysmetoder användes för att undersöka blodplättar från patienter med huvudsakligen heterozygota mutationer i någon av generna *UNC13D*, *STX11* och *STXBP2*. Majoriteten (67%) av dessa patienter hade blodplättar som visade tecken till nedsatt förmåga att släppa ut granulae, vilket skulle kunna tala för att heterozygota mutationer i dessa gener bidrar till en medfödd blödningsbenägenhet. Det krävs dock ytterligare studier för att slutgiltigt bevisa ett sådant orsakssamband.

I **Artikel II** undersöktes den underliggande orsaken till en brist på koagulationsfaktorn fibrinogen som påvisats hos individer i en svensk familj. Utredningen visade att familjemedlemmarna med brist på fibrinogen hade en mycket ovanlig heterozygot mutation i genen *FGB* som låg till grund för tillståndet. Så vitt vi vet har denna specifika mutation endast beskrivits en gång tidigare, och då i en familj på Nya Zeeland.

I **Artikel III** undersöktes betydelsen av mutationer i gener som är centrala för kroppens produktion av kollagen. Kollagen är ett protein som bidrar till stabilitet i kroppens bindväv, en mycket utbredd typ av vävnad som bl.a. finns i hud, kärlväggar och senor. Man vet sedan tidigare att den ärftliga bindvävssjukdomen Ehlers-Danlos syndrom ger upphov ökad blödningsbenägenhet, i tillägg till typiska symtom som exempelvis töjbar hud och överrörliga leder. Ehlers-Danlos syndrom beror på kollagendefekter som bl.a. orsakas av heterozygota mutationer i generna *COL1A1*, *COL3A1*, *COL5A1* och *COL5A2*. Det visade sig att anmärkningsvärt många av våra patienter med misstänkta medfödda blödningsrubbningar hade

heterozygota mutationer i minst en av dessa gener. För att undersöka betydelsen av dessa mutationer utfördes analyser som specifikt mäter produktionen och nedbrytningen av det kollagen som bildas med hjälp av dessa gener. Eftersom C-vitamin har betydelse för kroppens produktion av kollagen undersöktes även nivåerna av C-vitamin i blodet hos patienterna. Kollagenanalyserna kunde inte visa på någon koppling mellan mutationerna och en påverkad kollagenomsättning. Däremot påvisades ett intressant samband mellan låga nivåer av C-vitamin i blodet och mer uttalade blödningssymtom.

I **Artikel IV** undersöktes orsaken till ett extremt lågt antal blodplättar sedan födseln hos en nu 17-årig pojke. Redan vid förlossningen drabbades han av en hjärnblödning och har därefter uppvisat många olika typer av blödningssymtom. Genom åren har flertalet utredningar gjorts för att försöka finna orsaken till blodplättsbristen, dock utan framgång. Med hjälp av genetiska analyser visade vi tillslut att patienten var sammansatt heterozygot för två sannolikt mycket ovanliga mutationer i genen *GNE*. Denna gen är avgörande för kroppens produktion av sialinsyra. Sialinsyra behövs på trombocyternas yta för att säkerställa en normal livslängd hos trombocyterna i cirkulationen. Analyserna som utfördes visade tydligt hur patienten hade mycket låga nivåer av sialinsyra på sina blodplättar. Således kunde mutationerna i genen *GNE* fastställas som orsaken till patientens låga antal blodplättar. Ett behandlingsförsök gjordes med influensaläkemedlet Tamiflu, då det fanns en teoretisk grund för att denna behandling eventuellt skulle kunna öka nivåerna av sialinsyra på blodplättarna och på så vis öka antalet blodplättar i cirkulationen. Ingen tydlig effekt av behandlingen kunde dock observeras. Ett lågt antal blodplättar orsakat av mutationer i genen *GNE* har tidigare endast beskrivits ett fåtal gånger. Således utgör studien en karaktärisering av ett mycket ovanligt tillstånd, där även behandling med Tamiflu utvärderats för första gången.

Sammanfattningsvis bidrar denna avhandling till den samlade kunskapen om genetiska orsaker till medfödda blödningsrubbningar. Kunskap om specifika mutationers betydelse är en förutsättning för att genetisk diagnostik ska kunna öka andelen av patienter som erhåller en korrekt diagnos i framtiden.

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