Geke Poolen

Shaping the Thrombotic Cascade

From clinical risk prediction to targeted modulation of coagulation

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ISBN: 978-94-6537-772-8
DOI: https://doi.org/10.33540/3572

Printing: Ridderprint, ridderprint.nl
Layout and design: W. Aalberts, persoonlijkproefschrift.nl

The research described in this thesis was conducted at the University Medical Center Utrecht and at Synapse Research Institute.

Financial support for printing of this thesis was kindly provided by Federatie van Nederlandse Trombosediensten and Stago BNL.

Shaping the thrombotic cascade

From clinical risk prediction to targeted modulation of coagulation

De stollingscascade als aangrijpingspunt

Van klinische risicovoorspelling tot gerichte modulatie van de bloedstolling
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. ir. W. Hazeleger,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op

woensdag 2 september 2026 des middags te 2.15 uur

door

Gerrie Carina Poolen

geboren op 9 juli 1997
te Nijkerk

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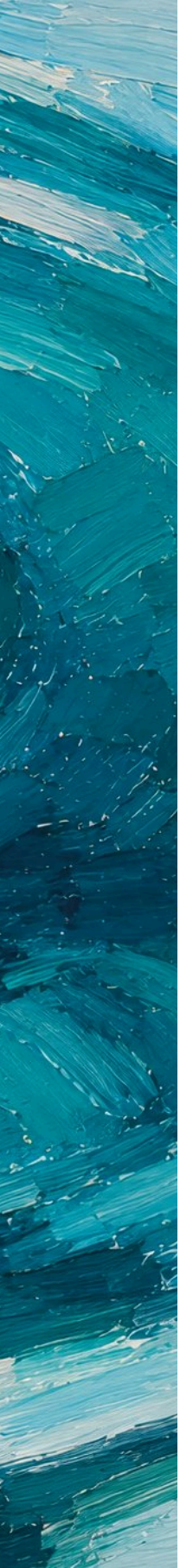
Everyone who drinks this water will be thirsty
again, but whoever drinks the water I give them
will never thirst. Indeed, the water I give them will
become in them a spring of water welling up...

John 4:13–14 (NIV)

TABLE OF CONTENTS



CHAPTER 1

General Introduction

GENERAL INTRODUCTION

The hemostatic balance

The hemostatic system plays a crucial role in maintaining vascular integrity by balancing clot formation and resolution (1). Upon vascular injury, platelets and coagulation factors rapidly interact to form a stable clot that prevents excessive bleeding. Simultaneously, natural anticoagulant mechanisms ensure that clotting remains localized and help avoid pathological thrombosis (2). This dynamic balance between procoagulant and anticoagulant forces is essential for survival.

Hemostasis is classically divided into three phases (**Figure 1**):

- **Primary hemostasis** centers around platelets. Following vascular injury, platelets are rapidly recruited, become activated, and secrete the contents of their storage granules, including platelet factor 4 (PF4/CXCL4) and beta-thromboglobulin (β-TG/CXCL7). They then adhere to the damaged vessel wall via collagen and von Willebrand Factor (VWF) and aggregate to form a platelet plug that temporarily seals the breach and prevents further blood loss (3).

- **Secondary hemostasis** involves activation of the coagulation cascade, a series of complex reactions between clotting factors that ultimately leads to the formation of thrombin (4, 5). Thrombin then converts fibrinogen into fibrin, and the resulting fibrin fibers stabilize the platelet plug.

- **Fibrinolysis** – also referred to as tertiary hemostasis – is the controlled degradation of the clot once vessel integrity has been restored.

Clot formation and resolution are tightly regulated. Natural anticoagulants such as antithrombin, protein C, Protein S and thrombomodulin inhibit coagulation, while the fibrinolytic system degrades fibrin clots (6). In addition, the endothelium is crucial for inhibiting platelet activation through surface expression of ecto-ADPase and the release of prostacyclin (PGI_2) and nitric oxide (NO) (7, 8). These regulatory mechanisms act in concert with procoagulant processes to ensure that clots form when needed and resolve once their function is fulfilled.

Dysregulation of this balance can lead to either bleeding or thrombosis. A detailed understanding of these regulatory mechanisms is essential for improving the diagnosis and management of coagulation disorders.

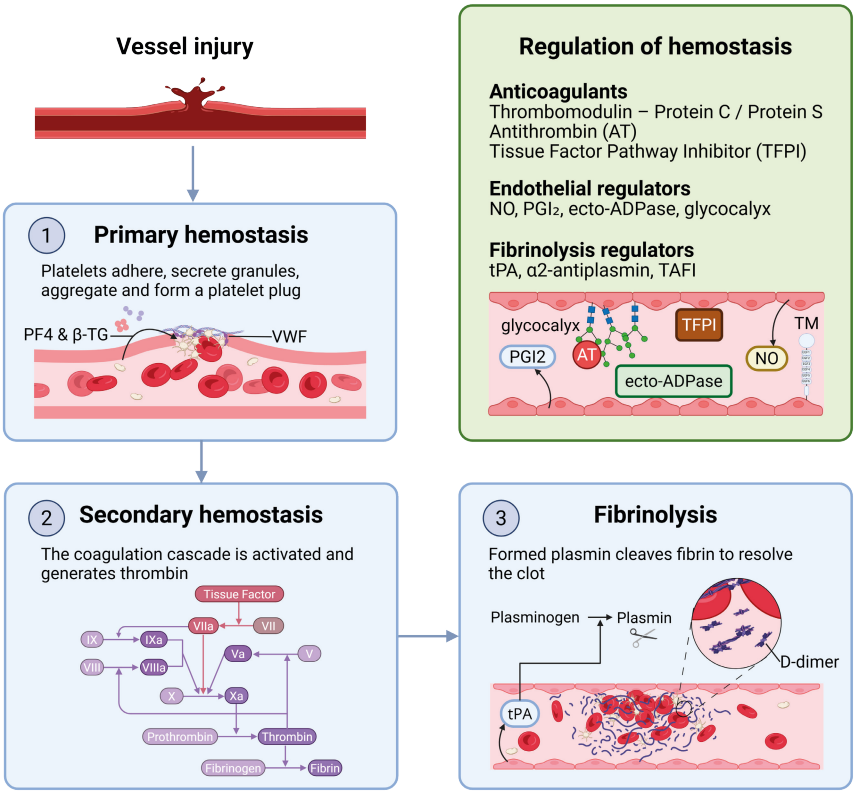


Figure 1. Schematic overview of hemostasis

Hemostasis consists of three phases initiated by vascular injury: (1) primary hemostasis, during which platelets adhere to the damaged vessel wall (via von Willebrand factor [VWF] and collagen), secrete their granules (containing PF4 and β-TG), aggregate, and form a platelet plug; (2) secondary hemostasis, in which the coagulation cascade is activated, generating thrombin that converts fibrinogen into fibrin, stabilizing the clot; and (3) fibrinolysis, which resolves the clot through plasmin-mediated fibrin degradation. Natural regulatory mechanisms, including anticoagulants (antithrombin [AT], tissue factor pathway inhibitor [TFPI], the protein C/thrombomodulin/protein S system), fibrinolysis regulators (thrombomodulin-activatable fibrinolysis inhibitor [TAFI], tPA, α2-antiplasmin), and endothelial-regulators (platelet inhibitors: PGI_2, NO, ecto-ADPase, and the glycocalyx), help maintain hemostatic balance and prevent pathological thrombosis. Fibrin breakdown products such as d-Dimer are clinically used to detect thrombosis and monitor fibrinolysis. Figure created with BioRender.com

Clinical relevance: Thrombosis and its complications

Disruption of the hemostatic balance can lead to excessive clotting, of which venous thromboembolism (VTE) is a frequent clinical manifestation. VTE encompasses deep vein thrombosis (DVT), in which clots form in the deep veins of the legs, and pulmonary embolism (PE), which typically results from embolization of a DVT-derived clot to the lungs. In rare cases, PE may also arise *in situ* within the pulmonary vasculature. VTE affects approximately 1 in 12 people, with around 30,000 new cases annually in the Netherlands (9-11).

DVT often presents with leg pain and swelling but may progress silently. A dislodged clot can travel to the lungs and cause PE, a life-threatening complication. PE can acutely impair oxygenation and carries a mortality rate of approximately 10% despite treatment (12, 13). VTE is now recognized not only as an acute event but also a chronic and recurrent condition. Up to 60% of DVT patients develop post-thrombotic syndrome (PTS), characterized by persistent pain, edema, and in severe cases, venous ulcers (14). Similarly, PE survivors may develop chronic thromboembolic pulmonary hypertension (CTEPH), a debilitating condition with progressive respiratory and cardiovascular impairment (15-17).

VTE is commonly classified as provoked or unprovoked, based on the presence of transient or persistent risk factors, such as recent surgery, trauma, immobilization, active cancer, hormone therapy (e.g. oral contraceptives or hormone replacement therapy), pregnancy or inherited thrombophilia (18). In contrast, unprovoked VTE, which accounts for approximately one-third of all cases, occurs without an identifiable trigger (19). This classification has important clinical implications: unprovoked VTE is associated with a higher risk of recurrence compared with provoked VTE (20, 21). Accordingly, treatment guidelines recommend limited-duration anticoagulation for provoked VTE, whereas indefinite anticoagulation is often considered for unprovoked cases (22).

Thrombosis may also occur in hematologic disorders such as immune thrombocytopenia (ITP). Although ITP is characterized by low platelet counts and bleeding risk, it paradoxically seems to also increase thrombotic risk, especially in patients treated with thrombopoietin receptor agonists such as eltrombopag (23, 24). The underlying mechanisms remain incompletely understood but may involve altered platelet function, endothelial activation, and an imbalance in coagulation regulation.

Recurrence is a major challenge in VTE management. Approximately 10% of patients experience a new thrombotic event within the first year after discontinuing anticoagulation, increasing to 25% within five years (25). Anticoagulation remains the cornerstone of treatment and prevention and is typically prescribed for 3–6 months following a first event (22, 26-28). While prolonged treatment reduces the risk of recurrence, it also increases the risk of major bleeding, which may carry a higher case fatality rate (29-31). Therefore, careful risk stratification is crucial to balance the benefits and harms of extended therapy.

To improve individualized care in VTE, there is a need for accurate prediction models that can identify patients at high risk of recurrence. Such models can help avoid unnecessary extended anticoagulation in low-risk patients, thereby reducing bleeding complications, while ensuring that high-risk patients receive adequate long-term therapy to prevent recurrence. This is particularly relevant for patients with unprovoked VTE, who might otherwise receive indefinite anticoagulation by default. Enhancing risk stratification is essential to optimize long-term outcomes through personalized anticoagulation strategies.

Challenges in risk stratification and prediction

Several prediction models have been developed, some of which are currently used in clinical practice to assess the risk of recurrence in individuals with VTE. The primary goal of these models is to identify patients who may safely discontinue anticoagulation and, conversely, to also identify those who may benefit from extended anticoagulation therapy. They typically incorporate clinical factors such as age, sex, body mass index (BMI), history of VTE, and certain comorbidities. Well-known examples include the DASH score (32), the D-dimer model (33), HERDOO2 (34, 35), L-TRRiP (36), the Vienna Prediction Model (VPM) (37-39) and VTE-Predict (40).

While these prediction models provide a practical and easily applicable framework for risk assessment, their predictive accuracy remains limited (41, 42). They often lack sufficient specificity and generalizability, leading to both over- and undertreatment. For example, in the randomized VISTA trial of 883 patients with unprovoked VTE, treatment duration based on the VPM did not reduce overall recurrence risk compared to usual care. However, it demonstrated good discrimination (c-statistic 0.76) and may safely support discontinuation of anticoagulation in patients with low predicted risk (39). Similarly, the HERDOO2 rule prospectively identified low-risk women with a recurrence rate of about 3% per year after stopping treatment, though it is not applicable to men and misses some high-risk individuals (35). Other models like DASH and LTRRiP have only modest discrimination (c-statistics ~0.6–0.65), and none have been definitively shown to improve clinical outcomes when used in practice (43-45). Overall, while these models can help refine risk stratification, their limited accuracy underscores the need for better tools to support individualized decision-making.

Although predictive models do not require a mechanistic understanding to be effective, the limited accuracy of current tools for predicting VTE recurrence suggests that integrating biological markers could enhance performance (46). These markers provide additional layers of information by capturing biological variation that clinical variables may overlook. However, it is important to emphasize that the predictive value of such biomarkers does not imply a causal relationship (47). Their utility lies in improving individual risk stratification, rather than advancing mechanistic understanding.

Functional assays and biomarkers in coagulation

Biomarkers, broadly defined as measurable biological indicators that distinguish individuals with differing risk of recurrence, may include plasma protein concentrations, cellular markers, and functional assays such as thrombin generation (47). These tools hold promise for enhancing individualized thrombotic risk prediction (48, 49). A classical approach has focused on measuring protein levels in plasma, a strategy widely used in recurrence prediction. However, despite many promising associations, relatively few biomarkers have been translated into routine clinical practice, due to inconsistent findings across studies and the substantial effort required for clinical validation.

Notably, certain biomarkers, such as D-dimer and Factor VIII, which reflect underlying coagulation activity, are already incorporated into certain prediction models and have been shown to improve their discriminatory power (50, 51). These established examples illustrate the practical value of integrating biologically informative markers into clinical algorithms.

Other potential biomarkers may include additional coagulation factors, as well as markers that reflect other components of Virchow's triad, the classical framework explaining thrombosis (46, 52). For example, endothelial proteins shed from inflamed or dysfunctional endothelium, such as soluble thrombomodulin or von Willebrand Factor, and platelet proteins, such as PF4/CXCL4 or β-TG/CXCL7, released during platelet activation in hypercoagulable states, may serve as markers of vascular injury and thrombogenic potential. Additionally, inflammatory proteins such as C-reactive protein may reflect underlying pathological processes and serve as biomarker of thrombotic risk.

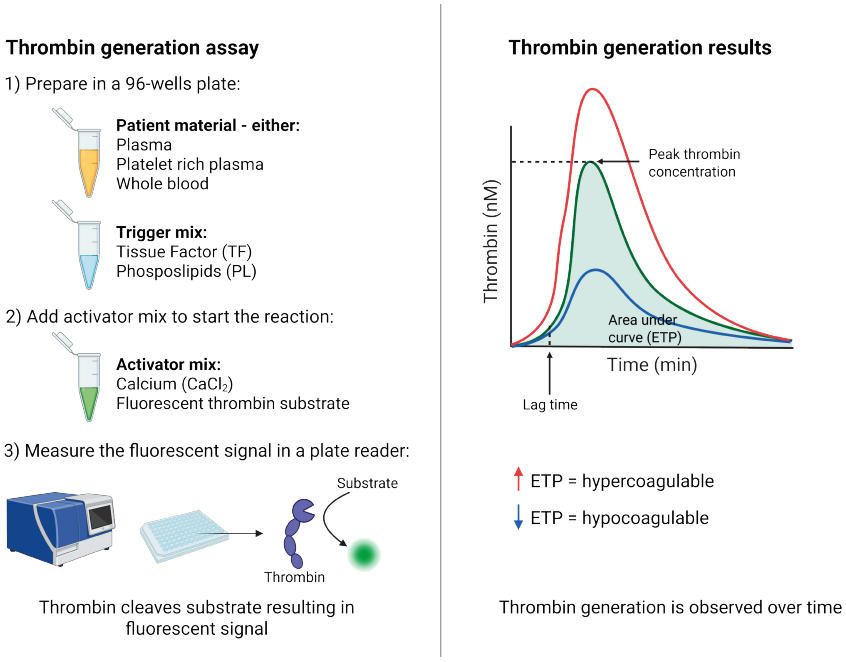


Figure 2. Thrombin generation assay and interpretation
Thrombin generation is measured by mixing patient plasma (or whole blood) with a trigger solution containing Tissue Factor (TF) and phospholipids (PL), and an activator solution containing calcium and a fluorescent substrate. A calibrator sample without TF and PL is run alongside each patient sample for baseline correction. Thrombin produced in the sample cleaves the fluorescent substrate, generating a real-time signal that produces a thrombin generation curve. This curve reflects the balance between procoagulant and anticoagulant factors. An increased endogenous thrombin potential (ETP) indicates a hypercoagulable state, while a decreased ETP suggests an increased risk of bleeding. Figure created with BioRender.com

Unbiased mass spectrometry and other high-throughput techniques enable the simultaneous measurement of numerous proteins, providing a comprehensive view of the circulating proteome (53, 54). These approaches not only allow the quantification of established or suspected biomarkers but also facilitate the discovery of novel or unexpected biomarkers.

Another promising domain of biomarkers is functional assays, which capture more than just the concentration of individual proteins. A well-established example in coagulation research is the thrombin generation assay (55, 56). This assay quantifies in vitro thrombin formation over time. Depending on the context, it can be performed in platelet-poor plasma, platelet-rich plasma, or whole blood (57). It can be further modified using different activating reagents or by adding anticoagulant proteins such as activated protein C (APC) or thrombomodulin. The assay provides parameters potentially associated with recurrence risk, such as endogenous thrombin potential (ETP) and lag time. For instance, patients with bleeding disorders often have low ETP, while elevated ETP may be linked to an increased thrombotic tendency (58, 59).

Unlike measuring the concentration of a single or multiple protein(s), thrombin generation assesses the dynamic process of coagulation, potentially providing a more comprehensive picture of a patient's hemostatic balance. Thrombin generation can further be divided into two phases: prothrombin conversion and thrombin inactivation. This analytical method is known as thrombin dynamics (60-62).

Together, these methods hold promise for identifying biomarkers of VTE recurrence and improving risk prediction in affected patients.

Modulation of thrombomodulin: a key coagulation protein

Thrombomodulin plays a central role among the molecular regulators of thrombin generation in vivo. Its unique diagnostic and therapeutic potential make it a compelling candidate for further investigation. Thrombomodulin is a membrane protein primarily expressed on endothelial cells (63, 64). It modulates coagulation by binding thrombin, which then alters thrombin's substrate specificity to activate two key regulatory proteins: protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) (**Figure 3**). Activated protein C (APC) inactivates Factors Va and VIIIa, serving as a critical negative feedback mechanism that limits further thrombin formation (65). Meanwhile, activated TAFI stabilizes clots by removing lysine residues from partially degraded fibrin. These lysine residues normally serve as highaffinity binding sites for plasminogen and tissuetype plasminogen activator (tPA), amplifying plasmin generation; their removal reduces plasminogen and tPA binding and thereby slows fibrinolysis (66).

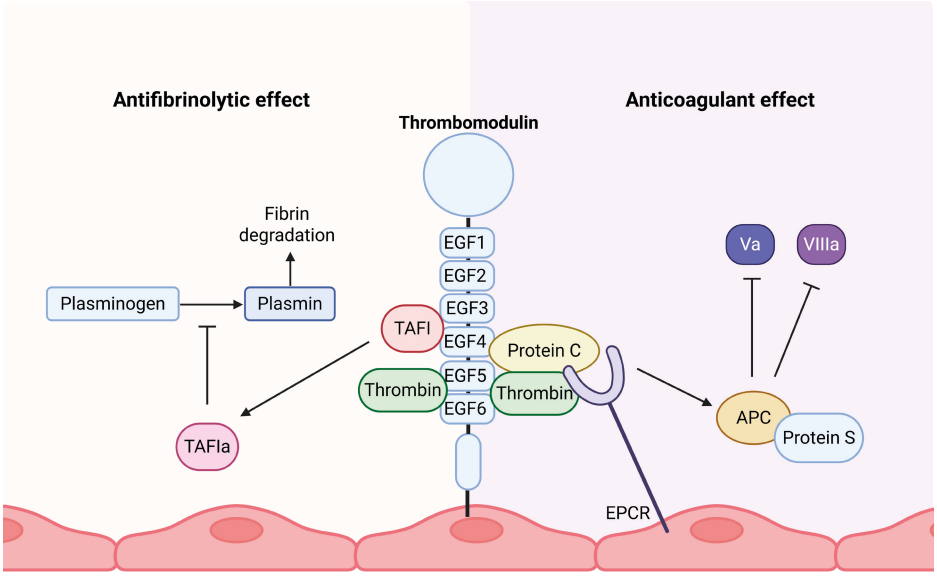


Figure 3. Thrombomodulin modulates thrombin activity via anticoagulant and antifibrinolytic pathways

Thrombomodulin (TM) is an endothelial transmembrane protein that binds thrombin via its EGF-like domains 5 and 6. Once bound, thrombin undergoes a functional switch and activates protein C (APC) when protein C binds TM at EGF-like domain 4, a process facilitated by the endothelial protein C receptor (EPCR). Activated protein C, in complex with its cofactor protein S, inactivates coagulation factors Va and VIIIa, thereby downregulating thrombin generation. Simultaneously, TM-bound thrombin activates thrombin-activatable fibrinolysis inhibitor (TAFI), which binds to EGF-like domains 3 and 4. Thrombin cleaves TAFI to generate its active form, TAFIa, which attenuates fibrinolysis by removing C-terminal lysine residues from fibrin. This impairs plasminogen binding and subsequent plasmin generation. Together, these mechanisms underpin the anticoagulant and antifibrinolytic functions of thrombomodulin. Figure created with BioRender.com

Given its crucial regulatory role in coagulation, thrombomodulin holds considerable diagnostic and therapeutic potential (67). In the diagnostic setting, thrombomodulin can be used to assess the functionality of the protein C pathway in patients plasma or whole blood (68, 69) often through thrombin generation assays (70). In these assays, thrombomodulin serves as a cofactor to evaluate the efficiency of thrombin-mediated activation of protein C. For example, in patients with liver cirrhosis, this method reveals a clear hypercoagulable state, highlighting its clinical utility (71). Incorporating thrombomodulin into a lipid membrane or surface may more closely replicate its physiological presentation on endothelial cells. This strategy could enhance the physiological relevance and sensitivity of functional assays used to evaluate coagulation status.

In therapeutic settings, certain genetic mutations can lead to elevated levels of soluble thrombomodulin, which are associated with bleeding tendencies in affected individuals

(72, 73). In such cases, selectively inhibiting thrombomodulin activity may offer clinical benefit by restoring the hemostatic balance (74). Additionally, thrombomodulin-mediated activation of TAFI can help stabilize clots, suggesting a potential therapeutic avenue for patients with bleeding disorders (75).

In contrast, in thrombotic conditions, enhancing thrombomodulin activity may be advantageous. Administration of recombinant thrombomodulin promotes protein C activation and thereby suppresses thrombin generation. This therapeutic strategy is already in clinical use in Japan for the treatment of sepsis-associated coagulopathy (76).

To further improve the efficacy of recombinant thrombomodulin, advanced delivery strategies are under investigation. One such approach involves coupling thrombomodulin to liposomes, which can extend its circulatory half-life and enhance stability (77). Moreover, modifying the lipid composition of these liposomes may influence the preferential activation of thrombomodulin-mediated pathways, potentially allowing for more targeted modulation of coagulation and fibrinolysis (78).

Together, these insights highlight thrombomodulin as a critical regulator of hemostasis with significant diagnostic and therapeutic implications. Its dual role in modulating both anticoagulant and antifibrinolytic pathways underscores its relevance across a spectrum of bleeding and thrombotic disorders. Ongoing efforts to refine functional assays and develop targeted delivery strategies may further enhance the clinical utility of thrombomodulin-based approaches.

OUTLINE OF THIS THESIS

Venous thromboembolism and immune thrombocytopenia are complex coagulation disorders that pose substantial clinical challenges. Improving the prediction of recurrent thrombotic events, deepening mechanistic understanding of prothrombotic states, and developing more effective diagnostic and therapeutic tools remain critical unmet needs. The overarching goal of this work is to contribute to these goals by advancing clinical decision-making, enhancing our understanding of the coagulation cascade, and supporting the development of novel strategies for diagnosis and treatment in patients with coagulation disorders.

This thesis is divided into two parts:

The **first part** of this thesis focuses on clinical applications in thrombotic risk prediction and pathophysiology.

- **Chapters 2-4** investigate several biomarker domains to improve prediction of recurrent venous thromboembolism: **Chapter 2** focuses on platelet and endothelial markers, **Chapter 3** employs unbiased mass spectrometry and **Chapter 4** examines thrombin generation with thrombin dynamics.
- **Chapter 5** explores the prothrombotic state in patients with immune thrombocytopenia, with a focus on altered thrombin generation and platelet activation.

The **second part** of this thesis delves into the molecular and biochemical aspects of coagulation, with a special emphasis on the membrane protein thrombomodulin.

- **Chapter 6** describes the design and development of liposomal formulations with thrombomodulin conjugated to a lipid surface, with the aim of enhancing thrombin generation assays for diagnostic applications.
- **Chapter 7** examines the in vitro therapeutic potential of thrombomodulin inhibition using a VhH targeting its EGF-like domains 3–6.

Finally, **Chapter 8** discusses the findings presented throughout this thesis, placing them in a broader clinical and scientific context and outlining directions for future research.

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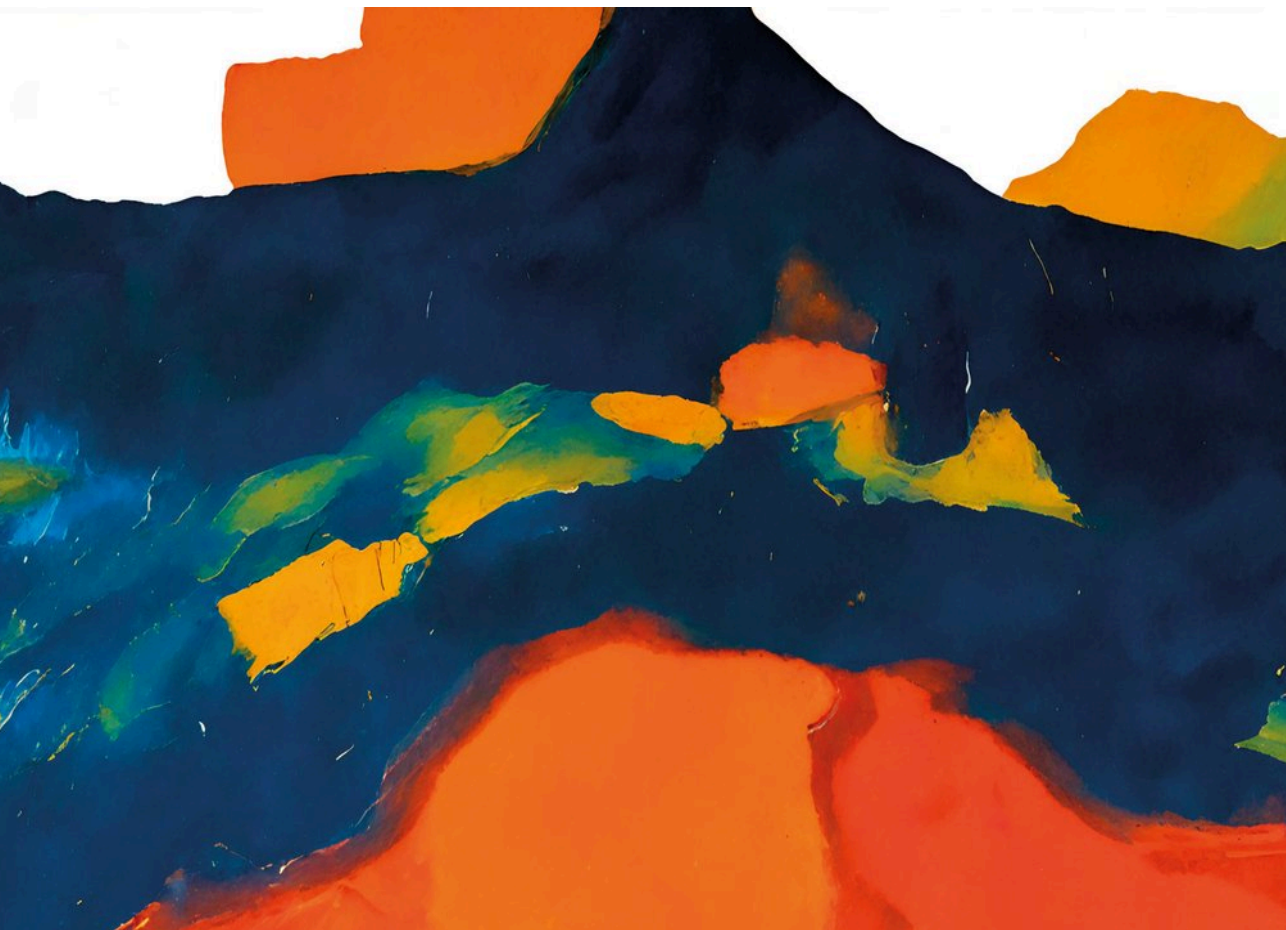
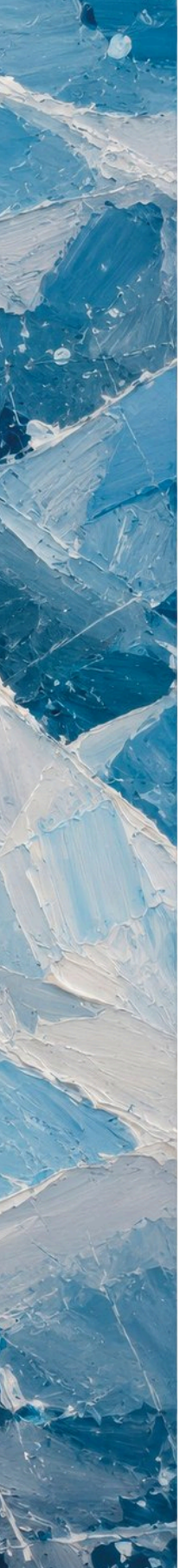
PART I

Clinical applications of coagulation biomarkers and thrombin generation





CHAPTER 2

Elevated levels of (active) von Willebrand Factor during anticoagulation are associated with early recurrence of venous thromboembolism

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J Thromb Haemost. 2025 Aug;23(8):2558-2567.

ABSTRACT

Background

Venous thromboembolism (VTE) can recur shortly after stopping anticoagulation, highlighting the need for reliable biomarkers to identify high-risk patients during treatment.

Objectives

To determine whether platelet and endothelial markers predict VTE recurrence.

Methods

We used data and samples from the randomized controlled VISTA trial (2011–2015), which included patients with unprovoked VTE who were treated with vitamin K antagonists (VKA) for six months. After treatment cessation, patients were followed for two years to assess recurrence. This dataset formed the basis for the current prospective analysis, in which plasma levels of VWF, aVWF, soluble P-selectin, soluble thrombomodulin, CXCL4, and CXCL7 were measured before and after cessation of anticoagulation. Associations between biomarker levels and recurrence were analyzed using Cox regression.

Results

Plasma samples from 629 patients with a first unprovoked VTE who discontinued VKA were analyzed. Recurrence occurred in 75 patients (12%); 11 (15%) within one month, 29 (39%) within three months and 46 (61%) after 3 months. Elevated levels of VWF and aVWF were associated with an increased risk of recurrence per 10-unit increase (VWF: HR: 1.03, 95%CI: 1.01-1.06; aVWF: HR: 1.02, 95%CI: 1.00-1.05). Associations were stronger for early recurrence (<3 months), while (a)VWF levels were not associated with late recurrence (>3 months). Stratification by sex showed VWF and aVWF were associated with increased recurrence risk in men, but not in women.

Conclusions

Elevated levels of VWF and aVWF during anticoagulation were associated with early recurrence in men and might serve as biomarkers for predicting VTE recurrence.

KEYWORDS

Anticoagulation – Biomarkers – Recurrence – Venous Thromboembolism – von Willebrand Factor

INTRODUCTION

Recurrence of venous thromboembolism (VTE) is an important clinical concern when deciding on the (dis)continuation of anticoagulation after standard treatment. Recurrence rates are estimated at 10% in the first year after discontinuation of anticoagulation, increasing to 25% in year five (1). Some VTE patients even experience recurrence within just one month after discontinuation of anticoagulation. This emphasizes the need for prognostic markers to identify patients at increased risk of early recurrence in whom cessation of anticoagulation could be avoided.

Currently, prognostic factors used in prediction models of VTE recurrence are mostly clinical characteristics such as sex, age and location of index event (2, 3). Occasionally, laboratory markers are also used as indicator of recurrence risk. Laboratory markers that are used, such as D-dimer, often have to be determined after cessation of anticoagulation. Preferably, recurrence risk is determined reliably before cessation of anticoagulation, to prevent patients from discontinuing anticoagulation when they are at high-risk of early recurrence. This can possibly decrease the number of early recurrences, while also stimulating therapy adherence, since patients do not need to stop and restart anticoagulation. To date, there are no laboratory markers to reliably predict recurrence risk during anticoagulation.

Platelet and endothelial activation, as well as dysfunction, play pivotal roles in VTE pathophysiology (4-6). Markers of these processes, such as von Willebrand Factor (VWF), active VWF, CXCL4, CXCL7, soluble P-selectin (sP-selectin) and soluble thrombomodulin (sTM), might be associated with recurrent VTE. Most of these markers have not been studied extensively in relation to VTE recurrence. An older study from 1982 found increased levels of β-thromboglobulin/CXCL7 in people with recurrent deep vein thrombosis (DVT) (7). More evidence is available for VWF. In a cohort of patients referred for thrombophilia testing increased VWF levels predicted new venous events (8). In the MEGA study higher levels of VWF antigen, determined after anticoagulation in most patients, were also associated with increased incidence of VTE recurrence (9). Studies on sP-selectin report conflicting results (10-13). Thus, evidence that these markers are useful in the prediction of recurrent VTE is still inconclusive and more evidence is needed.

This study aims to determine the association of circulating platelet activation markers (CXCL4, CXCL7 and sP-selectin) and endothelial cell activation markers: (sTM, VWF and aVWF) with VTE recurrence risk. These markers were measured both during anticoagulation treatment and after its cessation. Improving risk prediction during anticoagulation is crucial because early identification of high-risk patients could help prevent unnecessary discontinuation of anticoagulation, thereby reducing the likelihood of early recurrences. The study also seeks to identify factors that influence these associations, such as time to recurrence, sex differences, and the timing of biomarker measurement (during or after cessation of anticoagulation). Identifying biomarkers associated with recurrence could improve risk prediction which can aid in identification of high-risk patients in whom

cessation of anticoagulation might be harmful, as well as low-risk patients in whom cessation is probably safe.

MATERIAL AND METHODS

In the current study, samples from the VISTA trial were used. The study design and main results of the VISTA trial have been reported earlier (14). All participants provided written informed consent, and the study protocol was approved by the Medical Ethics Review Committee (METC) of the UMC Utrecht. In short, VISTA was an unblinded, randomized trial designed to investigate the impact of implementing the Vienna Prediction Model (VPM) (15). Patients with a first unprovoked VTE were recruited from nine local thrombosis services in the Netherlands between 2011 and 2015. During the first six months of standard treatment with vitamin K antagonists (VKA), patients were randomized into two groups: the intervention arm, where the decision to continue or discontinue anticoagulation was based on the VPM risk prediction, and the control arm, which followed usual care. Patients were followed for two years after cessation of anticoagulation, with the primary outcome being recurrence of VTE. Patients with provoked VTE, such as those with recent surgery, plaster casts, pregnancy, or postpartum status, were excluded. Patients with a history of VTE within the past 10 years, active malignancy, or other indications for anticoagulant treatment (e.g., atrial fibrillation or prosthetic heart valves), were also excluded. VTE cases related to oral contraceptives and hormone replacement therapy were included, since they were considered as unprovoked due to past classification ambiguity. Blood samples were collected twice: once before discontinuation of anticoagulation (T0) and again one month after VKA discontinuation (T1). Citrated plasma samples were then stored at -80°C.

Study Design

The primary aim of this study is to evaluate whether of sP-selectin, sTM, CXCL4, CXCL7, VWF and aVWF are associated with VTE recurrence, comparing patients with recurrence to those without recurrence. The secondary objectives include: (1) assessing whether these associations differ for early recurrences; defined as recurrences occurring within one month and three months after discontinuation of anticoagulation; (2) examining potential sex differences in these associations by comparing biomarker levels between male and female patients; and (3) investigating the impact of sample withdrawal timing on biomarker levels to determine whether sampling during or after anticoagulation therapy yields better predictive value for recurrence risk.

For the current study, patients without available samples from T0 and patients who continued VKA were excluded. Time of follow-up was determined from the date of VKA discontinuation to the date of recurrent VTE event, the last available follow-up date, or the end of the study period, whichever came first. All patients were censored at 800 days. Recurrent VTE was defined as proximal deep vein thrombosis (DVT) or fatal or nonfatal pulmonary embolism (PE), confirmed by compression ultrasonography for DVT and by

computed tomography pulmonary angiography (CTPA) for PE. Sex was recorded based on self-report.

Platelet and endothelial markers

Plasma levels of sP-selectin, sTM, CXCL4, CXCL7, VWF:Ag and aVWF were determined with enzyme-linked immunosorbent assay (ELISA). The ELISA for aVWF was developed in-house to specifically detect the open conformation of VWF by targeting the A1 domain (16). Plates were coated overnight with one of the following capture antibodies: mouse anti-human P-selectin/CD62P, mouse anti-human TM, mouse anti-human CXCL4, mouse anti-human CXCL7 (*R&D systems, Minnesota, United States*) or rabbit anti-human VWF (*Dako Denmark, Glostrup, Denmark*) or anti-human aVWF VHH (Clone A11, produced in-house). After coating, plates were washed and incubated with a blocking buffer (e.g., 1% BSA in PBS or a suitable commercial blocking solution) for 1 hour at room temperature to minimize nonspecific binding. Plasma samples were diluted as follows: 1/10 for sP-selectin, 1/8 for sTM, 1/1000 for CXCL4, 1/1000 for CXCL7, 1/160 for VWF and 1/20 for aVWF. The diluted samples were added to the wells and incubated for 1 hour on the coated plates. Each plate included a negative control, where only buffer was added, to correct for background signal. After thorough washing of the plates, the following detection antibodies were added: mouse anti-human P-selectin, biotinylated mouse anti-human TM, goat anti-human CXCL4, goat anti-human CXCL7 (*R&D systems, Minnesota, United States*) or rabbit anti-human VWF-HRP (for both VWF and aVWF) *(Dako Denmark, Glostrup, Denmark)*. After incubation with detection antibodies, plates were washed again. For VWF and aVWF detection, substrate (luminol with freshly added stable peroxide) was added, and luminescence was measured immediately using a multiplate reader. For CXCL4 and CXCL7, anti-goat IgG-HRP was added, while Streptavidin-HRP was added to the sTM and sP-selectin plates (R&D Systems, Minnesota, United States). Again, plates were washed after incubation. The plate detecting sP-selectin was subsequently incubated with TMB (*Tebu-bio, Heerhugowaard, the Netherlands*). Colorimetric reactions were stopped with H_2SO_4, and absorbance was measured in SpectraMax iD3 at 450nm. The other plates were incubated with luminol and were measured similarly to the plates detecting (a)VWF. All concentrations were determined by fitting the optical density (OD) of a calibration curve using non-linear regression in GraphPad Prism 10, allowing for interpolation of unknown concentrations after subtraction of the background signal obtained from the negative control wells.

FVIII activity was measured on the STA-R Evolution analyzer (Diagnostica Stago, Asnières, France) using a one-stage coagulation assay with reagents from Diagnostica Stago (Asnières, France).

Statistical analysis

Missing data were addressed using complete case analysis. Patients with missing samples did not differ significantly from those with complete data in baseline characteristics or outcomes. Descriptive statistics were used to summarize the characteristics of the study

population. Differences between groups for continuous variables were assessed using independent sample T-tests or Mann-Whitney U test, depending on whether the data were normally or non-normally distributed. For categorical variables, the Chi-square test was used. Changes in biomarker levels over time were analyzed using multilevel regression analysis.

Both univariate and multivariate Cox regression analyses were conducted to assess the relationship between recurrence of venous thromboembolism (VTE) and laboratory markers. The multivariate models were adjusted for age, sex (male or female), location of the index event (pulmonary embolism [PE], distal DVT, or proximal DVT), and hormone use (including both oral contraceptives and hormonal replacement therapy). We analyzed the biomarkers as continuous variables and changed the scale of biomarker units (e.g., from 1 ng/ml to 10 ng/ml) to avoid small or difficult-to-interpret hazard ratios, as reported in the results tables. Additionally, Kaplan-Meier survival curves were generated to visualize the recurrence of VTE across tertiles of biomarkers. Biomarker tertiles were defined by the 33rd percentile and the 66th percentile of each specific biomarker. In a separate analysis, we examined the correlation between (a)VWF and FVIII and evaluated the impact of adding FVIII to the Cox regression model to assess if FVIII modulates the predictive value of (a) VWF for VTE recurrence.

The significance level was set at $p < 0.05$ for all statistical tests. All statistical analyses were two-tailed, and results are presented as numbers (%), means with standard deviations (SD) or as medians with interquartile ranges (IQR), where appropriate. Hazard ratios are reported with their 95% confidence intervals. Statistical analyses were performed with R Studio (version 2024.04.2) and GraphPad Prism (version 10) for was used for Kaplan-Meier curves.

RESULTS

Patient characteristics and recurrence during follow-up

Of the 883 patients enrolled in the VISTA study, 629 were included in the present analysis. Patients were excluded if they continued anticoagulation or lacked baseline samples (**Figure 1**). Those who continued anticoagulation were more often male (70% vs. 44%) and more likely to have presented with pulmonary embolism (67% vs. 50%), reflecting higher predicted recurrence risk. Baseline characteristics of the included patients are shown in **Table 1**. The mean age of the patients was 56 years (range: 18-85) and 276 participants were women (44%). Forty-five percent of women were using hormones (oral contraceptives and/or hormone replacement therapy) at the time of their first VTE. First events were mostly pulmonary embolism (PE) (50%), followed by proximal deep vein thrombosis (DVT) (35%) and distal DVT (15%). During the two-year follow-up, recurrence occurred in 75 patients (12%). Of these, 29 (39%) had early recurrence: 11 (15%) within one month and 18 (24%) between one and three months after VKA discontinuation. The median time to recurrence was 147 days and median time of follow-up was 24 months (range 1-26 months). Patients with recurrence were older (59 vs. 55 years, p=0.03) and more often male. Recurrence occurred in 15% of men and 8% of women (men: n=52, women: n=23, p=0.02). Among women, 3% of those using hormones during the first event had a recurrence, compared to 13% of those not using hormones (n=4 vs. n=19, p=0.01).

Table 1. Baseline characteristics of study participants

	Total N = 629	No recurrence N = 554	Recurrence N = 75	P-value
Age, mean (SD)	56 (14)	55 (14)	59 (12)	0.03
Women, N (%)	276 (44)	253 (46)	23 (31)	0.02
Hormone use, N (% of women)	125 (45)	121 (22)	4 (5)	0.01
1st VTE Type, N (%)				0.76
Distal DVT	91 (15)	82 (15)	9 (12)	
Proximal DVT	223 (35)	197 (35)	26 (35)	
PE	315 (50)	275 (50)	40 (53)	
BMI, mean (SD)	27.7 (5)	27.7 (5)	28.1 (4)	0.48

This table presents the baseline characteristics of study participants. The variable "VTE Type" describes the location of the first venous thromboembolism (VTE) event. Hormone use is reported as a percentage of women only. Means were compared using Student's t-test, while categorical variables were analyzed with Pearson's chi-squared test. Note that there are three missing values for body mass index (BMI). Abbreviations: BMI = body mass index; VTE = venous thromboembolism.

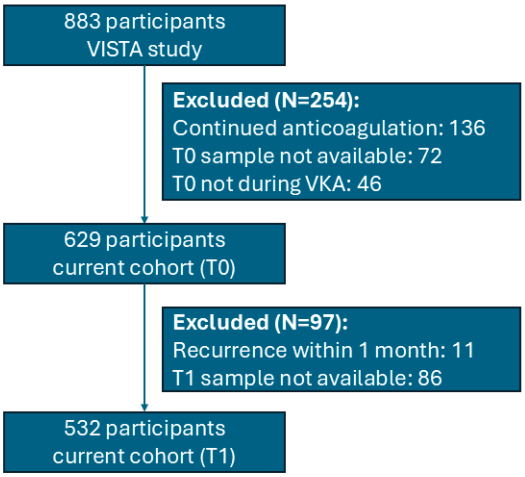

Figure 1. Patient flow and exclusion criteria for analysis
Patient flow diagram showing the inclusion and exclusion of participants. Of the 883 participants in the VISTA study, 629 were enrolled in the current cohort, and 532 were included in the analysis at 1 month after anticoagulation cessation (T1). Reasons for exclusion at both time points are indicated.

Elevated VWF and aVWF during anticoagulation are associated with increased risk of recurrence

Prior to VKA discontinuation (T0), we measured six platelet and endothelial markers: VWF, aVWF, CXCL4, CXCL7, sP-selectin and thrombomodulin. Biomarker levels were compared between patients who experienced recurrence and those who did not. VWF levels were significantly higher in patients with recurrence compared to those without (195% vs. 164%, p=0.04). There was also a trend toward higher aVWF levels in the recurrence group (aVWF: 142% vs. 131%, p=0.07). The other markers, CXCL4, CXCL7, sP-selectin and TM, were similar in patients with and without recurrence (**Table 2**).

We calculated hazard ratios (HRs) to assess the association between these biomarkers and the risk of recurrence. For VWF levels, analyzed as a continuous variable, each 10-unit increase was associated with a 3% higher risk of recurrence (HR: 1.03, 95% CI: 1.01–1.06, p=0.02). Similarly, each 10-unit increase in aVWF levels was linked to a 2% higher recurrence risk (HR: 1.02, 95% CI: 1.00–1.05, p=0.03). No significant associations were observed between the other biomarkers and recurrence (**Supplemental Table 1**). In the multivariate analysis, adjusted for sex, age, location of the first VTE event and hormone use, the HRs for VWF and aVWF remained similar. However, while VWF continued to show a significant association with recurrence risk, aVWF was no longer significant (HR: 1.02, p=0.14). Kaplan-Meier curves, with recurrence-free survival stratified by biomarker tertiles, showed a clear separation for VWF and aVWF; with more recurrences observed in the highest tertile. In contrast, the curves for the other biomarkers showed considerable overlap between tertiles (**Figure 2**).

Table 2. Biomarker levels at T0 (during anticoagulation therapy)

	No recurrence	Recurrence		Early recurrence (<1 month)		Early recurrence (<3 months)	
	Median (IQR)	Median (IQR)	P-value	Median (IQR)	P-value	Median (IQR)	P-value
VWF (%)	164 (122-222)	195 (152-227)	0.04	218 (130-384)	0.09	204 (153-278)	0.02
aVWF (%)	131 (100-178)	142 (111-190)	0.07	165 (133-394)	0.04	176 (118-212)	0.01
sP-selectin (ng/mL)	47 (34-61)	44 (36-63)	0.57	43 (39-93)	0.50	48 (38-63)	0.40
CXCL4 (ng/mL)	1363 (777-2053)	1312 (730-1853)	0.37	1883 (1107-2662)	0.25	1526 (1049-2530)	0.29
CXCL7 (ng/mL)	729 (479-1053)	672 (457-935)	0.25	868 (641-1304)	0.24	854 (623-1162)	0.25
TM (ng/mL)	3.3 (2.7-3.9)	3.4 (2.9-4.3)	0.09	3.6 (3.0-4.8)	0.20	3.5 (3.1-4.7)	0.04

This table presents the levels of biomarkers measured at T0, prior to the discontinuation of vitamin K antagonists (VKAs). Data are reported as median with interquartile range (IQR). Biomarker levels are shown separately for the no recurrence group, recurrence group, early recurrence within 1 month, and early recurrence within 3 months. Units for each biomarker are indicated in the table headers (e.g., percentages, ng/mL). Comparisons of biomarker levels to the no recurrence group were conducted using the Mann-Whitney U test, with corresponding p-values also presented in the table. Abbreviations: VKA = vitamin K antagonist.

Since VWF levels are closely related to FVIII levels, we assessed the contribution of FVIII to recurrence risk. Moderate positive correlations were observed between FVIII and VWF (r = 0.52), and between FVIII and aVWF (r = 0.43). FVIII levels were associated with two-year recurrence risk (HR: 1.04, 95% CI: 1.01-1.08 per 10-unit increase). Inclusion of FVIII in multivariate analyses attenuated the association between VWF and recurrence risk, suggesting that FVIII partially mediates this relationship.

Predictive value of VWF and aVWF differs for early and late(r) recurrences

Next, we examined the association between biomarkers and early recurrence, defined as recurrence within 1 or 3 months after VKA discontinuation. Median and mean levels of VWF and aVWF were notably higher in patients who experienced early recurrence (**Table 2**). For recurrences within 1 month, both VWF and aVWF were significantly associated with increased recurrence risk (VWF: HR: 1.08, 95% CI: 1.02-1.13; aVWF: HR 1.06, 95% CI: 1.03-1.09 per 10-unit increase). Similarly, for recurrences within 3 months, VWF and aVWF remained significantly associated with recurrence (VWF: HR: 1.06, 95% CI: 1.02-1.09; aVWF: HR: 1.04, 95% CI: 1.02-1.07 per 10-unit increase). Adjustment for FVIII did not attenuate these associations, and FVIII was not associated with early recurrence (HR: 1.00, 95% CI: 0.93-1.08). In contrast, baseline VWF and aVWF levels were not associated with late recurrence (>3 months) (VWF: HR: 1.01, 95% CI: 0.97-1.05; aVWF: HR: 1.00, 95% CI 0.96-1.04) (**Figure 3 and Supplemental Table 1**), while FVIII levels showed a significant association with late recurrence (FVIII: HR: 1.05, 95% CI: 1.00-1.09). Although the association between sP-selectin and early recurrence did not reach statistical significance, a trend was observed—particularly for recurrence within one month—with a 6% increase in risk per 10 ng/ml increase (**Supplemental Table 1**).

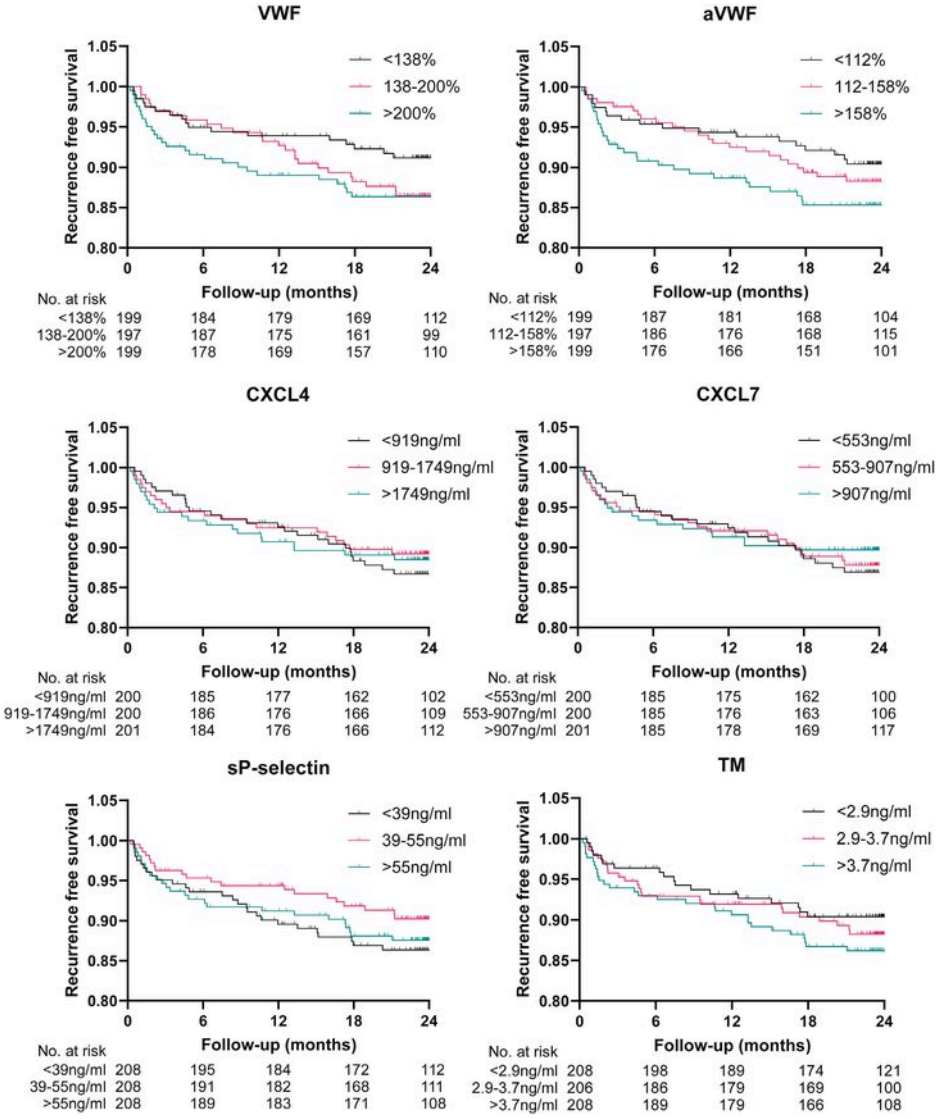

Figure 2. Kaplan-Meier curves for recurrence-free survival by biomarker tertiles

Kaplan-Meier curves illustrating the proportion of patients remaining recurrence-free, stratified by biomarker tertiles (based on the 33rd and 66th percentiles as cut-offs, indicated in the legends). The X-axis represents follow-up time in months, and the Y-axis shows the proportion of patients remaining recurrence-free. The survival probabilities are displayed for patients in the following tertiles: green represents the highest tertile, pink corresponds to the middle tertile, and black denotes the lowest tertile. Censoring events are indicated by a thin line above the curves. The tables below each curve show the number of patients at risk at indicated time point for each tertile.

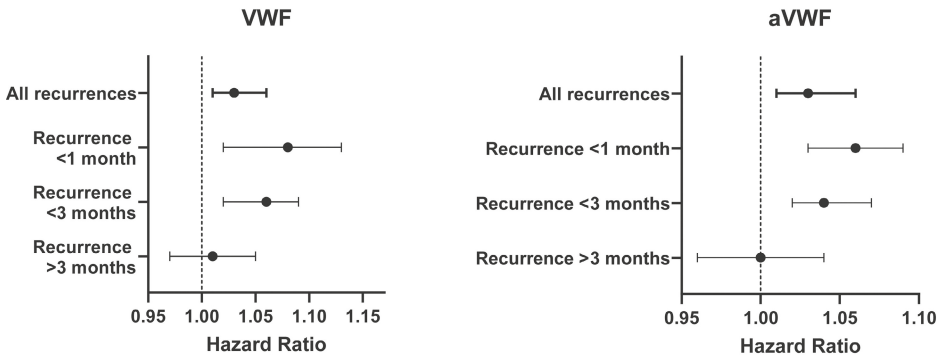

Figure 3. Hazard ratios of VWF and aVWF stratified by time to recurrence
Forest plots displaying hazard ratios (HRs) for VWF and aVWF, stratified by time to recurrence. Each plot represents the associations between biomarker levels and recurrence risk across four time frames: all recurrences, recurrences within 1 month, recurrences within 3 months and recurrences after 3 months. The HRs are presented with 95% confidence intervals (CIs) indicated by horizontal lines. The dotted vertical line at HR = 1.0 represents the null hypothesis of no effect.

Predictive value of VWF and aVWF differs for men and women

We stratified the data by sex to assess any potential sex differences in our cohort. Overall, women were younger (52 vs. 58 years, p=0.001), had a higher BMI (28.3 vs. 27.2, p=0.003) and a higher prevalence of PE compared with men (58% vs. 44%, p=0.002). After excluding hormone-related VTE cases, women were older than men (62 vs. 58, p=0.01). Men experienced recurrence more often than women; however, when hormone-related cases were excluded, there was no significant difference in recurrence risk between the sexes (13% of women vs. 15% of men).

Baseline levels of VWF, CXCL4, and CXCL7 were similar between men and women. sP-selectin, TM and aVWF were lower in women compared with men. After excluding hormone-related VTE cases, only TM levels remained significantly lower in women. Plasma levels of (active) VWF were associated with increased risk of recurrence in men (VWF: HR 1.04, 95% CI: 1.01-1.06; aVWF: HR 1.02, 95% CI: 1.00-1.04), but not in women (VWF: HR 0.98, 95% CI: 0.92-1.05; aVWF: HR: 0.99, 95% CI: 0.91-1.07). Hazard ratios in women did not change after excluding women receiving hormones (VWF: HR 0.97, 95% CI: 0.90-1.04; aVWF: HR 0.99, 95% CI: 0.91-1.08) (**Figure 4**).

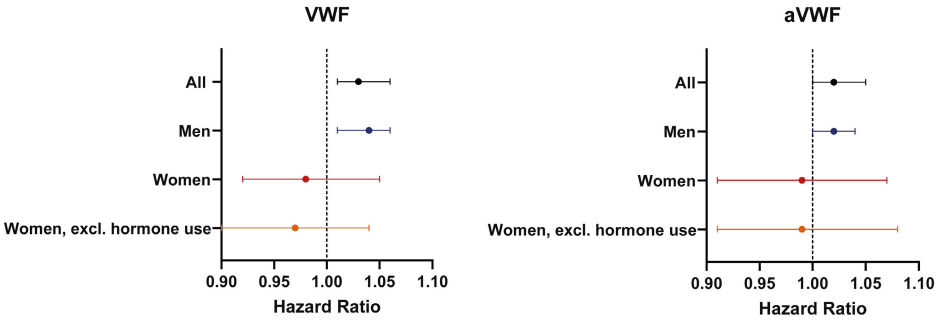

Figure 4. Hazard Ratios of VWF and aVWF stratified by sex

Forest plots displaying hazard ratios (HRs) VWF and aVWF, stratified by sex. Each plot illustrates the association between biomarker levels and recurrence risk for men, women and women who were not taking hormones during the first VTE event (women, excl. hormone use). HRs are presented with 95% confidence intervals (CIs), indicated by horizontal lines. The dotted vertical line at HR = 1.0 represents the null hypothesis of no effect.

Predictive value of (a)VWF differs on timing of measurement (during vs. 1 month after cessation of anticoagulation)

The analysis of biomarkers at 1 month after cessation of anticoagulation (T1) was performed in the remaining 532 patients (85% of the cohort), of whom 54 experienced a recurrence within the two-year follow-up period. Patients who experienced recurrence within the first 30 days (n=11) were excluded from the analysis at T1, as they reached the study endpoint before blood samples at this time point were collected. Despite considerable intra-patient variability, all biomarkers remained stable over time, with no significant increases or decreases in biomarker levels observed. However, among patients who experienced a recurrence, VWF levels were lower at T1 (mean difference: -23, p=0.04). VWF levels at this time point were not significantly associated with recurrence within 3 months after cessation of anticoagulation (HR: 1.02, 95% CI: 0.97-1.07, p = 0.42). In contrast, aVWF levels at T1 were significantly associated with recurrence within 3 months (HR: 1.05, 95% CI: 1.00-1.11, p = 0.04).

DISCUSSION

We found that elevated levels of VWF and aVWF during anticoagulation were associated with an elevated risk of recurrence. We identified three factors that influenced the association between these biomarkers and recurrence in our cohort: 1) Time to recurrence: VWF and aVWF were associated with early recurrence only; the levels were not associated with recurrence after three months. 2) Sex: these associations were only present in men and not women. 3) Timing of the measurement: the association of VWF with early recurrence was absent if measured a month after VKA discontinuation. Therefore, VWF and aVWF are potential markers that might help estimate recurrence risk before cessation of anticoagulation.

Other studies have also identified that elevated VWF levels associate with increased recurrence risk. Our findings are in accordance with these studies, but the studies differ from our study in several ways. A nested case-control study, among individuals referred for antiphospholipid (aPL) testing, identified VWF as a predictor of new venous events (8). Since the study included all patients who required aPL testing, not everyone had experienced a first VTE event, making it difficult to specifically discuss recurrence. In total, only five patients with recurrent venous events were included, and it is unclear if VWF levels were measured during or after anticoagulation. Furthermore, the MEGA study also showed incidence rates of recurrence increased gradually with increasing levels of VWF:Ag (9). In the MEGA study, samples were collected approximately three months after discontinuation of anticoagulation in most patients and a considerable proportion of the first events was provoked VTE. Besides that, VWF levels and their associations were not stratified by sex. Our study demonstrates that VWF levels are predictive also during anticoagulation, and we identified possible sex differences that were not considered in these previous studies.

This study focuses on predicting thrombotic recurrence risk and does not establish causal relationships or conclusions about the underlying etiology. However, based on our findings, we hypothesize that VWF's contribution to early recurrence risk may extend beyond its association with FVIII. VWF and FVIII are closely linked in the coagulation process, with elevated VWF levels often correlating with higher FVIII concentrations. While VWF stabilizes FVIII in circulation by protecting it from rapid degradation, it also plays a direct role in platelet adhesion and activation, both crucial in thrombus formation. Previous studies have shown that increased FVIII is a significant risk factor for venous thromboembolism (VTE) recurrence (17, 18). In contrast, our findings indicate that both VWF and its activated form (aVWF) were significantly associated with early recurrence, even after adjusting for FVIII. This suggests that VWF's role in recurrence risk may extend beyond its relationship with FVIII. Further support for this hypothesis comes from a trend toward increased soluble P-selectin (sP-selectin) levels in patients with early recurrence, indicating additional platelet activation, though this did not reach statistical significance. Moreover, the predictive role of aVWF—the open conformation of VWF that facilitates platelet binding—further strengthens the notion that VWF's contribution to recurrence risk may be more related to platelet activation than to FVIII stabilization. It is also important to consider the influence of ABO blood group on VWF levels, as individuals with non-O blood types typically exhibit higher VWF levels, which could contribute to an increased thrombotic risk (19, 20). While blood group data were not included in this study, this factor may explain some of the observed associations and warrants further investigation in future research.

The other markers we measured; CXCL4, CXCL7, sP-selectin and thrombomodulin (TM), were not associated with recurrence. One earlier study showed thrombomodulin was elevated in patients with acute DVT events (21). Our results seem to indicate that there is no association of thrombomodulin levels with recurrence and the same counts for CXCL4 and CXCL7. We identified four studies that looked at the association of sP-selectin with

recurrence that had contrasting results. Two studies reported an association between sP-selectin levels and recurrence and two others found no association between sP-selectin and recurrence. This can be explained by different inclusion and exclusion criteria, number of recurrences in the study and/or different study populations. In general, these biomarkers were studied as risk factors for first VTE events before and often associated with these first VTE events. In our cohort, we show that these biomarkers are not necessarily predictive of a secondary event as well.

Our findings indicate that the association between VWF and recurrence is influenced by the time to recurrence. Specifically, we showed VWF and aVWF were associated with early recurrence but not with late recurrence. A possible explanation for the stronger association seen with early recurrence is that VWF reflects ongoing pathological processes that are more pronounced close to a recurrent event. In contrast, recurrent events occurring long after biomarker measurement may not yet be detectable as these pathological processes are not occurring yet, thereby weakening the association between the biomarker and recurrence risk.

Sex also influenced the association between VWF and VTE recurrence in our cohort. It is well established that risk factor relationships in various cardiovascular diseases differ between men and women (22, 23). A previous study also identified sex differences in the association of D-dimer with VTE recurrence (24). A potential explanation for the observed sex differences could lie in the hormone use among women. However, when we excluded hormone-related VTE cases the association of VWF with recurrence remained non-significant in women. Additionally, one study indicated that oral contraceptives do not seem to impact VWF levels (25). Another explanation for the lack of significant associations in women could be the small sample size; only 23 women experienced recurrence, which limits statistical power. It is also important to note that VWF levels are generally higher in men than in women and levels tend to increase with age (26, 27). However, multivariate analysis showed that associations remained when we corrected for age and sex. Furthermore, we observed similar VWF levels of men and women in our cohort, and there was no strong correlation of VWF with age.

The associations between VWF and recurrence also varied depending on the timing of biomarker measurement – before or after VKA discontinuation. Other studies also highlight the importance of timing, noting that associations can differ at various time points (28). An example is D-dimer, where negative results during anticoagulation may be inaccurate; levels can become positive shortly after VKA discontinuation (29). Additionally, D-dimer levels depend on the type of anticoagulation therapy; DOAC or VKA (30). This suggests that anticoagulation might affect biomarker levels. However, it is unlikely that VKAs influence the production of VWF in endothelial cells. Furthermore, we showed that VWF levels do not differ significantly between measurements taken during and after anticoagulation. Yet, the predictive value of VWF measured during and after anticoagulation was significantly

different. This discrepancy might be explained by patients who experienced recurrence within the first month after VKA discontinuation – prior to the second measurement. These patients had the highest VWF levels influencing the association more strongly. Additionally, we observed that patients with 'later' recurrence showed a decrease in VWF levels between T0 and T1, which was not observed in non-recurrent patients.

Several limitations of this study warrant acknowledgment. First, our follow-up period was limited to two years, meaning that recurrence status of patients beyond this timeframe remains unknown. It is possible that some patients may have experienced recurrences after this period, which could influence biomarker associations. However, as VWF was not associated with late recurrences, this limitation is unlikely to have significantly affected our conclusions. Second, the study included women with hormone-related VTE, a factor that is now considered a provoking risk factor rather than unprovoked VTE. This might have led to a lower recurrence risk among women and influenced statistical power in sex-specific analyses. Third, we excluded patients who continued anticoagulation, most of whom were considered high-risk. As a result, we may have underestimated the recurrence risk in this cohort. Fourth, the lack of platelet count data prevented adjustment for differences in platelet numbers when analyzing platelet activation markers. As platelet counts can influence biomarker levels, future studies should include this parameter to better assess the contribution of platelet activation in recurrence risk. Lastly, all patients in this study were treated with VKAs, whereas direct oral anticoagulants (DOACs) are now more commonly prescribed. While the impact of DOAC use on the associations observed in our study is uncertain, it is unlikely that VKAs influence VWF levels, suggesting that similar associations may be observed with DOACs.

The strength of this study is the prospective nature of our cohort. Furthermore, this study was a nationwide, general population study over multiple centers with a relatively large sample size, leading to better generalizability. Besides that, we have samples from two different timepoint, both during and after anticoagulation. Determining biomarker levels reliably during anticoagulation is beneficial for patients, since they are not exposed to the risk of recurrence in the meantime.

In summary, we measured endothelial and platelet markers in a prospective cohort of VTE patients to find potential markers for VTE recurrence. We identified that VWF and aVWF may be such markers measurable during anticoagulation, especially for early recurrence in men. Incorporating VWF and aVWF into prediction models may hold promise for identifying patients at high risk of early recurrence during anticoagulation, offering guidance on the safety of discontinuing anticoagulant therapy.

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Acknowledgement

We gratefully acknowledge the technical assistance of Harmen Middelveld, Veronica Schmalschlager and Jente Schoenaker with the sample measurements. The VISTA trial received funding from the Netherlands Organisation for Health Research and Development (ZonMw grant number 171002214). The current study was funded by the institutes of the authors.

Authorship contribution

Geke C. Poolen – Formal analysis, Investigation, Data curation, Writing – Original Draft, Review & Editing, Visualization, Project Administration
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Bas de Laat - Conceptualization, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition
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Declaration of interest

Geke C. Poolen – No conflict of interest.
Rolf T. Urbanus – The institution of RT Urbanus has received research funding from Hemab Therapeutics.
Mark Roest – Employee of Synapse Research Institute, part of Stago SAS.
Geert-Jan Geersing – No conflict of interest.
Bas de Laat - Employee of Synapse Research Institute, part of Stago SAS.
Roger E.G. Schutgens – The institution of REG Schutgens has received speakers' fees and/or research grants from Bayer, CSL Behring, Hemab Therapeutics, Novartis, NovoNordisk, Octapharma, Roche, Sobi and Takeda.

Supplementary Table: Hazard ratios of Cox regression of biomarkers with recurrence during anticoagulation therapy (T0).

		Recurrence			Early recurrence (<1 mo)			Early recurrence (<3 mo)			Late recurrence (>3 mo)		
	Units increase	HR	CI95%	P-value	HR	CI95%	P-value	HR	CI95%	P-value	HR	CI95%	P-value
VWF	10%	1.03	1.01-1.06	0.02	1.08	1.02-1.13	0.004	1.06	1.02-1.09	0.001	1.01	0.97-1.05	0.59
aVWF	10%	1.02	1.00-1.05	0.03	1.06	1.03-1.09	0.0002	1.04	1.02-1.07	0.001	1.00	0.96-1.04	0.96
sP-selectin	10 ng/mL	1.02	0.96-1.07	0.57	1.06	0.99-1.14	0.08	1.04	0.98-1.11	0.21	0.99	0.91-1.09	0.89
CXCL4	100 ng/mL	0.99	0.97-1.01	0.33	1.02	0.97-1.07	0.40	1.01	0.98-1.04	0.49	0.96	0.93-1.00	0.04
CXCL7	100 ng/mL	0.97	0.92-1.03	0.34	1.05	0.94-1.18	0.38	1.03	0.96-1.11	0.37	0.92	0.85-0.99	0.03
TM	1 ng/mL	1.08	0.98-1.19	0.14	1.14	0.90-1.44	0.27	1.10	0.93-1.30	0.25	1.07	0.94-1.22	0.32

This table presents the univariate results of Cox regression analyses examining the association between biomarker levels measured during anticoagulation therapy (T0) and the risk of recurrence. Hazard ratios (HRs) are reported with 95% confidence intervals (CIs) and corresponding *P* values. Associations are shown separately for overall recurrence, early recurrence within 1 month, early recurrence within 3 months, and late recurrence after 3 months. The unit of increase for each biomarker (e.g., 1 unit, 10 units, or 100 units) is specified in the table and reflects the change in hazard associated with that increase.
aVWF, active von Willebrand factor; sP-selectin, soluble P-selectin; TM, thrombomodulin; VWF; von Willebrand Factor.

CHAPTER 3

Plasma proteomics does not identify biomarkers of venous thromboembolism recurrence during and after anticoagulation: results from the VISTA study

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J Thromb Haemost. 2026 Apr;24(4):1365-1376.

ABSTRACT

Background

Venous thromboembolism (VTE) is typically treated with anticoagulation for 3-6 months. Decisions about extending treatment require balancing recurrence and bleeding risk. Current prediction models based on clinical markers lack sufficient discriminative accuracy, emphasizing the need for reliable biomarkers.

Objective

To identify plasma biomarkers associated with VTE recurrence during and after anticoagulation.

Method

We conducted a case-cohort study using data and samples from the VISTA trial, which followed patients with a first VTE treated with vitamin K antagonists (VKA) for six months. Follow-up for recurrence was two years. Recurrence cases (n=96) were compared with a randomly selected subcohort (n=192). Plasma was collected in the final month of VKA therapy and one month after cessation. Unbiased mass spectrometry-based proteomics was used to analyze plasma proteins.

Results

Principal component analysis revealed no global proteomic differences between patients with and without recurrence. In addition, no statistically significant changes in plasma protein levels were associated with recurrence neither during- nor after treatment. While paired sampling demonstrated the variability in inter-individual response upon initiation and cessation of VKA-treatment, including VKA-dependent coagulation proteins (e.g., prothrombin (F2), coagulation factor VII (F7), F9, F10, vitamin K-dependent protein S (PROS1), PROC, PROZ), there were no statistically significant differences in protein level alterations associated with recurrence.

Conclusion

While plasma proteomics captured anticoagulation effects, it did not reveal biomarkers predictive of VTE recurrence, underscoring the need for alternative approaches.

KEYWORDS

Biomarkers - Mass spectrometry - Proteomics - Recurrence - Venous thromboembolism

INTRODUCTION

Venous thromboembolism (VTE) is a serious condition with a high risk of recurrence; nearly 10% of unprovoked VTE's recur within the first year after cessation of anticoagulation, increasing to 25% after five years (1). A central challenge in VTE management is determining the optimal duration of anticoagulation, specifically whether extended therapy is necessary or whether anticoagulation can be safely discontinued (2, 3). Currently, patients are treated with anticoagulation, such as vitamin K antagonists (VKA) or direct oral anticoagulants (DOAC), for a period of three to six months (4, 5). After this initial treatment period, the decision to continue or stop treatment is guided by an individual's estimated risk for VTE recurrence or for bleeding. Accurate prediction of VTE recurrence risk is therefore critical to inform treatment duration and improve clinical outcomes.

Several prediction models have been developed to assess the risk of recurrence, most of which rely primarily on clinical factors such as age, sex and the location of the index event. Given their limited predictive accuracy (6) incorporation of laboratory biomarkers is a promising approach to improve risk stratification tools. Among biomarkers studied to date, D-dimer and factor VIII (F8) have shown the most consistent associations with recurrent VTE, with D-dimer widely used in clinical practice (7). However, several challenges limit their clinical utility, including the influence of ongoing anticoagulant therapy. Therefore, a "washout period" after treatment cessation is required to obtain reliable measurements (8-10). This interruption may expose patients to recurrence risk during the brief period off therapy (11, 12) and can also complicate treatment adherence when anticoagulation is restarted (13). An additional issue is the inconsistency in associations of proposed biomarkers with VTE recurrence, including von Willebrand factor (VWF) (14-16), C-reactive protein (CRP) (17), Factor IX (F9) (18, 19), Factor XI (F11) (18, 20), and fibrinogen (21, 22), which contributes to ongoing uncertainties which biomarkers could reliably improve risk prediction.

We recently showed that within the context of thrombosis data-independent acquisition (DIA)-based plasma proteomic profiling can identify disease- and treatment-related changes (23). Therefore, to identify biomarkers for VTE recurrence using discovery-based, unbiased mass spectrometry, we conducted a case-cohort study within the VISTA study of patients with a unprovoked VTE. Our aim was to identify proteins associated with VTE recurrence that could ultimately improve clinical decision-making regarding the duration of anticoagulation therapy through more accurate risk prediction.

METHODS

Study design and population

The study population consisted of patients enrolled in the VISTA trial, described in detail elsewhere (24). The VISTA study was a randomized controlled trial that recruited 883 patients with a first unprovoked VTE through nine local thrombosis services across the Netherlands. Patients with previous surgery or limb-casting within 3 months prior to the VTE event, as well as those who were pregnant or in the puerperium, were excluded. Patients with active malignancy, a history of VTE within the past 10 years, or another indication for long-term anticoagulation (e.g., atrial fibrillation, prosthetic heart valves) were also excluded. Women with hormone-associated VTE were eligible, as at the time of study initiation there was ongoing debate whether these events should be classified as provoked or unprovoked. All patients were treated with VKAs for six months. The primary outcome was recurrent VTE during a two-year follow-up period after cessation of anticoagulation. The trial was approved by the medical ethics committee of UMC Utrecht in 2011, and all patients provided written informed.

For the current analysis, we conducted a case-cohort study. This design was chosen because it allows efficient analysis of biomarker data by measuring only cases and a randomly selected subcohort, avoiding the need to assay the entire cohort while still enabling unbiased estimation of associations between plasma biomarkers and recurrent VTE. This approach combines the strengths of a cohort study with the efficiency of a nested case-control design and is particularly useful when laboratory analyses are costly or sample volume is limited (25, 26). All 96 patients who experienced recurrent VTE during follow-up were included as cases; plasma samples collected during VKA therapy were available for 87 of these patients. A subcohort of 192 patients, twice the number of cases, was randomly selected from the full trial cohort, regardless of recurrence status, in line with the case-cohort design. Of these, 167 had plasma samples available collected during VKA therapy. A second plasma sample, collected one month after cessation of VKA therapy, was available for 61 recurrent cases and 132 subcohort patients.

Citrated plasma was collected during the final month of VKA therapy and one month after cessation. Plasma was isolated by centrifuging whole blood at 2000*g* for 10 minutes at room temperature (RT), followed by a second centrifugation of the plasma under the same conditions. To assess the normalization of plasma protein levels after anticoagulation, citrated plasma was also collected from 20 anonymous healthy controls using the same protocol. All plasma samples were stored at -80 °C until analysis.

Plasma proteomics

All plasma samples were thawed at 37 °C and 10 µL were transferred into a 96-well format. Every plate included triplicates of pooled aliquots from 30 healthy donors (Sanquin, the Netherlands) used to monitor analytical performance. Ten µL plasma samples were diluted in 590 µL 100 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl,

Life Technologies, UK) (pH 8.0) (1:60 ratio), afterwards 9 µL of the diluted plasma was resuspended in five µL 20mM Tris(2-carboxyethyl)phosphine (TCEP, Thermo Fisher Scientific, Rockford, IL), 80mM chloroacetamide (CAA, Sigma Aldrich, St Louis, MO) in 100mM Tris-HCl (pH 8.0) and then heated at 95 °C for 5 minutes to denature, reduce and alkylate the plasma proteins. Once the samples were cooled to RT, 200 ng (1:50 ratio) MS-grade Trypsin Gold (Promega, Madison, WI) in 30 µL 50 mM Tris-HCl (pH 8.0) was added and samples were continuously shaken at 700 rpm at 25 °C overnight for digestion. Subsequently, the samples were acidified with 5 µL 10% trifluoroacetic acid (TFA, Thermo Fisher Scientific, Rockford, IL) to quench digestion and stored at -20 °C until analysis.

Next, 500 ng of peptides were loaded onto EvoTip Pure tips (EvoSep, Denmark) according to manufacturer's guidelines. Subsequently, peptides were analyzed using an EvoSep One Liquid Chromatography (LC, EvoSep, Denmark) system coupled to a timsTOF HT (Bruker, Germany) mass spectrometer, which was operating in data independent acquisition using parallel accumulation-serial fragmentation (diaPASEF). Peptides were separated using the pre-defined 60 samples per day (SPD)[27] method with on a 8 cm x 150 µm C18 (1.5 µm, EV1109, EvoSep, Denmark) column and 0.1% FA in water (Biosolve, the Netherlands) as mobile phase A and 0.1% FA in acetonitrile (ACN) (Biosolve, the Netherlands) as mobile phase B. For acquisition, an in-house py_diAID optimized (28) diaPASEF method was used. MS1 ranged from 100-1700 *m/z*, and MS2 acquisition with a cycle time of 1.85 seconds. In total, 28 MS2 scans with mass and ion mobility windows ranged from 350.17-1500.8 *m/z* and 0.70-1.45 1/k0 respectively, with 100 ms accumulation time, 100% duty cycle and collision energy as a linear function of ion mobility.

The .d files were processed in DIA-NN (version 1.8.1) (29) with a mass accuracy of 15 ppm, a 1 – 4 precursor charge range, a 300-1800 precursor *m/z* , a minimum peptide length of 6 amino acids, a maximal peptide length of 30 amino acids with an *in silico*-predicted spectral library from the reviewed human proteome database (Swiss-Prot Database, 20423 entries, downloaded on 8 August 2023). We allowed up to 2 missed cleavages and 3 variable modifications, including methionine oxidation and protein N-terminal acetylation. We enabled match between runs, disabled heuristic protein inference, did not allow for shared spectra, used single-pass mode as neural network classifier with robust LC (high precision) for the quantification strategy and protein inference based on the genes.

Data analysis

Protein group values were filtered out if based on only one precursor per protein group and considered reliably quantified if present in at least 50% of the samples in one of the groups (healthy controls and patients stratified for recurrence and treatment). Label free quantification (LFQ) values were then $\log_2$ transformed (**Table S2A**) and imputed with a normal distribution (downshift of 1.8 and width of 0.3, **Table S2B**) for statistical analysis. Coefficients of variation (CVs) were calculated based on non-$\log_2$ transformed data.

The agreement between available laboratory data and plasma protein levels determined with mass spectrometry was assessed using Spearman correlation. Available laboratory data included prothrombin and fibrinogen activity levels measured on the STA-R analyzer, following the manufacturer's recommendations (Diagnostica Stago, France) as well as von Willebrand factor (VWF:Ag), CXCL4 and CXCL7 quantified with ELISA (30, 31). D-dimer levels were measured locally, both as part of routine clinical care and, in the intervention arm of the VISTA study, to inform risk stratification using the Vienna Prediction Model.

Principal component analysis (PCA) was performed with prcomp. Statistical analysis was performed using limma (32) with a block on individuals. A Benjamini-Hochberg correction was applied to control the false discovery rate, with adjusted p-value below 0.05 considered significant; these were plotted against the effect size (log_2-fold change, LFC).

For analysis of the VKA-treatment effect, 148 patients with complete paired samples were included. The individual-specific effect size was calculated by subtracting the log_2 transformed protein levels during treatment from those after treatment (**Table S2C**) and statistically compared between individuals with and without recurrence using limma. Peptide coverage of F2 per sample was determined by mapping each peptide to the FASTA with seqinr (33) with an in-house script.

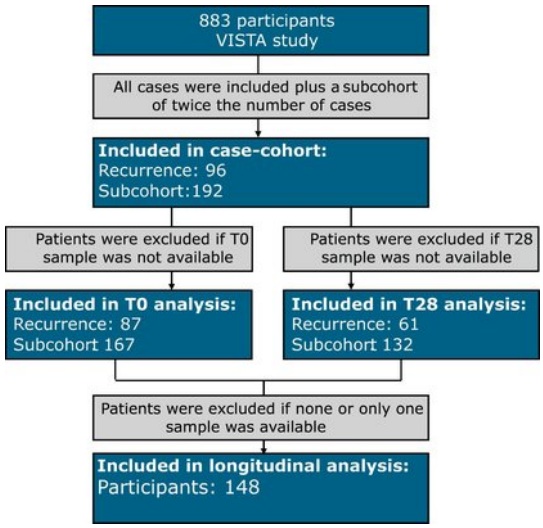

Figure 1. Flowchart of participant selection and sample availability in the VISTA study following case-cohort design

Of the 883 participants in the VISTA study, 96 recurrence cases and 192 subcohort patients were selected for the current case-cohort analysis. For the T0 analysis, samples were available for 87 cases and 167 subcohort patients. For the T1 analysis, samples were available for 61 cases and 132 subcohort patients. A total of 148 patients had both T0 and T1 samples available during which they received VKA-treatment at T0 and had stopped treatment at T1, these were included for longitudinal analysis.

RESULTS

Patient characteristics and VTE recurrence

Of the 883 patients enrolled in the VISTA study and treated with VKA for a first VTE, 245 participants with available samples were selected for the current case-cohort analysis (**Figure 1**). Additionally, a complementary analysis was conducted to compare cases and controls; the number of participants in each group is detailed in the supplemental information (**Figure S1**). The study population had a median age of 57 years (IQR: 48-67), and 68% were male (**Table 1**). The index VTE event consisted of distal deep vein thrombosis (DVT) in 10.4% of participants, proximal DVT in 36.5%, and pulmonary embolism (PE) in 53.1%. The median duration of VKA therapy was 187 days (IQR: 181–207). Median D-dimer levels measured during anticoagulation were 270 ng/mL (IQR: 190–385), increasing to 455 ng/mL (IQR: 265–1240) after cessation of anticoagulation.

The timing of sample collection and sample availability were similar between groups, although the recurrence group had fewer T1 samples, likely due to recurrence occurring before the second blood withdrawal (**Table 1**).

Table 1. Patient characteristics in case-cohort design

	Cases (N=96)	**Subcohort (N=192)**
Age, Median (IQR)	56.5 (48.0-67.0)	55.0 (46.8-64.3)
Male, No. (%)	65 (67.7%)	122 (63.5%)
Hormonal therapy, No. (%)	10 (10.4%)	34 (17.7%)
Type of primary VTE, No. (%)		
Distal VTE	10 (10.4%)	29 (15.1%)
Proxymal VTE	35 (36.5%)	70 (36.5%)
PE	51 (53.1%)	93 (48.4%)
Recurrence, No. (%)	96 (100%)	21 (10.9%)
Available samples at T0, No. (%)	87 (90.6%)	167 (87.0%)
D-Dimer levels at T0, Median (IQR)	270 (190-385)	283 (205-385)
Missing, No. (%)	40 (41.7%)	93 (48.4%)
Available samples at T1, No. (%)	61 (63.5%)	132 (68.8%)
D-Dimer levels at T1, Median (IQR)	455 (265-1240)	390 (240-670)
Missing, No. (%)	32 (33.3%)	55 (28.6%)
Number of days between T0 and T1, Median (IQR)	49.0 (34.0-64.0)	49.0 (35.8-72.3)
Missing, No. (%)	29 (30.2%)	56 (29.2%)
Days to recurrence, Median (IQR)	147 (48.0-477)	211 (37.8-510)
Missing, No. (%)	29 (30.2%)	56 (29.2%)
Time on VKA, Median (IQR)	187 (181-207)	184 (179-198)
Missing, No. (%)	9 (9.4%)	32 (16.7%)

Proteomic depth and agreement with quantitative conventional laboratory test

In total, 413 samples were analyzed in a random order with a plasma proteomics workflow across different sample preparation plates. To monitor the reproducibility of relative quantification across the different sample preparation plates, reflecting analytical performance, each plate contained three wells of a quality control pooled sample. The quality control samples revealed 423 quantified proteins (94%) with a coefficient of variation below 30% at a median CV of 8.8 (**Figure 2A**). These also included biomarkers for VTE recurrence postulated in literature, which were stable across the QC samples (**Figure 2B**). Across all patient samples 479 proteins were quantified, with similar proteomic depth between timepoints and recurrence (**Figure 2C**). To further assess the robustness of quantification in this cohort, protein levels as measured with MS were correlated with conventional immune-assay based test results. These showed a good correlation for prothrombin to F2 (**Figure 2D**), VWF to VWF (**Figure 2E**), fibrinogen to FGA (**Figure**

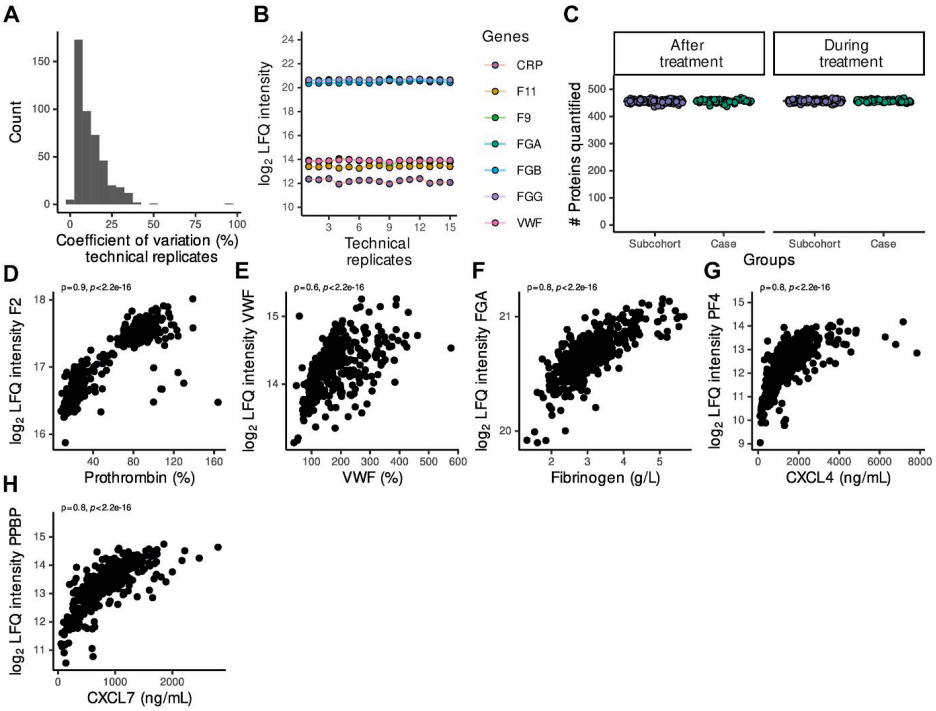

Figure 2: Proteomic depth and agreement with laboratory measurements

(A) Histogram representing the coefficients of variation for all proteins quantified in quality control samples (QCs). (B) Label-free quantification (LFQ) intensity levels of literature suggested biomarkers (C-reactive protein; CRP, coagulation factor XI; F11, coagulation factor IX; F9, fibrinogen alpha chain; FGA, fibrinogen beta chain; FGB, fibrinogen gamma chain; FGG, von Willebrand factor; VWF) across QC samples. (C) proteomic depth of samples from cases (green) and subcohort (purple) stratified by treatment at time of sampling. (D-H) Scatter plot shows the correlation between proteins quantified using laboratory measurement (y-axis) and MS acquisition (x-axis) for prothrombin (D), von Willebrand factor (VWF, E), fibrinogen (F), C-X-C motif chemokine 4 (CXCL4, i.e. platelet factor 4 (PF4), G) and C-X-C motif chemokine 7 (CXCL7, i.e. platelet basic protein (PPBP), H).

2F), CXCL4 to platelet factor 4 (PF4, **Figure 2G**) and CXCL7 to pro-platelet basic protein (PPBP, **Figure 2H**) with all correlation coefficients (ρ) above 0.6. Overall, we found high reproducibility, as highlighted by the technical replicates, and robust data, as determined by agreement with conventional immune-assay based testing.

No association between plasma protein levels with recurrent VTE during VKA

To study changes associated with recurrence during VKA treatment, we examined the global plasma proteome using principal component analysis (PCA, **Figure 3A**), variation analysis (**Figure 3B**) and statistical analysis (**Figure 3C-E**). We observed no differences associated with recurrence between cases and subcohort (**Figure 3A**) or cases and controls (**Figure S2A**) during treatment in the PCA, indicating that the global plasma proteome variation was not based on recurrence. In both the case-cohort (**Figure 3B**) and case-control (**Figure S2B**) we observed expected inter-individual biological variation irrespective of VTE recurrence, e.g. serum amyloid A-1 protein (SAA1), serum amyloid A-2 protein (SAA2), immunoglobulin heavy constant delta (IGHD), immunoglobulin heavy constant epsilon (IGHE), apolipoprotein-a (LPA), pregnancy zone protein (PZP). Cases were statistically compared to subcohort (**Figure 3C**), and additional sub-analysis were performed because most recurrences occur in the first months after cessation of anticoagulation and sex differences in recurrence have been reported. Analysis of patients with early recurrence (≤ 90 days, n = 178, 32 recurrence and 146 without recurrence, **Figure 3D**) and stratified for sex (**Figure 3E**) are shown. In all three cases, no significant differences in protein levels were found: neither with case-cohort design (**Figure 3C – 3E**) nor case-control design (**Figure S2C-E**). In summary, no plasma protein signatures, including previously suggested biomarkers for recurrence (CRP, F9, F11, FGA, FGB, FGG and VWF) (**Figure 3F**), associated with VTE recurrence during VKA-treatment.

Reduction of GLA-domain containing proteins during treatment

The effect of VKA on the plasma proteome was assessed by comparison of protein levels during and after treatment. Based on principal component analysis, no separation of samples during or after VKA-treatment was observed (**Figure 4A**). However, 17 proteins were significantly different (BH-adjusted p-value < 0.05), of which two with an absolute $\log_2$-fold change ≥ 1 (**Figure 4B – Table S2D**). The cluster of proteins with the lowest p-value (BH-adjusted p-value < 10^{-40}) contained all vitamin-K dependent, Gla-domain containing coagulation factors (F2, F7, F9, F10, protein C (PROC), proteins S (PROS1) and protein Z (PROZ)), along with complement 4 binding protein (C4BP)-A and C4BPB (binding partners of protein S) and protein Z inhibitor (SERPINA10) (**Figure 4B, 4C**). For proteins in this group, levels returned to those observed in the control group after discontinuation of VKA treatment (**Figure S3**). Although the levels of protein C receptor (PROCR), coagulation factors FXIIIA (F13A1) and FXIIIB (F13B), and apolipoprotein C1 (APOC1), -C2 (APOC2) and catalase (CAT1) changed significantly after cessation of VKA therapy, differences between on- and off-treatment and differences with healthy controls were less consistent (**Figure 4C, S3**). For serum amyloid P-component (APCS) and PROCR, levels were increased during

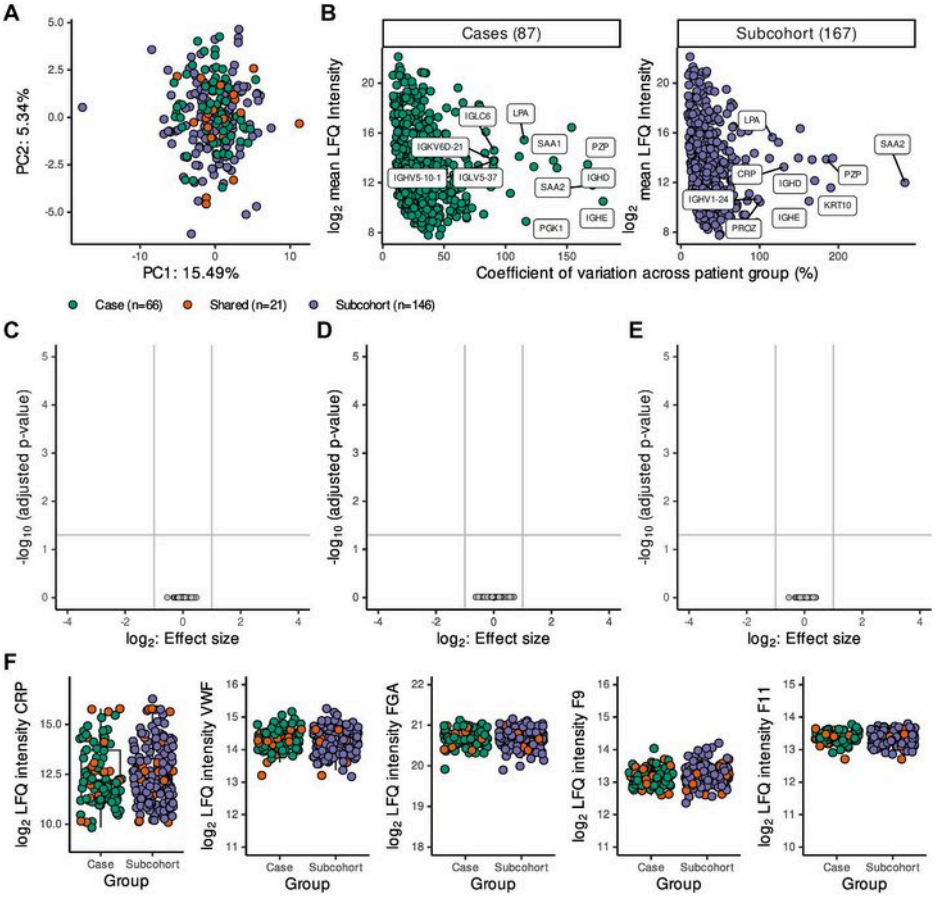

Figure 3. Protein associations with risk for recurrent VTE during VKA-treatment

(A) Principal component analysis scores plot based on samples collected during VKA-treatment, colours represent cases (green), subcohort (purple) and patients presented in both groups (orange). (B) Biological coefficient of variation (CVs) of the plasma proteins measured in cases (green) and subcohort (purple) plotted against the mean LFQ intensities. Genes with the highest CVs are labelled. (C) Volcano plot with proteins significantly different between patients with and without recurrent VTE during VKA treatment. (D) Volcano plot with proteins significantly different between patients with early recurrence (< 90 days) and without recurrent VTE during VKA treatment. (E) Volcano plot with proteins significantly different between patients with and without recurrent VTE during VKA treatment stratified for gender. (F) Levels of literature suggested biomarkers (C-reactive protein; CRP, coagulation factor XI; F11, coagulation factor IX; F9, fibrinogen alpha chain; FGA, fibrinogen beta chain; FGB, fibrinogen gamma chain; FGG, von Willebrand factor; VWF) during VKA treatment in cases (green), subcohort (purple) and patients presented in both groups (orange).

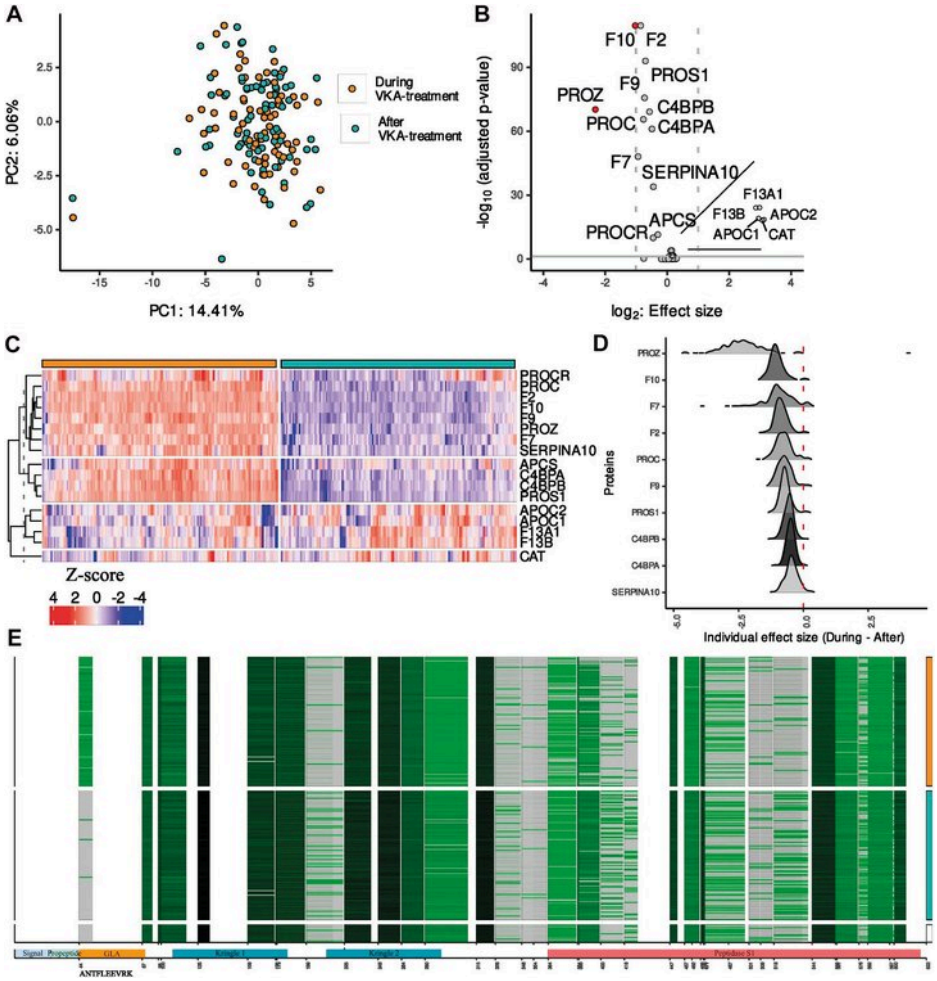

Figure 4. Effect of VKA on the global plasma proteome in patients at risk for recurrent VTE

(A) Principal component analysis scores plot based on all patient samples, colours represent samples collected during VKA-treatment (brown) and after VKA-treatment (turquoise). (A) Volcano plot showing proteins significantly different during and after VKA treatment (n=148). (C) Heatmap with Z-scored levels of all significantly different proteins during (brown) and after VKA-treatment (turquoise). (D) The effect of VKA-treatment expressed by the distribution in relative difference in intensity for patients with paired samples available (n=148). the red dashed line indicates no change. (E) Prothrombin amino acid sequence coverage per individual patient during (brown) and after VKA-treatment (turquoise) along with healthy controls (white).

versus after cessation of VKA treatment (**Figure 4C**). However, only for APCS, levels remained increased compared to the control group after cessation of VKA treatment (**Figure S3**).

To assess the effect size of the reduction in Gla domain–containing proteins between individuals, we compared the distribution of protein level differences (**Figure 4D**). This revealed a protein-specific effect of VKA treatment, which was highest for PROZ and F10, followed by F7, F2, PROC, F9, PROS1 as well as C4BPA, C4BPB and SERPINA10. Finally, sequence coverage of all GLA-domain containing proteins revealed detection of a non-gamma-carboxylated peptide from the GLA domain for F2 (43-ANTFLEEVRK-53), which could only be detected in individuals during VKA treatment and not in healthy controls nor patients after VKA discontinuation (**Figure 4E**). This highlights the feasibility of DIA-based MS profiling to capture meaningful treatment-specific differences in plasma.

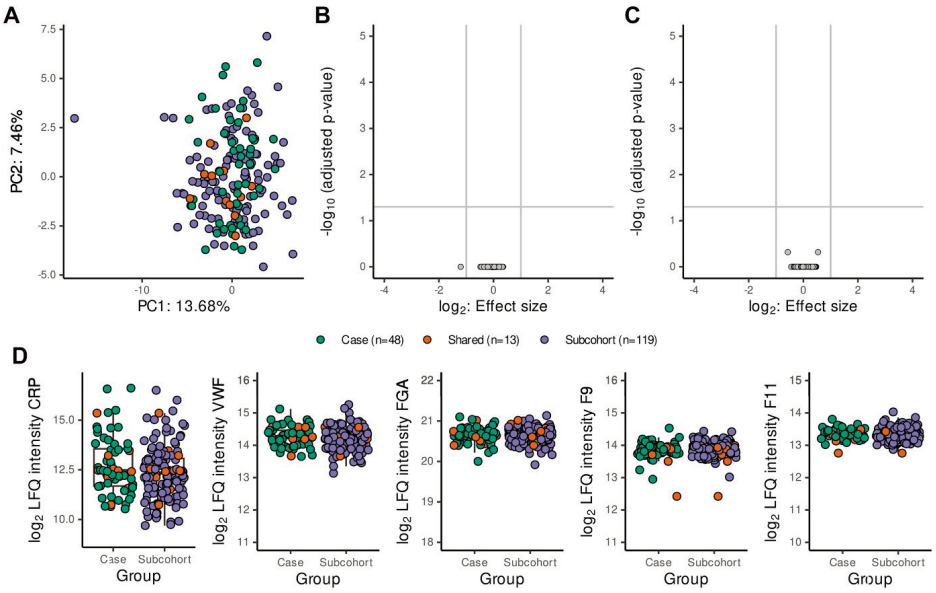

Figure 5. Protein associations with risk for recurrent VTE after VKA-treatment
(A) Principal component analysis scores plot based on samples collected during VKA-treatment, colours represent cases (green), subcohort (purple) and patients presented in both groups (orange). (B) Volcano plot with proteins significantly different between patients with and without recurrent VTE after VKA treatment. (C) Volcano plot with proteins significantly different between patients with and without recurrent VTE based on the effect of VKA discontinuation. (D) Levels of literature-reported biomarkers (C-reactive protein; CRP, coagulation factor XI; F11, coagulation factor IX; F9, fibrinogen alpha chain; FGA, fibrinogen beta chain; FGB, fibrinogen gamma chain; FGG, von Willebrand factor; VWF) after VKA treatment in cases (green), subcohort (purple) and patients presented in both groups (orange).

No association between plasma protein levels with recurrent VTE after VKA
Finally, we studied the changes associated with VTE recurrence after cessation of VKA treatment. Global plasma proteome analysis after cessation of VKA therapy also did not show variation based on recurrence status (**Figure 5A**). These observations were also made in the global plasma proteome using a case-control design (**Figure S4A**). In addition, no proteins were significantly associated in VTE after treatment (**Figure 5B**). An additional sub-analysis identified no significant differences associated to recurrence in the relative changes in protein levels prior and after cessation of treatment (**Figure 5C**). These observations were also made using a case-control design (**Figure S4B-C**). We identified no differences in plasma protein levels that were previously suggested as biomarkers for recurrence (CRP, F9, F11, FGA, FGB, FGG, VWF) (**Figure 5D**).

DISCUSSION

In this study, we applied an unbiased proteomics approach to identify plasma biomarkers associated with recurrent venous thromboembolism (VTE). In line with previous research (23, 34), we observed clear proteomic differences in VKA-dependent proteins between samples collected during and after anticoagulation treatment, supporting the technical validity of our approach. However, we did not identify plasma proteins associated with VTE recurrence risk.

A key methodological feature of this study is the use of a case-cohort design, which offers an efficient and robust approach for biomarker discovery (35, 36). By including all patients who experienced recurrence and a randomly selected subcohort from the full VISTA cohort, we were able to estimate associations with recurrence while minimizing the number of samples subjected to proteomic analysis. This design preserves the advantages of prospective data collection and reduces selection bias compared to traditional case-control studies. To assess the robustness of our findings, we also performed a conventional case-control analysis using the same dataset. Even with this approach, we did not identify proteins that were significantly associated with recurrence. The lack of identification of recurrence-associated biomarkers across the two study design strategies suggests that the plasma proteomic differences between patients with and without recurrence, if any, are small, subtle, transient or biologically diffuse. Future biomarker studies focused on improved ability to detect subtle changes in protein levels may therefore benefit from complementary designs, including longitudinal sampling in proximity to recurrence events, in larger cohorts, potentially combined with targeted proteomics.

In line with previous findings, we captured the reduction of all C4BPB and VKA-dependent proteins during anticoagulation in comparison to healthy controls, which is most clear for PROZ and F2[23]. The current study design, including samples during and after cessation of VKA-treatment enabled for the distinction between inter-individual variation of protein levels. These individual-specific effect sizes showed that, although the extent of reduction

is heterogenous between proteins, the relative effect size was comparable between the individual patients as the outliers for PROZ were the same as for F2. Differences may be due to the number of potential γ-carboxylated residues in the GLA-domains and their effect on the secretion of the proteins as it has been reported that incomplete carboxylation of PROZ and PROS1 may result in degradation, while incompletely γ-carboxylated F9 may still be secreted into circulation (37). Especially for PROZ, which contains 13 glutamic acid residues, this might explain the discrepancy between extent of reduction and associations with functionality. Although this study did not capture the modified residues on these proteins, we did identify a non-modified peptide in the GLA-domain of F2, indicating the complete absence of γ-carboxylation (38, 39). This might suggest the potential of monitoring this unmodified peptide of prothrombin for therapeutic drug monitoring in VKA therapy. In addition to decreased VKA-dependent coagulation proteins, we also identified secondary alterations in protein levels with small effect sizes which were consistent for APCS, C4BPA, PROCR and SERPINA10, but inconsistent for APOA1, APOC1, F13A1, F13B and CAT. Interestingly, for APCS, protein levels were lower during treatment, albeit at similar levels as healthy controls, and increased upon cessation. APCS has been described to interact in a calcium-dependent manner with fibronectin (40) and to inhibit the regulatory function of the C4-BP complex through binding (41, 42). Complex assembly of C4-BPA, C4-BPB, PROS1 with APCS might explain these observations (43).

Although previous biomarker studies have reported associations between the recurrence of VTE and levels of plasma proteins (14-17, 19-22, 44), our study did not reveal significant differences in these markers. This is consistent with the lack of consensus and reproducibility between studies (45) and in line with our own previous research (23). The lack of agreement in biomarker studies may, in part, be attributed to variations in study design and inclusion of specific subgroups of patients, differences in pre-analytical handling of plasma samples, protein quantification strategies and data normalization. Additional targeted MS-based and proximity extension assay-based proteomic approaches, capturing putative biomarkers not identified in the current study (46, 47), are routinely applied. The complementary nature of these approaches, in part due to their distinct characteristics (48), suggests that the search for biomarkers may benefit from combined application of these complementary quantitative approaches. Additionally, expanding beyond the proteome could identify other relevant biomarkers, for example in metabolic or lipidomic profiles (49). Alternatively, qualitative approaches, allowing for assessment of protein function or enzymatic reactions, may provide a novel perspective on thrombosis at the molecular level. For instance, proteolytic signatures in plasma can capture peptide products derived from pro- and anticoagulant events within and beyond the classical coagulation system (50, 51). Alternatively, proteoforms, including polymorphic variants in proteins (52) and post-translational modifications (53, 54), may reveal recurrence-associated changes not captured in a quantitative proteomics design.

A major strength of this study is the use of a well-characterized, prospective cohort from a randomized controlled trial allowing for systematic follow-up and standardized sample collection at clinically relevant time points. Samples were collected just before cessation of anticoagulation; the critical moment in clinical practice when the decision whether to continue therapy is made, and one month after cessation; when D-dimer levels can be reliably measured. Furthermore, the availability of paired plasma samples during and after anticoagulation provided a unique opportunity to investigate biomarkers at both time points, as well as temporal changes in the proteome. Moreover, the unbiased MS-based approach enabled the detection of a broad range of proteins without prior assumptions, allowing for a discovery-based approach to identify unexpected biomarkers that may be suitable for verification in other cohorts and validation in clinical setting. In the current MS-based plasma proteomics study, the quantified proteins correlated well with conventional laboratory measurements, highlighting the high agreement between quantification methods and reliability of the data. Additionally, using technical replicates of the same QC sample we found high reproducibility across the entire experiment, indicating consistent data quality. Nonetheless, several limitations should be considered when interpreting the results of this study. First, the case-cohort design was partially affected by missing samples in the study. However, these were missing at random, and there were no significant differences between patients with and without available samples. Second, the relatively small number of recurrent events and the overall sample size may have limited statistical power to detect potential biologically meaningful, yet subtle protein differences between groups. Larger cohorts with more events would increase the ability to identify such differences with greater confidence. This may be achieved through the combination of multiple, subtype-specific cohorts to increase the verification rate of putative biomarkers (55). Third, low-abundance or highly transient proteins may have been missed by our unbiased MS-based proteomics approach. For example, our global approach did not quantify platelet-derived growth factor β (46), deglycase DJ-1 (44) and leptin (47), which have previously been postulated as potential biomarker based on plasma proteomic studies that include capture of low-abundant proteins through protein depletion and by proximity-based proteomic assays. Fourth, we did not systematically collect information on whether recurrent events were provoked or unprovoked; we recorded only whether it was a DVT or PE. Most recurrence studies follow a similar approach, and only a small proportion of recurrences are provoked, suggesting this is unlikely to substantially affect our findings.

In conclusion, our discovery-based approach offered novel opportunities for therapeutic effect monitoring yet did not identify plasma protein biomarkers predictive of VTE recurrence. These findings underscore the complexity of VTE recurrence prediction and suggest that protein abundance alone may not fully capture relevant biological risk factors.

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Acknowledgement

We acknowledge all the patients that participated in the VISTA trial.

Author contributions

E.R.S, G.P. equally contributed to this work and are co-first authors. E.R.S and D.M.S. performed sample preparation, MS analysis, DIA-NN analysis. E.R.S. performed proteomics data analysis. E.R.S. and G.P. wrote the manuscript. E.R.S., G.P. R.U., and M.v.d.B. designed figures. G.P. selected patients, collected patient samples and clinical data. E.R.S. and C.v.d.Z. collected plasma samples from Sanquin healthy controls and maintained the mass spectrometer. A.J.H. and T.T.v.D. proteomics data analysis. G.J.G., R.S., R.U., M.v.d.B. and S.C.C. designed study and critically revised article. R.U. and M.v.d.B. supervised research and wrote the manuscript. All authors approved the final manuscript.

Disclosure of Conflict of Interest

Authors declare no conflicts of interest.

Funding

The VISTA trial received funding from the Netherlands Organisation for Health Research and Development (ZonMw grant number 171002214).

Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

SUPPLEMENTAL FIGURES

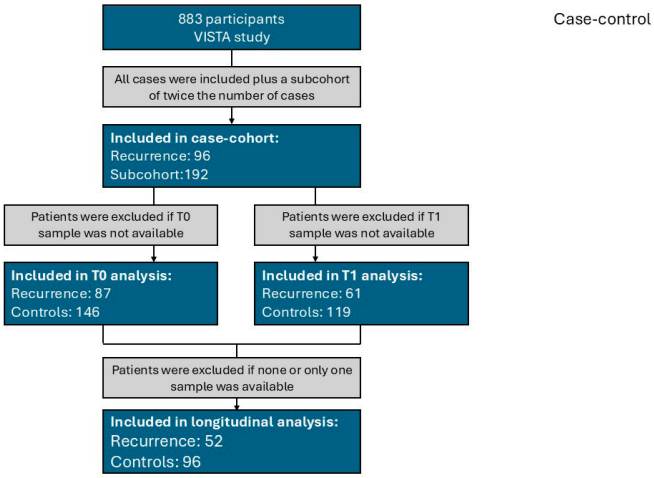


Figure S1. Flowchart of participant selection and sample availability in the VISTA study with case-control design

Of the 883 participants in the VISTA study, 96 recurrence cases and 192 subcohort patients were selected for the current case-cohort analysis. For the T0 analysis, samples were available for 87 patients with recurrence and 146 patients without recurrence. For the T28 analysis, samples were available for 61 patients with recurrence and 119 patients without recurrence. A total of 148 patients had both T0 and T1 samples available during which they received VKA-treatment at T0 and had stopped treatment at T1, these were included for longitudinal analysis.

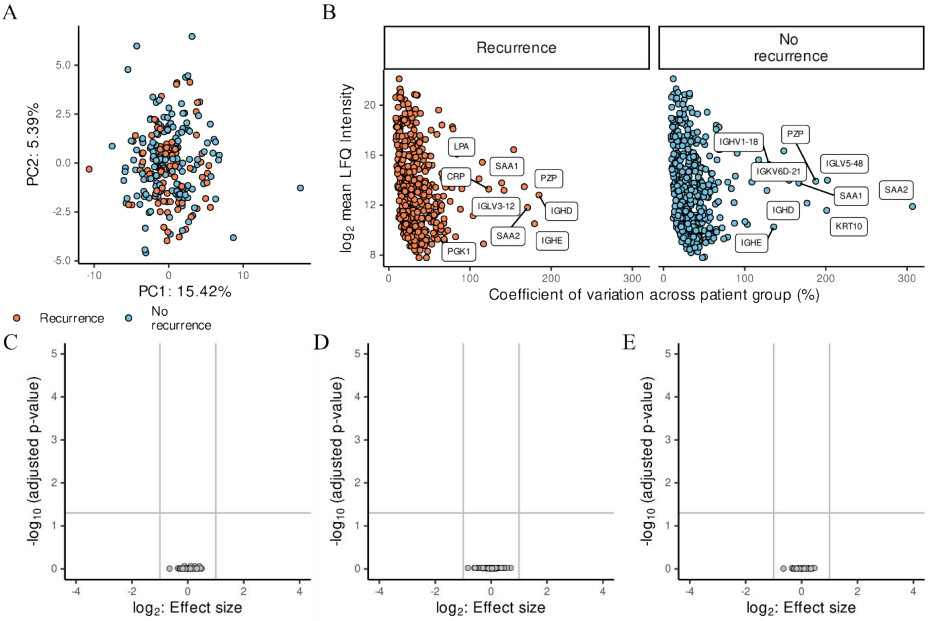


Figure S2 Protein associations with risk for recurrent VTE during VKA-treatment

(A) Principal component analysis scores plot based on samples collected during VKA-treatment, colours represent patients with recurrence (green) and patients without recurrence (orange). (B) Biological coefficient of variation (CVs) of the plasma proteins measured in patients with recurrence (orange) and without recurrence (blue) plotted against the mean LFQ intensities. Genes with the highest CVs are labelled. (C) Volcano plot with proteins significantly different between patients with and without recurrent VTE during VKA treatment. (D) Volcano plot with proteins significantly different between patients with early recurrence (< 90 days) and without recurrent VTE during VKA treatment. (E) Volcano plot with proteins significantly different between patients with and without recurrent VTE during VKA treatment stratified for gender.

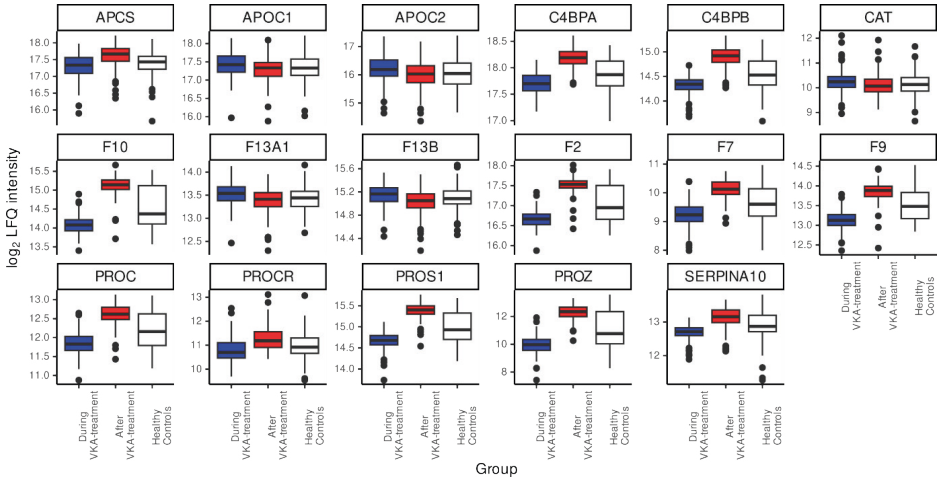


Figure S3. Protein levels changes between on- and off VKA-treatment
Levels of all significant proteins (BH-adjusted p-value < 0.05) in patients during (red) and after VKA-treatment (blue) and in healthy controls (white).

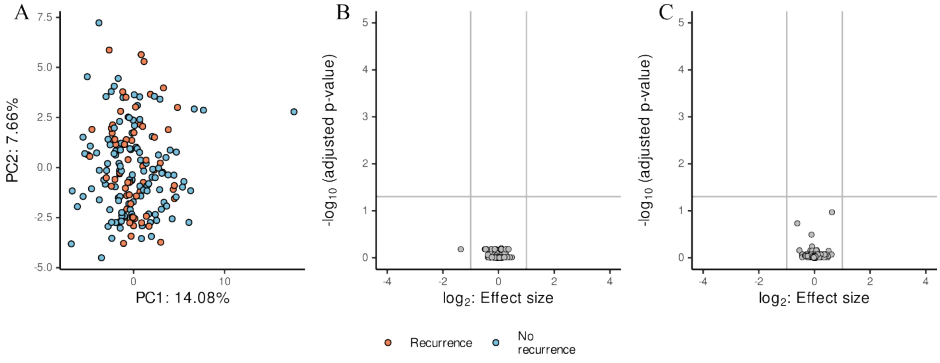
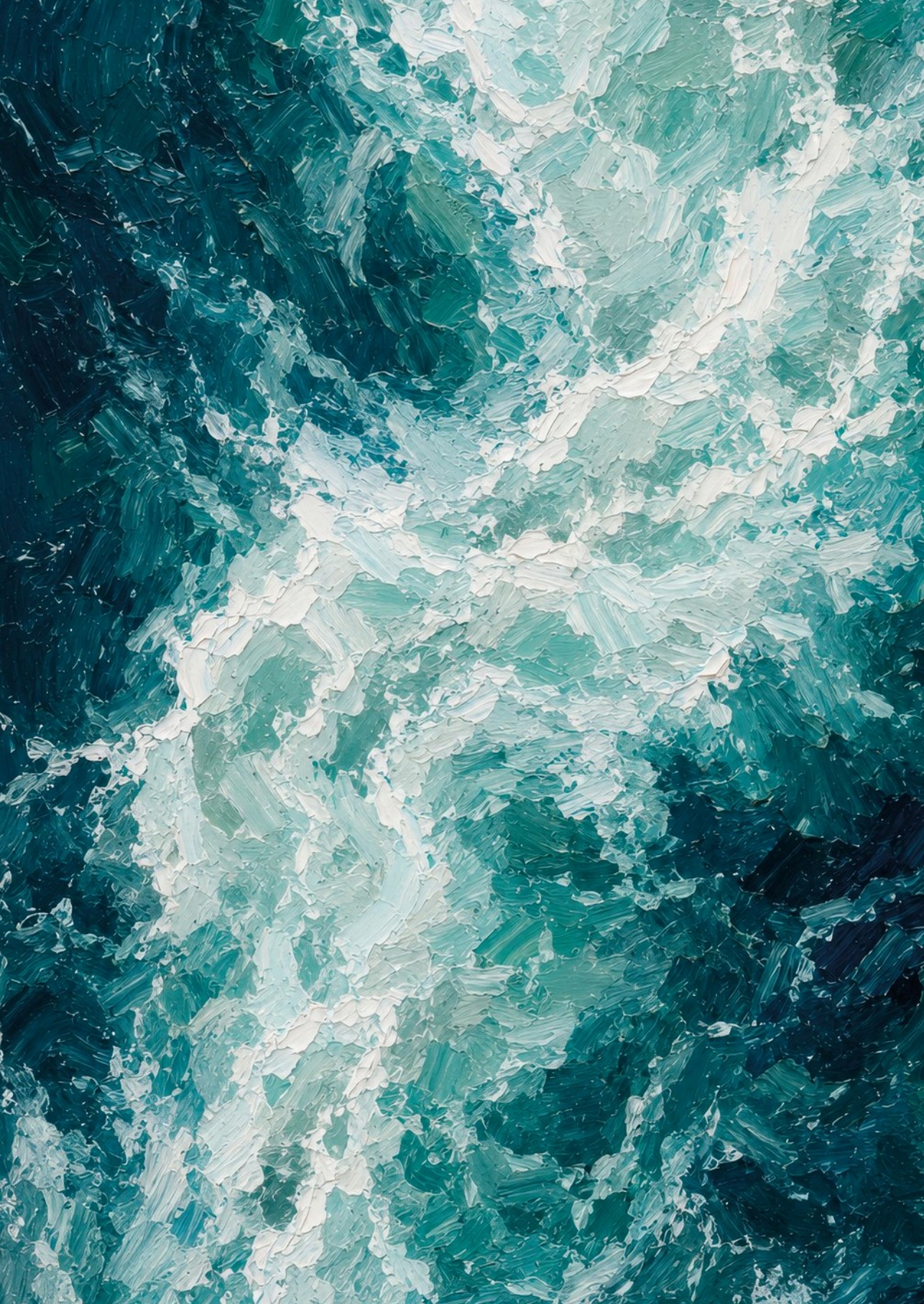


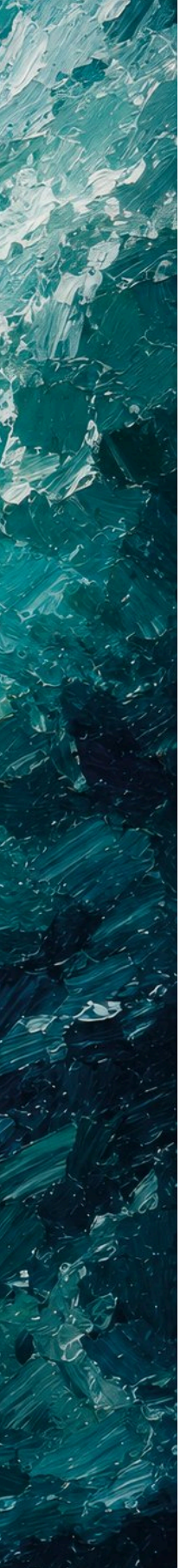
Figure S4. Protein associations with risk for recurrent VTE after VKA-treatment
(A) Principal component analysis scores plot based on samples collected during VKA-treatment, colours represent patients with recurrence (green) and patients without recurrence (orange). (B) Volcano plot with proteins significantly different between patients with and without recurrent VTE after VKA treatment. (C) Volcano plot with proteins significantly different between patients with and without recurrent VTE based on the effect of VKA discontinuation.

SUPPLEMENTAL TABLES

Table S1. Patient characteristics in case-control design

	Recurrence (N=92)	No recurrence (N=153)
Age, Median (IQR)	57.0 (48.0-67.3)	56.0 (48.0-64.0)
Male, No. (%)	61 (66.3%)	97 (63.4%)
Hormonal therapy, No. (%)	10 (10.9%)	26 (17.0%)
Type of primary VTE, No. (%)		
Distal VTE	9 (9.8%)	23 (15.0%)
Proximal VTE	33 (35.9%)	52 (34.0%)
PE	50 (54.3%)	78 (51.0%)
Recurrence, No. (%)	92 (100%)	0 (0%)
Available samples at T0, No. (%)	87 (94.6%)	146 (95.4%)
D-Dimer levels at T0, Median (IQR)	270 (190-385)	290 (210-380)
Missing, No. (%)	40 (43.5%)	72 (47.1%)
Available samples at T1, No. (%)	61 (66.3%)	119 (77.8%)
D-Dimer levels at T1, Median (IQR)	450 (260-1270)	390 (240-668)
Missing, No. (%)	29 (31.5%)	31 (20.3%)
Number of days between T0 and T1, Median (IQR)	49.0 (34.0-64.0)	50.0 (36.0-76.0)
Missing, No. (%)	26 (28.3%)	33 (21.6%)
Days to recurrence, Median (IQR)	147 (47.0-454)	NA
Time on VKA, Median (IQR)	187 (181-206)	184 (180-200)
Missing, No. (%)	7 (7.6%)	21 (13.7%)





CHAPTER 4

Thrombin generation and thrombin dynamics are associated with recurrent venous thromboembolism after anticoagulation withdrawal

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J Thromb Haemost. 2026 Apr;24(4):1438-1447.

ABSTRACT

Background

Recurrent venous thromboembolism (VTE) is a major concern after stopping anticoagulation. Thrombin generation (TG) parameters—including thrombomodulin (TM) sensitivity—have been proposed as biomarkers of recurrence, but findings remain inconsistent. The role of thrombin dynamics (TD) parameters, including prothrombin conversion and thrombin inactivation, is unclear.

Objective

To evaluate whether TG and TD parameters are associated with VTE recurrence.

Methods

In 589 patients from the prospective VISTA study, TG was measured during anticoagulation and one month after stopping VKA therapy. TM sensitivity was quantified as the percentage inhibition of endogenous thrombin potential (ETP) and peak thrombin concentration. TD parameters were derived computationally from TG curves. Cox regression was used to evaluate associations with recurrent VTE over two years, including subgroup analyses excluding hormone-related events.

Results

During the two-year follow-up, 63 patients experienced recurrent VTE. In multivariable Cox regression adjusted for age, sex, index event type, and hormone use, reduced thrombin inactivation by antithrombin (T-AT) was associated with recurrence (per 100-unit decrease: HR: 1.10, 95% CI: 1.00-1.20). In patients without hormone-related VTE, lower ETP and reduced TM-mediated inhibition of peak thrombin concentration were also significantly associated with recurrence (ETP per 100-unit decrease: HR: 1.11, 95% CI: 1.01-1.21; TM sensitivity per 10% decrease: HR: 1.26, 95% CI: 1.00-1.58). Other TG parameters were not significantly associated with recurrence.

Conclusions

Reduced ETP, TM sensitivity and thrombin inactivation were associated with VTE recurrence in people with unprovoked VTE. These biomarkers may have potential to identify patients at higher risk of recurrence.

KEYWORDS

Biomarkers – Blood Coagulation Tests – Cohort Studies – Recurrence – Venous Thromboembolism

INTRODUCTION

Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common and potentially life-threatening condition caused by blood clots in veins. Standard anticoagulant treatment consists of anticoagulation for three to six months. Within the first year after discontinuation of anticoagulation approximately 10% of patients experience a recurrence (1). Recurrent VTE significantly increases mortality and the risk of long-term complications, such as post-thrombotic syndrome, which can severely affect patients' quality of life (2-4). Identifying individuals at high risk for recurrence is essential to support treatment decisions, including the possibility of extended anticoagulation to reduce the risk of future thrombotic events.

Biomarker-based risk prediction could help guide clinical decisions on whether to continue or discontinue anticoagulation therapy (5, 6). Several biomarkers have been explored for their potential to predict VTE recurrence, including D-dimer levels and more advanced coagulation assays such as thrombin generation and clot lysis time assays (7, 8). The thrombin generation (TG) assay measures an individual's capacity to generate thrombin, a central enzyme in the coagulation cascade. High endogenous thrombin potential (ETP), a key parameter of TG, has been associated with an increased risk of VTE recurrence in some studies (9-12). However, other studies have either failed to confirm this association or found an inverse relationship, highlighting variability in the results (13-15). While TG shows promise, standard TG parameters may not fully capture the complexity of clot formation and dissolution. To address this limitation, thrombin dynamics analysis provides a more detailed approach by using an algorithm to study both pro- and anticoagulant processes underlying thrombin generation. This computational model divides the TG curve into two main processes: prothrombin conversion and thrombin inactivation (16, 17). By doing so, it allows for a more nuanced assessment of the balance between pro- and anticoagulant factors.

In this study, we investigated the association between thrombin generation and thrombin dynamics parameters with the recurrence of VTE using samples and data from the VISTA study, a randomized trial conducted across nine thrombosis services in the Netherlands with 883 patients (18). By identifying novel biomarkers associated with VTE recurrence, we aim to improve early risk assessment and inform clinical decision-making, ultimately personalizing treatment strategies for high-risk patients.

METHODS

We included patients from the VISTA study. The detailed study protocol and primary results have been described elsewhere (18). In short, the VISTA study was an unblinded randomized trial, designed to evaluate the implementation of the Vienna Prediction Model (VPM), a risk prediction tool for recurrent VTE (19). Patients with a first unprovoked VTE were enrolled between 2011 and 2015 at nine thrombosis services throughout the Netherlands.

All patients initially received standard treatment with Vitamin K antagonists (VKA) for approximately six months. After randomization, the decision to continue or discontinue anticoagulation was guided by VPM risk estimates in the intervention arm or usual care in the control arm. The primary outcome was recurrent VTE during approximately two years of follow-up. All participants provided written informed consent, and the Medical Ethics Review Committee (METC) of UMC Utrecht approved the study protocol.

Blood samples were collected at two predefined time points: T0, immediately before cessation of VKA therapy (after approximately 5–6 months of anticoagulant treatment), and T1, one month after discontinuation of anticoagulation (approximately 7 months after the index VTE event). Platelet-poor plasma was prepared by centrifuging whole blood at 2,000 g for 10 minutes at room temperature, followed by a second centrifugation of the plasma under the same conditions to ensure platelet counts $<10\times10^9$/L. Plasma samples were stored at -80°C until analysis.

Patients with provoked VTE, such as patients with recent surgery, use of plaster casts, pregnancy, or in the postpartum period, were excluded from the study. Individuals with a VTE history in the past decade, active malignancies, or other indications of anticoagulant therapy—like atrial fibrillation or the presence of prosthetic heart valves—were also excluded. VTE cases associated with oral contraceptive use and hormone replacement therapy were included, as these were previously categorized as unprovoked due to classification uncertainties at the time of inclusion.

Current study design

The current cohort included all patients with available blood samples collected one month after cessation of anticoagulation (T1), irrespective of randomization arm. Patients were excluded if they were still receiving anticoagulation at the time of T1 sampling or if the T1 sample was unavailable. For analysis at the T0 (during anticoagulation), patients were excluded if the sample was unavailable or if T0 sampling did not occur during VKA treatment.

Recurrent VTE was defined as proximal DVT or PE, confirmed by compression ultrasonography for DVT and computed tomography pulmonary angiogram (CTPA) for PE, and accompanied by initiation of anticoagulation therapy. Outcome assessment was performed by reviewing all available hospital discharge information. Follow-up time was calculated from the date anticoagulant therapy was stopped to the date of recurrent VTE, the last follow-up visit, or the end of the study period, whichever came first. Patients without a recurrence were censored at 800 days of follow-up.

Coagulation factors

Prothrombin activity, antithrombin and fibrinogen, were measured with the STA-R Evolution coagulation analyzer (Diagnostica Stago, Asnières, France). Fibrinogen was quantified with the Clauss assay. Functional α_2-macroglobulin (α_2M) activity was determined with an in-house chromogenic assay, as previously described (Synapse Research Institute, Maastricht, the Netherlands) (17). D-dimer levels were measured locally, either as part of routine clinical care or, for participants in the VISTA study intervention arm, to support risk stratification using the Vienna Prediction Model.

Thrombin generation

Thrombin generation (TG) was measured with Calibrated Automated Thrombinography (CAT), as previously described (20, 21). For each patient, TG was measured with PPP Reagent Low and PPP reagent (Diagnostica Stago, Asnières, France), with or without recombinant soluble thrombomodulin (TM, 1.5 nM). TG was initiated by FluCa substrate mix containing ZGGR-AMC substrate (Bachem, Basel, Switzerland, 417 µM final concentration) and calcium chloride (16.7 mM final concentration). Measurements were performed in half-volume format and in duplicate (22).

Thrombin dynamics

The computational approach used to determine prothrombin conversion and thrombin inactivation has been described previously (17, 23). In brief, the TG curve reflects the net result of two opposing processes that occur simultaneously: prothrombin conversion and thrombin inactivation.

Thrombin inactivation is estimated using a validated kinetic model based on plasma levels of antithrombin (AT), alpha-2-macroglobulin (α_2M), fibrinogen and free thrombin, the latter derived from the TG curve. The model consists of differential equations that describe the rate of thrombin inactivation at each point in time:

$$1)\ \frac{d(T\text{–}AT)}{dt} = k_{AT} \cdot [AT]_t \cdot [T_{free}]_t$$

$$2)\ \frac{d(T\text{–}\alpha_2M)}{dt} = k_{\alpha_2M} \cdot [\alpha_2M]_t \cdot [T_{free}]_t$$

$$3)\ \frac{-d(T_{free})}{dt} = k_{AT} \cdot [AT]_t \cdot [T_{free}]_t + k_{\alpha_2M} \cdot [\alpha_2M]_t \cdot [T_{free}]_t$$

Using this inactivation model, the total amount of thrombin inactivated at each point during the TG measurement can be calculated. By combining this with the observed TG curve, the underlying prothrombin conversion profile can be inferred as the sum of thrombin inactivation and net thrombin generation.

The following thrombin dynamics parameters are derived:

- PC_{tot}: total amount of prothrombin converted (AUC)
- PC_{max}: maximal prothrombin conversion rate (peak height)
- T-AT: total amount thrombin inactivated by antithrombin
- T-α_2M: total amount thrombin inactivated by alpha-2-macroglobulin
- TDC: thrombin decay constant, reflecting the rate of overall thrombin inactivation, independent from the TG curve

These parameters are computationally inferred from the TG curve and reflect the underlying processes rather than direct experimental measurements. The model has been previously validated against experimental measurements of prothrombin conversion (17).

Statistical analysis

All analyses were performed using R (version 4.5.0), and figures were created with GraphPad Prism (version 10.4). A two-sided p-value < 0.05 was considered statistically significant. Baseline characteristics were compared between patients with and without recurrence using the t-test for continuous variables and the chi-squared test for categorical variables. TG parameters were presented as medians with interquartile ranges (IQR) and compared using the Wilcoxon Rank-Sum Test. Thrombomodulin (TM) sensitivity was assessed by calculating the percentage inhibition of TG, measured as reductions in endogenous thrombin potential (ETP) and peak thrombin, after TM addition, which reflects activated protein C (APC) pathway function.

For thrombin dynamics (TD) analysis, missing values for coagulation factors (fibrinogen [n=2], and **α_2**-macroglobulin [n=43]) were imputed using the mean. Missing TG data were not imputed, as the full TG curve is required for analysis and imputing the entire curve would not be reliable. Continuous TG and TD variables were scaled prior to analysis to facilitate clinically interpretable hazard ratios (HRs), as indicated in the text and results tables. Associations between TG and TD markers and recurrence were assessed using Cox proportional hazards regression, with HRs and 95% confidence intervals (CIs) reported. Multivariable models were adjusted for age, sex, hormone use and type of index event (distal or proximal DVT, or PE), which represent risk factors of VTE recurrence, to evaluate the independent contribution of the study parameters. Sensitivity analyses were conducted by excluding patients with hormone-related VTE. To further explore the observed associations between T-AT and recurrent VTE, additional Cox models were performed adjusting T-AT for potential explanatory variables, including antithrombin levels, prothrombin levels, and ETP as covariates.

RESULTS

Baseline characteristics of the study cohort

A total of 589 patients were included in the present analysis from the original VISTA cohort of 883 patients, based on the eligibility criteria shown in **Figure 1**. Of the 294 excluded patients, 136 continued anticoagulation beyond standard treatment, 151 had no available plasma sample after cessation of anticoagulation (time point T1), and 7 had T1 samples drawn while still on anticoagulant treatment due to date inconsistencies. Included and excluded patients were similar in age, recurrence rate, and type of first VTE, suggesting minimal selection bias (**Supplemental Table 1**).

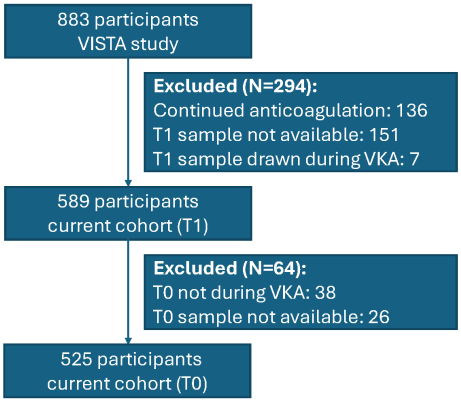

Figure 1. Flow diagram of participant selection for current analysis
The diagram depicts the number of patients initially enrolled in the VISTA study and the subsequent exclusion criteria applied to derive the final cohort for analysis. T1 samples were collected one month after cessation of anticoagulation, and T0 samples were collected during anticoagulation treatment.

During the two-year follow-up, 63 of 589 (10.7%) patients experienced a recurrent VTE event. The median time to recurrence was 199 days (IQR: 68-489) after stopping anticoagulation. Baseline characteristics were generally similar between patients with and without recurrence. However, patients who developed a recurrence tended to be older, were more often male, and were more likely to have had PE as their index event, although these differences were not statistically significant. Comorbidities and BMI were similar between patients with and without recurrence (**Table 1**).

Notably, half of the included women (50%) were using hormones at the time of their first VTE. Recurrence occurred in 6% of hormone users (8 of 132) compared to 12% of non-users (55 of 457). This lower recurrence rate in hormone-associated VTE supports classifying these cases as provoked events, which justifies their exclusion in subsequent subgroup analyses focusing specifically on unprovoked VTE.

Table 1: Baseline characteristics of the cohort

	Total	Patients without recurrence	Patients with recurrence	P-value
	N = 589	N = 526	N = 63	
Age, mean (SD)	55 (14)	55 (14)	58 (12)	0.11
Women, N (%)	266 (45)	243 (46)	23 (37)	0.19
Hormone use, N (% of women)	132 (50)	124 (51)	8 (35)	0.07
Type of 1st VTE, N (%)				0.30
Distal DVT	85 (15)	80 (15)	5 (8)	
Proximal DVT	214 (36)	190 (36)	24 (38)	
PE	290 (49)	256 (49)	34 (54)	
BMI, mean (SD)	28 (5)	28 (5)	27 (5)	0.24
Comorbidities, N (%)				
CVA	10 (2)	10 (2)	0 (0)	
Myocardial infarction	13 (2)	11 (2)	2 (3)	
Angina pectoris	5 (1)	5 (1)	0 (0)	
Heart failure	3 (1)	3 (1)	0 (0)	
Diabetes type 2	35 (6)	31 (6)	4 (6)	
COPD	24 (4)	22 (4)	2 (3)	
Asthma	36 (6)	31 (6)	5 (8)	
Smoking	83 (14)	77 (15)	6 (10)	
Varices	127 (22)	108 (21)	19 (30)	

Data are presented as mean (SD) for continuous variables (age, BMI) and as number (%) for categorical variables (sex, hormone use, index event type, comorbidities). Participants are grouped by recurrence status. Abbreviations: BMI: body mass index, COPD: chronic obstructive pulmonary disease, CVA; cerebrovascular accident, DVT; deep vein thrombosis, PE: pulmonary embolism.

Thrombin generation during anticoagulation is uniformly suppressed

Thrombin generation during anticoagulation was assessed in patients with available T0 samples collected while on VKA treatment (N=525). During anticoagulation, thrombin generation was strongly suppressed by VKA treatment, with endogenous thrombin potential (ETP) values around 320 nM·min. No significant differences in thrombin generation parameters were observed between patients with and without recurrence during anticoagulation (**Supplementary Table 2**).

Lower thrombin generation after anticoagulation in patients with recurrence

After cessation of anticoagulation, thrombin generation was assessed again under standard and low tissue factor (TF) conditions. In the overall cohort, ETP tended to be lower in patients who experienced recurrence compared to those who did not (median: 1402 vs. 1425 nM·min; p = 0.08; **Table 2**), although this difference was not statistically

significant. Peak thrombin concentration and lag time were similar between groups, and consistent results were seen under low TF conditions (1 pM). Prothrombin levels were lower in patients with recurrence (83.8 vs. 89.5; p = 0.046), whereas D-dimer levels were higher in patients with recurrence (450 vs. 360; p = 0.02).

When cases of hormone-associated (provoked) VTE were excluded, a significant difference emerged: patients with recurrent VTE had a lower ETP than those without recurrence (median: 1320 vs. 1402 nM·min; p = 0.046).

In univariable Cox regression, lower ETP was associated with increased risk of recurrence, with each 100-unit decrease linked to a 10% higher risk (HR: 1.10, 95% CI: 1.01-1.19). In the multivariable Cox model adjusted for age, sex, index event location and hormone use, this association showed a similar trend but was not statistically significant (HR: 1.08, 95% CI: 0.99-1.17). In the subgroup without hormone-related VTE, the association remained significant in the multivariable model (HR: 1.11, 95% CI: 1.01-1.21). Other TG parameters, including peak thrombin concentration and lag time, were not significantly associated with recurrence (**Table 3**).

Table 2. Thrombin generation parameters in patients with and without recurrent VTE Thrombin generation 1 month after stopping anticoagulation

	Patients without recurrence	**Patients with recurrence**	
	Median (IQR)	Median (IQR)	P-value
5pM TF			
ETP	1425 (1262-1623)	1402 (1127-1539)	0.08
Peak	262 (209-310)	265 (215-296)	0.51
Lagtime	3.0 (2.7-3.3)	3.0 (2.7-3.7)	0.40
1pM TF			
ETP	1362 (1191-1577)	1325 (1100-1542)	0.32
Peak	232 (150-301)	248 (158-293)	0.87
Lagtime	4.7 (4.0-5.5)	4.8 (4.0-5.7)	0.61
%Inhibition by TM			
ETP	32 (21-46)	30 (19-40)	0.34
Peak	20 (10-30)	15 (9-24)	0.10

Thrombin generation parameters measured at 1 pM and 5 pM tissue factor (TF) are presented as medians with interquartile ranges (IQR, 25th–75th percentile). Differences between patients with and without recurrence were assessed using the Wilcoxon rank-sum test; p-values are shown.

Table 3. Hazard ratios of thrombin generation parameters for risk of recurrent VTE

	Thrombin generation after anticoagulation			
	Univariable		Multivariable	
	HR (CI95%)	P-value	HR (CI95%)	P-value
5pM TF				
ETP (per 100 units ↓)	1.10 (1.01-1.19)	0.03	1.08 (0.99-1.17)	0.07
Peak (per 10 units ↓)	1.02 (0.99-1.05)	0.25	1.02 (0.99-1.05)	0.27
Lagtime (per min. ↑)	1.14 (0.97-1.34)	0.10	1.10 (0.93-1.30)	0.28
1pM TF				
ETP (per 100 units ↓)	1.06 (0.98-1.14)	0.13	1.05 (0.98-1.14)	0.16
Peak (per 10 units ↓)	1.00 (0.97-1.03)	0.97	1.00 (0.98-1.03)	0.75
Lagtime (per min. ↑)	1.08 (0.98-1.18)	0.12	1.06 (0.96-1.17)	0.25
%Inhibition by TM				
ETP	1.09 (0.93-1.28)	0.30	1.10 (0.93-1.29)	0.27
Peak	1.19 (0.97-1.47)	0.10	1.22 (0.98-1.50)	0.07

Univariable and multivariable Cox regression analysis of thrombin generation parameters measured at 1 pM and 5 pM tissue factor (TF). Hazard ratios (HRs) are presented with 95% confidence intervals (CIs) and p-values. Multivariable HRs are adjusted for age, sex, index event location and hormone use.

Reduced thrombomodulin response in recurrent VTE patients

In the overall cohort, thrombomodulin (TM)-mediated inhibition of ETP or peak thrombin concentration did not differ significantly between patients with and without recurrence, although there was a trend toward reduced inhibition in patients with recurrence. The median inhibition of peak thrombin concentration was lower in patients with recurrence (15%) than in those without recurrence (20%), although this was not statistically significant ($p = 0.10$, **Table 2**). Both univariable and multivariable Cox regression showed a trend towards higher recurrence risk associated with reduced inhibition of peak height, but statistical significance was not reached (table 3).

In the subgroup without hormone-related VTE, patients with recurrence had a lower TM response. Specifically, inhibition of peak thrombin concentration was lower in the recurrence group (16%) compared to the non-recurrence group (21%; $p = 0.04$). A similar trend was observed for ETP inhibition (29% vs. 34%, $p = 0.09$) (**Figure 2**).

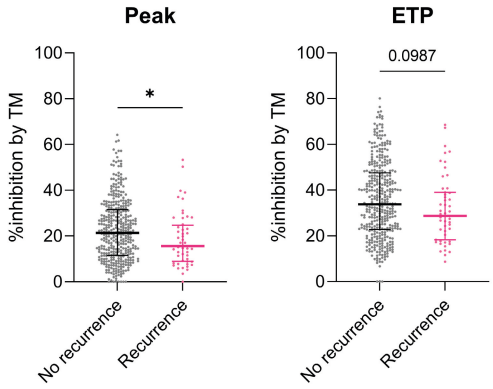

Figure 2. Thrombomodulin response in recurrent vs. non-recurrent unprovoked VTE patients

Scatter plot showing the TM-mediated inhibition of peak thrombin concentration and endogenous thrombin potential (ETP) in patients with and without recurrence, excluding hormone-related VTE cases. Non-recurrent patients are shown in black/gray, and recurrent patients in pink/magenta. Lines indicate median with IQR. Statistical significance is marked with an asterisk (*, $p < 0.05$); non-significant p-values are also shown.

In univariable Cox regression in this subgroup, each 10% decrease in TM-mediated inhibition of peak thrombin concentration was associated with a 27% higher risk of recurrence (HR: 1.27, 95% CI: 1.01-1.59). For ETP inhibition, each 10% decrease corresponded to a 16% higher recurrence risk, although this did not reach statistical significance (HR: 1.16, 95% CI: 0.98-1.39, unadjusted). In multivariable Cox regression, the association of TM-mediated inhibition of peak thrombin concentration remained significantly associated with recurrence (HR: 1.26, 95% CI: 1.00-1.58). These findings suggest impaired activation of the protein C (APC) pathway is associated with VTE recurrence, with the association being more pronounced for peak thrombin inhibition.

Reduced thrombin inactivation by antithrombin is associated with recurrent VTE

Baseline plasma levels of fibrinogen, α_2-macroglobulin and antithrombin (the input parameters of TD) were not individually associated with recurrent VTE (**Table 4**). Free thrombin concentration, the other input parameter of TD, cannot be directly analyzed in this way, because it varies dynamically during thrombin generation.

Computational thrombin dynamics revealed that impaired thrombin inactivation, rather than prothrombin conversion, was more strongly associated with recurrent VTE. In univariable Cox regression, a 100-unit decrease in thrombin inactivation by antithrombin (T-AT) was associated with an increased risk of recurrence (HR: 1.10, 95% CI: 1.01-1.19). This association became stronger after exclusion of hormone-related VTE cases (HR: 1.16, 95% CI: 1.06-1.27, unadjusted).

In contrast, total prothrombin conversion (PC_{tot}) and peak prothrombin conversion (PC_{max}) were not significantly associated with recurrence (PC_{tot} HR: 1.06, 95% CI: 0.99-1.15; PC_{max} HR: 1.01, 95% CI: 0.99-1.03, unadjusted). Other parameters of thrombin inactivation, the thrombin decay constant and thrombin inactivation by **α_2**-macroglobulin (T–**α_2**M), were also not associated with recurrence (**Table 4**).

Table 4. Hazard ratios of thrombin dynamics parameters for risk of VTE recurrence

	Thrombin dynamics after anticoagulation			
	Univariable		Multivariable	
	HR (CI95%)	P-value	HR (CI95%)	P-value
Input parameters				
Antithrombin	1.00 (0.98–1.02)	0.96	1.00 (0.98-1.02)	0.99
Fibrinogen	0.90 (0.61–1.31)	0.57	0.83 (0.56-1.24)	0.36
α_2-macroglobulin	1.02 (0.84–1.24)	0.87	1.00 (0.82-1.21)	0.96
Prothrombin conversion				
Total prothrombin converted (PC_{tot}) (per 100 units ↓)	1.06 (0.99-1.15)	0.09	1.06 (0.98-1.15)	0.14
Maximal prothrombin conversion rate (PC_{max}) (per 10 units ↓)	1.01 (0.99-1.03)	0.41	1.01 (0.99-1.03)	0.43
Thrombin inactivation				
Thrombin decay constant (per 1 unit ↑)	1.14 (0.13-9.79)	0.90	1.36 (0.16-11.93)	0.78
.. by antithrombin (T-AT) (per 100 units ↓)	1.10 (1.01-1.19)	0.02	1.10 (1.00-1.20)	0.04
.. by α2-macroglobuin (T-α_2M) (per 1 unit ↓)	1.01 (1.00-1.02)	0.11	1.01 (1.00-1.02)	0.16

Univariable and multivariable Cox regression analysis of thrombin dynamics parameters derived from thrombin generation curves measured at 5 pM tissue factor (TF). Hazard ratios (HRs) are reported with 95% confidence intervals (CIs) and p-values. Multivariable HRs are adjusted for age, sex, index event location and hormone use.

In multivariable Cox regression adjusted for age, sex, type of index event (distal/proximal DVT or PE), and hormone use, the association between T-AT and recurrence remained significant (HR: 1.10, 95% CI: 1.00-1.20). In a separate model adjusting T-AT for only antithrombin levels, the association with recurrence persisted (HR: 1.11, 95% CI: 1.03-1.21). However, when T-AT was adjusted individually for prothrombin levels or ETP the association was attenuated (adjusted for prothrombin: HR 1.07, 95% CI: 0.98-1.18; adjusted for ETP: HR 1.06, 95% CI: 0.94-1.18). T-AT and PC_{tot} were highly correlated (R=0.98); when both were included in the same model, the HR for T-AT was 1.50 (95% CI: 0.98-2.27) and for PC_{tot} 0.77 (95% CI: 0.55-1.08).

DISCUSSION

We found that lower thrombin generation, as reflected by a reduced ETP measured one month after cessation of anticoagulation, was associated with an increased risk of recurrent VTE. Reduced thrombin inactivation by antithrombin was also linked to higher recurrence risk. After excluding hormone-related VTE cases, focusing on unprovoked events, patients with recurrence showed a reduced response to thrombomodulin. These findings indicate that hemostatic markers differ between patients with and without recurrence, but further studies are needed to determine their utility in risk prediction models.

We observed that lower thrombin generation, particularly reduced ETP, was associated with an increased risk of recurrent VTE. This finding appears counterintuitive, as a prothrombotic state is typically associated with a higher thrombin potential. While no causal mechanism can be inferred from these observational data (24), one possible explanation is consumption of coagulation factors in these patients. Similar paradoxical relationships between thrombin generation and thrombotic risk have been reported other clinical contexts, including acute myocardial infarction and ischemic stroke (25-28). The consumption hypothesis suggests that lower ETP in these patients might reflect a state of ongoing, subclinical coagulation *in vivo,* leading to the consumption of coagulation factors such as prothrombin and Factor V (29, 30). Supporting this, we observed elevated D-dimer levels and lower prothrombin levels in the patients with recurrent VTE. However, further etiologic research is needed to clarify the mechanisms underlying these observations.

Previous studies have reported inconsistent associations between TG parameters and recurrent VTE risk. While several have reported that increased ETP correlates with a higher recurrence risk (9-11, 31), others have found no association or an inverse relationship, which aligns more closely with our findings (13-15, 31). These discrepancies likely reflect heterogeneity in study designs. Differences include the inclusion of provoked versus unprovoked events, and differences in the timing of blood sampling; whether during anticoagulation or after cessation, and whether samples were taken at the time of the acute event or weeks to months later. Other methodological factors, such as sample size, study design (e.g., case-control vs. prospective), and thrombin generation assay conditions—including tissue factor concentration, thrombomodulin use, and presence of inhibitors such as corn trypsin inhibitor—can also influence results. This is evident from the wide range of ETP and peak height values reported across studies, which reflects the known lack of standardization in thrombin generation assays (32, 33). Finally, variation in outcome definitions, follow-up durations, and recurrence rates (e.g., 11% in our study vs. ~30% in others) further complicates comparisons. These methodological differences complicate direct comparisons and limit the generalizability of individual findings. Our study addresses some of these challenges by standardizing timing of blood sampling post-anticoagulation and carefully excluding hormone-related VTE cases in sensitivity analyses. Nevertheless, the field would benefit from larger, multicenter studies with harmonized protocols to validate and refine the prognostic value of TG markers in recurrent VTE risk stratification.

The association between impaired thrombin inactivation by antithrombin (T-AT) and recurrent VTE cannot be directly explained by the individual TD input parameters. We hypothesize that reduced thrombin inactivation by antithrombin reflects a lower amount of thrombin formed, since the association disappears when either ETP or prothrombin levels are added to the model, but remains when adjusting for antithrombin levels. Notably, T-AT shows a stronger association with recurrence than total prothrombin conversion (PC_{tot}). When both T-AT and PC_{tot} are included in the same model, T-AT retains a stronger association; however, these two TD variables are highly correlated (R = 0.98), which may affect the stability and interpretation of the individual hazard ratios in the multivariable model.

Although our findings suggest potential utility of TG and TD parameters in risk stratification, several practical constraints limit their immediate clinical application. A key challenge is the sensitivity of TG-based biomarkers to anticoagulant therapy. Reliable measurements require a washout period after treatment cessation (34-36), as also demonstrated in the current study. This presents a clinical dilemma: patients may experience recurrent events during this brief interruption (37), and the need to restart anticoagulation after biomarker assessment could affect treatment adherence (38). Therefore, while TG and TD markers may improve risk prediction, they cannot be used to prevent early recurrences that occur shortly discontinuing anticoagulation. In addition, TG assays are not yet standardized across laboratories, and no validated cut-offs exist to guide clinical decision-making. Interpretation is further complicated by paradoxical associations, such as the link between lower ETP and higher recurrence risk, which challenge conventional assumptions about prothrombotic states.

This study has several strengths. It is based on a relatively large, well-characterized prospective cohort of patients with a first unprovoked VTE, with standardized follow-up and recurrence events confirmed using predefined clinical criteria. Thrombin generation was measured at two clinically relevant time points, during anticoagulation and one month after its cessation, providing insight into both treatment and post-treatment states. Additionally, thrombin dynamics parameters were derived using a validated computational model, allowing a more detailed assessment of coagulation regulation. However, several limitations should also be acknowledged. First, the follow-up period was limited to two years, potentially missing recurrences that occurred later. However, as most recurrences happen within the first few years after stopping anticoagulation, the impact of this limitation is likely modest (1, 39). Second, patients who continued anticoagulation were excluded. As these individuals were considered at higher risk of recurrence based on the VISTA protocol, their exclusion may have led to an underestimation of biomarker associations. Since highest-risk individuals were not represented, our study population reflects a lower- to intermediate-risk group, which limits the generalizability of our findings to higher-risk patients. Third, although our study focused on unprovoked VTE, we included women with hormone-related VTE, which are currently classified as provoked events. To

account for their distinct clinical profile, we also performed subgroup analyses excluding hormone-related cases.

In conclusion, our study demonstrates that both reduced thrombin generation (ETP), reduced TM sensitivity and reduced thrombin inactivation are associated with increased risk of recurrent VTE, especially in unprovoked cases. Given the variability in findings across studies, further research in larger, more diverse cohorts is needed to validate these associations and to clarify the potential predictive value of thrombin generation and thrombin dynamics parameters. At present, the evidence is insufficient to recommend their routine clinical use and their added predictive value over standard clinical models remains to be investigated.

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Acknowledgement

We gratefully acknowledge the technical assistance of Harmen Middelveld and Veronica with the sample measurements. The VISTA trial received funding from the Netherlands Organisation for Health Research and Development (ZonMw grant number 171002214). The current study was funded by the institutes of the authors.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to enhance the clarity of the writing and to verify grammatical accuracy. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Declaration of interest

Geke C. Poolen – No conflict of interest.
Romy M.W. de Laat-Kremers – Employee of Synapse Research Institute, part of Stago SAS.
Mark Roest – Employee of Synapse Research Institute, part of Stago SAS.
Geert-Jan Geersing – No conflict of interest.
Rolf T. Urbanus – The institution of RT Urbanus has received research funding from Hemab Therapeutics.
Bas de Laat - Employee of Synapse Research Institute, part of Stago SAS.
Roger E.G. Schutgens – The institution of REG Schutgens has received speakers' fees and/or research grants from Bayer, CSL Behring, Hemab Therapeutics, Novartis, NovoNordisk, Octapharma, Roche, Sobi and Takeda.

SUPPLEMENTARY INFORMATION

Supplemental Table 1. Baseline characteristics of included and excluded patients

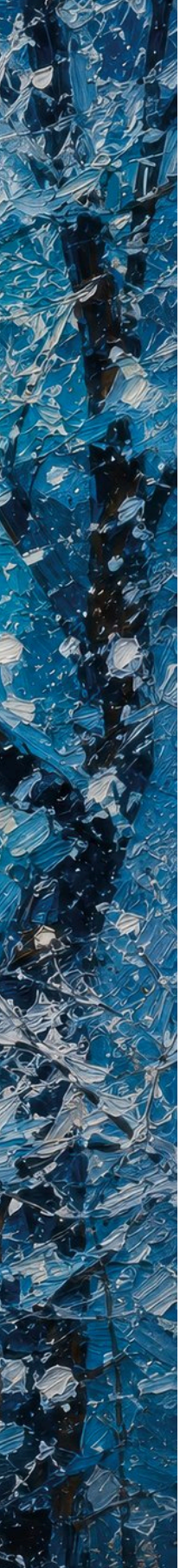
	Included patients	Excluded patients	Missing samples	Continued anticoagulation
	N = 589	N = 294	N = 151	N = 143
Recurrence, N (%)	63 (11%)	33 (11%)	26 (17%)	7 (5%)
Age, mean (SD)	55 (14)	56 (14)	55 (15)	56 (14)
Women, N (%)	266 (45%)	110 (37%)	64 (42%)	46 (32%)
Hormone use, N (% of women)	132 (50%)	51 (46%)	30 (46%)	21 (45%)
Type of 1st VTE, N (%)				
Distal DVT	85 (15%)	41 (14%)	27 (18%)	14 (10%)
Proximal DVT	214 (36%)	94 (32%)	52 (34%)	42 (29%)
PE	290 (49%)	159 (54%)	72 (48%)	87 (61%)

Data are presented as mean (SD) for continuous variables (age) and as number (percentage) for categorical variables (sex, type of first VTE, hormone use). Patients are grouped by inclusion status in the study cohort and split by exclusion reason.

Supplemental Table 2. Thrombin generation parameters during anticoagulation in patients with and without recurrent VTE
Thrombin generation during anticoagulation

	Patients without recurrence	Patients with recurrence	P-value
	Median (IQR)	Median (IQR)	
5pM TF			
ETP	320 (236-431)	309 (209-425)	0.41
Peak	60 (40-82)	57 (35-79)	0.55
Lagtime	6.0 (4.7-8.0)	6.3 (4.7-8.3)	0.51
1pM TF			
ETP	278 (197-381)	263 (161-372)	0.34
Peak	46 (27-72)	41 (26-70)	0.43
Lagtime	9.3 (6.9-13.4)	9.6 (7.2-13.0)	0.52

Thrombin generation parameters measured during anticoagulant treatment at 1 pM and 5 pM tissue factor (TF) are presented as medians with interquartile ranges (IQR, 25th–75th percentile). Differences between patients with and without recurrence were assessed using the Wilcoxon rank-sum test; p-values are reported.



CHAPTER 5

Evaluation of the procoagulant state in chronic immune thrombocytopenia before and after eltrombopag treatment- a prospective cohort study

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J Thromb Haemost. 2023 Apr;21(4):1020-1031.

ABSTRACT

Introduction

Thrombopoietin receptor agonists are frequently used in treating immune thrombocytopenia (ITP) owing to high response rates and good tolerability. ITP is associated with an increased risk of thrombosis. Whether treatment with eltrombopag further increases this risk is controversial. The mechanisms behind the thrombotic risk in ITP are unclear.

Aim

To assess platelet function and hypercoagulability in patients with ITP and the effect of eltrombopag thereon.

Methods

This prospective multicenter study assessed adult primary patients with ITP who were starting eltrombopag treatment. Platelet (re)activity and hypercoagulability were measured in whole blood or plasma before start and after 2 to 3 weeks of eltrombopag treatment and compared with those of controls. Change over time was assessed by mixed-effects models, and the results were corrected for multiple testing.

Results

We included 16 patients and 33 controls. At baseline, patients with ITP exhibited lower expression of glycoprotein VI, more activated platelets, and lower reactivity toward agonists compared with controls. β-Thromboglobulin levels reduced and thrombin generation peak height increased compared with those of controls. In line with this finding, patients with ITP showed high factor VIII (median, 217%; IQR, 174%-272%) and von Willebrand factor levels (median, 167%; IQR, 109%-198%). Eltrombopag treatment increased thrombin generation potential: lag time decreased and peak height and endogeneous thrombin potential increased. The latter changes were not significant after correction for multiple testing.

Conclusion

Patients with ITP in this study were in a hypercoagulable state, with preactivated platelets, increased thrombin generation potential, and increased levels of factor VIII and von Willebrand factor. Eltrombopag treatment further increased plasma thrombin generation potential but no other hemostatic parameters.

KEYWORDS

ITP - TPO-RA - Eltrombopag - Procoagulant - Thrombosis

INTRODUCTION

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by thrombocytopenia due to both peripheral platelet destruction and decreased platelet production by megakaryocytes (1, 2). Management focuses on increasing the platelet count to prevent severe bleeding. Most treatments act by inhibiting the immune system. Another strategy is to increase platelet production by stimulating the megakaryocytes with thrombopoietin receptor agonists (TPO-RAs). TPO-RAs are generally effective, with response rates between 50% and 90%, with relatively few side-effects (3). They are, therefore, increasingly favored over the traditional immunosuppressive strategies (4). Furthermore, TPO-RAs have a potential immune modulatory effect, which could lead to remission of the ITP after discontinuation of treatment (5-7). There are several TPO-RAs on the market, of which eltrombopag and romiplostim are most well known.

ITP is associated with increased thrombotic risk, with an incidence rate of 0.41 to 1.15 thromboembolic events per 100 patient-years (8). Whether treatment with TPO-RAs is associated with additional thrombotic risk is controversial (9-14). Two pooled analyses estimated an incidence rate of 4.2-7.3 thromboembolic events per 100 patient-years for romiplostim and 2.7 for eltrombopag (12, 15, 16). The latter incidence rate was based on data from a single trial rather than a pooled analysis. Conversely, a recent meta-analysis found no significantly increased risk in patients with ITP treated with TPO-RAs compared with those with ITP but not treated with TPO-RAs (risk ratio, 1.82; 95% CI, 0.78-4.24) (17). This may be owing to a lack of power, as the authors point out that the relative risk was greater than 1 in 10 of 11 studies.

Overall, the reason why TPO-RAs would cause thrombosis remains unclear (3, 18). Possible mechanisms are changes in platelet function or changes in secondary hemostasis or endothelial function. Parameters of platelet function that are associated with thrombosis include platelet reactivity to various agonists (including epinephrine, ADP, and thrombin receptor agonist peptide [TRAP]) and the expression of glycoprotein (GP)VI, $\alpha_{IIb}\beta_3$, and P-selectin (19-26). Parameters of secondary hemostasis or endothelial function that have been associated with thrombosis include thrombin generation (TG), factor (F)VIII, D-dimer, fibrinogen, and plasminogen activator inhibitor (27). The studies to date that assessed whether TPO-RAs cause hemostatic changes are heterogeneous in methodology and often find conflicting results, as reviewed in our recent systematic review (24). They generally combine different types of TPO-RAs and use many different methodological approaches to measure platelet function and hemostasis, and coagulation and endothelial function are mostly overlooked. Furthermore, not all studies were prospective, and none performed correction for multiple testing.

In this multicenter cohort study, we prospectively assessed hemostasis in patients with ITP and the effect of eltrombopag treatment to identify possible mechanisms that could lead to thrombosis. To do so, we measured platelet (re)activity, platelet desialylation, and

hypercoagulability before start and after 2-3 weeks of eltrombopag treatment, as other studies showed that any changes in hemostatic parameters occurred within weeks after initiation of the TPO-RA (24, 28).

METHODS

The "Changes in immune profiles and platelet function after start TPO-RA in ITP (I-ITP)" study was a prospective multicentre cohort study that included patients from 5 centres in the Netherlands: the University Medical Center Utrecht (UMCU), Meander Medical Center, St. Antonius hospital, Diakonessenhuis, and Slingeland hospital. Demographics and ITP specifications were extracted from the electronic patient files. This study was approved by the local medical ethical committee and was performed in accordance with the Good Clinical Practice guidelines.

The study follow-up comprised 2 venepunctures: one before start of the eltrombopag (time point 0 [t0]) and one after 2 to 3 weeks after start of the eltrombopag (time point 1 [t1]). All venepunctures and laboratory tests were performed at the University Medical Center Utrecht. Platelet (re)activity was assessed by GP expression with and without agonists, soluble P-selectin and β-thromboglobulin as proxies for in vivo platelet activation, and platelet desialylation, an autoantibody-mediated mechanism of platelet clearance in ITP that can also activate platelets (29). Hypercoagulability was assessed by plasma TG, D-dimer levels, thrombin–antithrombin complexes (TATc), and FV and FVIII (both determinants of the peak height of TG (30)) and von Willebrand factor (VWF) levels.

Subjects

We included patients (16 years or older) with a diagnosis of primary ITP who started treatment with eltrombopag as assigned by their local physician, with a current platelet count $<100 \times 10^9$/L, no indication for an underlying condition, absence of a history of persisting anaemia (defined as hemoglobin <6.0 mmol/L for men and women), and were willing and able to both understand the study information and give written informed consent. The inclusion period was from December 1, 2019, to January 1, 2022. Exclusion criteria were treatment with rituximab in the last 9 months and treatment with any immune-modulating drug other than corticosteroids in the last 3 months. These exclusion criteria were primarily applied for another subquestion regarding immunologic parameters, which will not be addressed in this article.

A control group of 33 people was recruited among employees and students of the UMCU and the faculty of medicine from Utrecht University.

Eltrombopag treatment

Eltrombopag was initiated, monitored, and, if necessary, stopped by the patient's local physician as part of routine care. We recorded the start dose and the dose at t1. If a patient

stopped eltrombopag treatment before t1, study participation was stopped. In those cases, the data were not used for the analyses assessing the effect of eltrombopag on platelet function and coagulation.

Follow-up and thrombosis

We followed up patients until 1 year after starting eltrombopag, until stopping eltrombopag, or until the time of analysis (February 1, 2022), whichever occurred first. At the end of the follow-up, we searched the patient electronic files to check whether thrombosis occurred during eltrombopag treatment.

Blood collection

Trisodium citrate anticoagulated whole blood was collected through venipuncture at t0 and t1. Whole blood was subjected to flow cytometric analysis between 1 and 4 hours after blood collection. Plasma samples were obtained through centrifugation at 2000*g* for 10 minutes, aliquoted, and stored at −80 °C until used.

Flow cytometry

Flow cytometry can be used in patients with low platelet counts (31). Whole blood was diluted 1:10 (vol:vol) in 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl pH 7.4 with or without platelet agonists, and fluorescent antibodies or lectins and incubated for 10 minutes at 37 °C. Samples were subsequently fixated for 20 minutes with 1.11% formaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH_2PO_4, 10.2 mM Na_2HPO_4, 1.15 mM KH_2PO_4, 4 mM EDTA, pH 6.8, and stored in the dark at 4 °C until analysis on a BD FACSCantoII. Platelet glycoprotein expression was assessed with anti–GPVI-eFluor660 (HY101; Thermo Fisher Scientific) for GPVI and anti–CD49-FITC (AK7; BD Pharmingen) for $\alpha_2\beta_1$. GPIb and $\alpha_{IIb}\beta_3$ (unstimulated) were excluded from the analyses because there was a problem in a part of the samples that were measured with a different batch of fluorescent antibodies. Platelet reactivity toward ADP (60, 30, and 15 µM) and thrombin receptor agonist peptide SFLLRN (TRAP; 25, 12.5, and 6.25 µM) was assessed by measuring platelet P-selectin expression (VhH clone B10.6-alexa647) as a proxy for α-granule secretion and fibrinogen binding (VhH clone C3-alexa488) as a proxy for αIIbβ3 activation. Fibrinogen-binding and P-selectin expression in the absence of agonists were used as baseline values. Background signal was assessed with isotype control VhH (clone R2). Platelet count does not influence the response toward agonists (32). Platelet desialylation was assessed through quantification of the exposure of terminal galactose residues on platelet glycans, which are normally capped with sialic acid. Terminal galactose was detected with the lectin *Ricinus communis* agglutinin fluorescein (Vector Laboratories). Background *R communis* agglutinin I binding was assessed in the presence of 500 mM galactose. Platelets were identified with forward and side-scatter and GPIbα (VhH clone 17-phycoerythrin or HIP1–activated protein C [APC]) or CD41-(HIP8-phycoerythrin) expression. Platelet glycoprotein and galactose expression were expressed as median fluorescent intensity (MFI) after subtraction of the background signal. Platelet reactivity was expressed as the area under the dose-response

curve. Data were reported when a minimum number of 1000 events was recorded in the platelet gate.

Plasma TG

TG was measured with calibrated automated thrombography in platelet-poor plasma, as described (33). Coagulation was initiated with 0.5-pM tissue factor (Innovin; Siemens) in the presence of 4 μM phospholipids (Coag reagent I; Avanti polar lipids) with and without 10 nM rabbit thrombomodulin (Prolytix, Essex Junction). Fluorescence was measured in SpectraMax iD3 with excitation at 390 nm and emission at 475 nm at 15-s intervals for 1 hour. From the fluorescence data, TG parameters were deduced as described previously (34). All measurements were performed in duplicate.

An APC sensitivity ratio was calculated by dividing the endogenous thrombin potential (ETP) from the TG with thrombomodulin by the ETP without thrombomodulin and dividing this ratio by the ratio in controls (mean ETP with thrombomodulin/mean ETP without thrombomodulin). A normalized APC ratio <1 indicates more sensitivity to protein C and >1 more resistance to protein C.

Enzyme-linked immunosorbent assays

Plasma levels of soluble P-selectin, TATc, and β-thromboglobulin were determined as described (35, 36). D-dimer levels were determined with Asserachrom D-Di (Stago) according to the manufacturer's instructions.

Coagulation factors and VWF assays

All coagulation factor and VWF assays were measured in the central ISO-15189 accredited laboratory of the UMC Utrecht. Coagulation factors V and VIII were measured using an ACL Top 750 LAS coagulation analyzer (Werfen Diagnostics). FV was determined with a 1-stage coagulation assay using FV-deficient plasma (Werfen diagnostics) and a prothrombin time reagent (HemosIL ReadiPlasTin; Werfen Diagnostics); FVIII was determined with Coamatic FVIII reagent (Werfen Diagnostics). VWF antigen (VWF:Ag) and vWF ristocetin (VWF:RCo) cofactor activity (VWF:GPIbR) were measured on an ACUstar chemiluminescence analyzer (Werfen Diagnostics).

Statistical analysis

Descriptive statistics were used to characterize the study population. Depending on the normality of the distribution of data, mean and SD or median and IQR were reported. The Mann–Whitney U-test was used to compare the hemostatic parameters at t0 and t1 with those of controls. The effect of eltrombopag on hemostatic parameters was assessed with mixed-effects models, with a random intercept and slope per patient (37). For our analyses, we added the random slope to consider that a parameter may change less if it is already elevated at baseline. These models were shown to model the data better according to the Akaike Information Criterion. However, we performed the analysis with

a random intercept only, and these results are reported in **Supplementary Table S3**. The parameters were estimated with restricted maximum likelihood estimation. The β values indicates the average change during treatment, while accounting for random variation in the baseline value and slope. The assumptions of all regression models were tested by assessing homoscedasticity and normality of the residuals. ELISA and TG data at t0 or t1 were missing in 2 patients owing to 2 lost samples; these patients were excluded from the respective analyses. Because we considered the data to be missing completely at random, a complete case analysis should give unbiased estimates (38). A sensitivity analysis was performed for variables that changed significantly during eltrombopag use to assess whether these changes were directly or indirectly due to changes in platelet count. This was performed by adding platelet count as a covariate to the mixed-effects model. A second sensitivity analysis was performed to assess whether there was a difference between platelet desialylation in patients with and without a response to eltrombopag. For this analysis, response was defined as a platelet count $>30 \times 10^9$/L at t1 or a doubling of the platelet count compared with that of baseline. Finally, descriptive statistics were used to compare the absolute values at t1 and the proportion of change between t1 and t0 of patients who developed thrombosis with those of the other patients. If applicable, a *p* value of less than .05 was considered statistically significant. The *p* values were corrected for multiple testing with the Bonferroni–Holm technique, which is similar to the Bonferroni correction but not as conservative (39, 40). All analyses were performed with RStudio version 2021.09.0.

Sample size

The data provided in previously conducted studies were unfortunately insufficient to perform a formal sample size calculation. We aimed to include 20 patients in this study, similar to previous studies. Because of a low inclusion rate, our final study population comprised 17 patients.

RESULTS

In total, 17 patients were included in the study, of whom 1 was excluded from the analyses owing to cessation of treatment before t1 because of an allergic reaction to eltrombopag. The characteristics of the remaining 16 patients are depicted in **Table 1**. The median time between start eltrombopag and t1 was 15 days (range, 12-21 days); 3 of the 16 patients (5%) had more than 2 weeks between t0 and start of eltrombopag (38, 68, and 187 days, respectively).

Table 1. Baseline characteristics

Values are given as median (interquartile range) or n (%).	
Total *N*	**16**
Age (years) *mean ±SD*	53,4 ±14,1
Female *N (%)*	7 (44)
ITP duration (years) *median (IQR)*	4 (3-14)
Splenectomy *N (%)*	2 (12)
Timepoint 0	
Platelet count (*10^9/L) *median (IQR)*	23 (9-27)
Eltrombopag starting dose *N (%)*	
25 mg every other day	1 (6)
25 mg/day	5 (31)
50 mg/day	10 (62)
Timepoint 1	
Platelet count (*10^9/L) *median (IQR)*	39 (10-119)
Dose at timepoint 1 *N (%)*	
25 mg every other day	1 (6)
25 mg/day	5 (31)
50 mg/day	6 (38)
75 mg/day	4 (25)
Response (platelet count ≥50 *10^9/L) at timepoint 1 *N (%)*	7 (44)

IQR: interquartile range, N: number, SD: standard deviation

Platelet function and coagulation in patients with ITP

Before start of eltrombopag, patients with ITP showed reduced expression of most GPs compared with controls (**Figure 1, Supplementary Table S1**). Platelets from patients with ITP showed signs of preactivation, with increased levels of both fibrinogen binding and P-selectin expression in unstimulated platelets, compared with those of controls, and were less responsive toward ADP and TRAP. Platelets from patients with ITP showed less desialylation than those from controls. Markers of systemic platelet activation, that is, plasma levels of sP-selectin and β-thromboglobulin, were reduced in patients with ITP (**Figure 2**). The differences in platelet GP expression and levels of β-thromboglobulin remained significant after correction for multiple testing. Patients with ITP showed a (borderline significant) higher peak in TG (**Figure 3**). Both FVIII and FV are determinants of peak height in TG (30). Although FV was normal, FVIII levels were remarkably high in patients with ITP (median [IQR]: 201% (171%-245%]), as were the levels of VWF:RCo (167% [109%-198%]) (**Figure 4**). We performed a sensitivity analysis to assess the effect of recent steroids use on FVIII. This analysis indeed showed higher FVIII and VWF:RCo levels in patients who used steroids up to 3 months before start eltrombopag. However, in particular, the FVIII levels remained high in patients without recent use of steroids: 250% (214%-206%) with steroids and 192% (172%-235%) without steroids for FVIII and 206% (179%-236%) and 112% (90%-160%) with steroids for VWF:RCo,respectively.

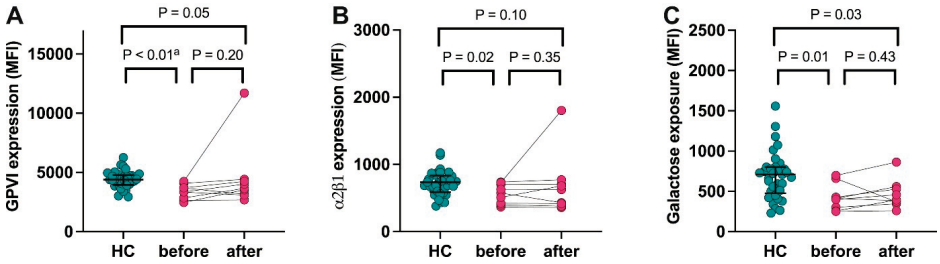

Figure 1. Platelet glycoprotein expression during eltrombopag treatment
Platelet glycoprotein expression and galactose exposure was analyzed with flow cytometry in patients with immune thrombocytopenia (ITP) before and 2-3 weeks after eltrombopag treatment (n = 9) and in healthy controls (HCs; n = 33). Expression of (A) glycoprotein VI and (B) α2β1 was assessed on resting platelets in whole blood. (C) Whole blood was incubated with FITC-labeled RCA-1 lectin and analyzed with flow cytometry. Background fluorescence was determined in the presence of an excess of galactose. Data represent galactose exposure on platelets after background subtraction. The horizontal line and error in the HC group indicate median and interquartile range. All data are expressed as median fluorescent intensity (MFI). The Mann–Whitney U-test was used to compare the hemostatic parameters in the ITP group before or after eltrombopag treatment with those of the control group. The effect of eltrombopag on hemostatic parameters was assessed with mixed-effects models, with a random intercept and slope per patient. [a]Statistical significance was retained after correction for multiple testing ($p < .05$).

Platelet function and coagulation during eltrombopag treatment

Platelet phenotype, reactivity, and activation status did not change after eltrombopag treatment, although many markers of platelet function and activation remained significantly different from those of controls (**Supplementary Table S1**). Moreover, platelet desialylation did not change significantly during eltrombopag treatment, and a sensitivity analysis showed no significant difference between responders and nonresponders to eltrombopag treatment (**Supplementary Table S4**). However, eltrombopag did induce changes in ETP, peak, and lag time with and without thrombomodulin. After correction for multiple testing, the changes in lag time remained significant and the peak with and without thrombomodulin was still higher than those in healthy controls ($p = .055$ and $p = .03$, respectively). Furthermore, the normalized APC sensitivity ratio, based on the ETP with thrombomodulin, changed from 1.18 (IQR, 1.64-1.03) before start to 1.66 (IQR, 1.74-1.04) after 2 to 3 weeks, indicating increasing resistance to protein C, but this change was not significant ($p = .14$). The sensitivity analysis showed that the changes in TG parameters remained significant after correction for the platelet count (**Supplementary Table S2**).

Thrombosis during follow-up

One patient experienced deep venous thrombosis in her leg during eltrombopag treatment, 5 months after initiation. For numerous of her plasma TG parameters as well as her VWF values, the change compared with baseline and/or the absolute value at t1 was among the most procoagulant (either highest or lowest, depending on the parameter) of all included patients: the increase in ETP and peak with and without thrombomodulin

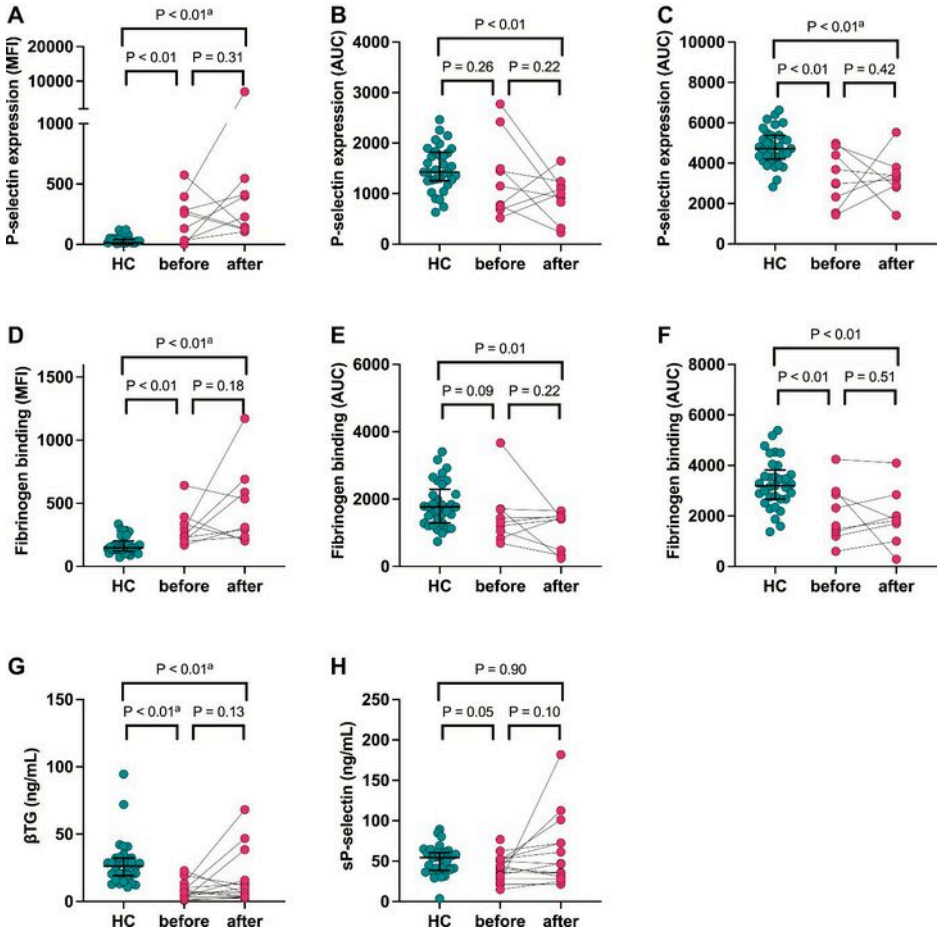

Figure 2. Platelet activation and reactivity during eltrombopag treatment

(A-F) Platelet reactivity toward agonists was analyzed with flow cytometry in patients with immune thrombocytopenia (ITP) (n = 9; magenta) before and 2-3 weeks after eltrombopag treatment and in healthy controls (HCs; n = 33; green). (A) P-selectin expression in unstimulated platelets. Data are expressed as median fluorescent intensity (MFI). (B) P-selectin expression after stimulation with 3 concentrations of ADP (15, 30, and 60 μM). Data are expressed as the area under the dose-response curve (AUC). (C) P-selectin expression after stimulation with 3 concentrations of thrombin receptor agonist peptide SFLLRN (TRAP; 6.25, 12.5, 25 μM). Data are expressed as the AUC. (D) Fibrinogen binding in unstimulated platelets. Data are expressed as MFI. (E) P-selectin expression after stimulation with 15, 30, or 60 μM ADP. Data are expressed as the AUC. (F) P-selectin expression after stimulation with 6.25, 12.5, or 25 μM TRAP. Data are expressed as the AUC. (G, H) Plasma levels of (G) β-thromboglobulin (βTG) and (H) soluble P-selectin (sP-selectin) were determined with ELISA in 16 patients with ITP and 33 HCs. Data were missing for 2 patients with ITP after eltrombopag treatment. The horizontal line and error in the HC group indicate median and interquartile range. The Mann–Whitney U-test was used to compare the hemostatic parameters in patients with ITP before or after eltrombopag treatment with those of the HCs. The effect of eltrombopag on hemostatic parameters was assessed with mixed-effects models, with a random intercept and slope per patient. [a]Statistical significance was retained after correction for multiple testing ($p < .05$).

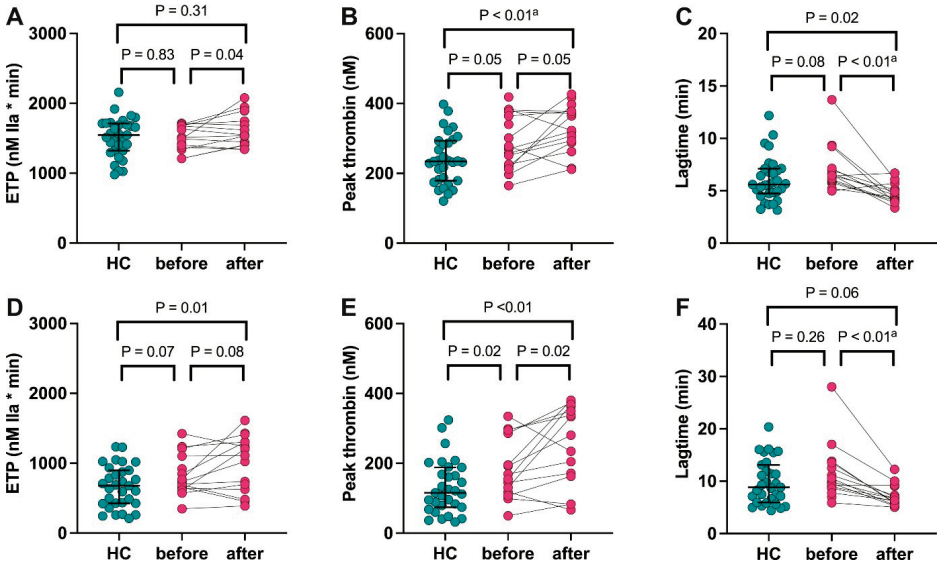

Figure 3. Thrombin generation during eltrombopag treatment

Thrombin generation was initiated with 0.5 pM TF and 4 μM phospholipids without (A-C) or with (D-F) 10 nM thrombomodulin in patients with immune thrombocytopenia (ITP) before and 2-3 weeks after eltrombopag treatment (n = 16) and in healthy controls (HCs; n = 33). Samples were missing in 2 patients with ITP after eltrombopag treatment. (A, D) Endogenous thrombin potential (ETP). (B, E) Peak thrombin concentration. (C, F) Lagtime. The horizontal line and error in the HC group indicate median and interquartile range. The Mann–Whitney U-test was used to compare the hemostatic parameters in patients with ITP before or after eltrombopag treatment with those of HCs. The effect of eltrombopag on hemostatic parameters was assessed with mixed-effects models, with a random intercept and slope per patient. [a]Statistical significance was retained after correction for multiple testing ($p < .05$).

was in the highest 10th percentile and the absolute value for ETP with and without thrombomodulin at t1 was in the highest 10th percentile. The decrease in lag time without thrombomodulin was in the lowest 10th percentile (ie, largest decrease) and the absolute values were in the lowest 10th and 20th percentiles, respectively. Moreover, the absolute values for VWF:RCo and VWF:Ag were in the highest 10th percentile, as was the increase in VWF:RCo. For other markers (D-dimer, TATc, sP-selectin, β-thromboglobulin, FVIII, and flow cytometry parameters), the change compared with that at baseline and the absolute values at t1 were similar to the other patients.

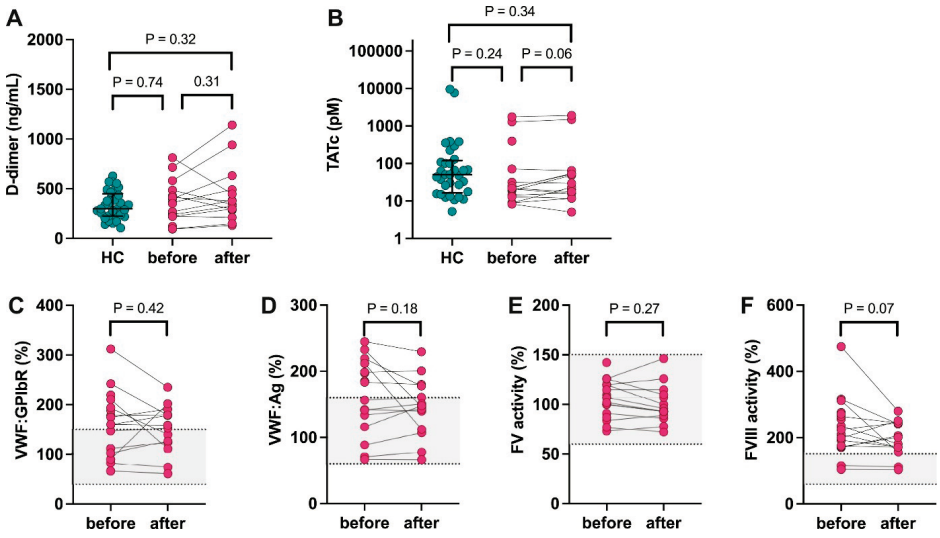

Figure 4. Coagulation and von Willebrand factor during eltrombopag treatment
(A) D-dimer and (B) thrombin–antithrombin complexes (TATc) were assessed with ELISA in patients with immune thrombocytopenia (ITP) before and after eltrombopag treatment (magenta; n = 16) and in healthy controls (HCs; green; n = 33). Samples were missing for 2 patients with ITP after eltrombopag treatment. The horizontal line and error in the HC group indicate median and interquartile range. (C) VWF activity (VWF:GPIbR) and (D) antigen (VWF:Ag) were assessed with chemiluminescence. (E) Factor (F)V and (F) FVIII activities were assessed with 1-stage clotting assays in plasma samples from patients with ITP (n = 16) before and 2-3 weeks after eltrombopag treatment. Samples were missing for 2 patients after eltrombopag treatment. Dotted lines and gray shaded area indicate normal reference values. The Mann–Whitney U-test was used to compare the hemostatic parameters in patients with ITP before or after eltrombopag treatment with those of HCs. The effect of eltrombopag on hemostatic parameters was assessed with mixed-effects models, with a random intercept and slope per patient.

DISCUSSION

We performed a prospective study to evaluate platelet (re)activity and coagulation in patients with ITP and the effect of eltrombopag on these markers. We found that patients with ITP show several signs of a hypercoagulable state, as evidenced by the presence of preactivated platelets, elevated plasma TG peak, and elevated levels of FVIII and VWF. Furthermore, eltrombopag treatment influenced all parameters of plasma TG, particularly the lag time, and possibly sensitivity to APC. These changes were independent of platelet count. Platelet function, FV, FVIII, and VWF were not influenced by eltrombopag treatment.

Hypercoagulable state in patients with ITP

We found high levels of FVIII and VWF in patients with ITP. Two studies also reported elevated levels of VWF in patients with ITP compared with those in controls (41, 42). Furthermore, a study in 1987 found increased FVIII-related antigen in patients with and without ITP and increased FVIII coagulant in the latter group, but these coagulation assays were not directly comparable with the ones used in this study (43). In this population, the peak height of the plasma TG was also elevated. Both FV and FVIII are determinants of peak

height (30). Because FV levels were normal, the increased peak height is likely mediated by the elevated FVIII levels. Elevated levels of FVIII and VWF and the peak height of TG are well-known risk factors for both venous and arterial thrombosis, so this finding may partially explain the occurrence of thrombosis in patients with ITP (27, 44-46).

It is unclear why VWF and FVIII levels were elevated in the patients with ITP. Recent steroid use may partly explain this observation because the levels were higher in these patients and steroid use does effect the endothelium in multiple ways (47). However, an additional explanation is necessary for the high FVIII levels in patients without recent steroid use. It is possible that the platelet number influences the levels of VWF and, subsequently, the levels of FVIII (43), but VWF levels in our study, particularly, in patients without steroids, were not elevated to the degree of the FVIII levels. Another hypothesis would be that ITP induces endothelial activation. Three recent studies found evidence for endothelial dysfunction in patients with ITP. One found increased levels of VWF:Ag and vascular cellular adhesion molecule in patients with ITP compared with those of matched controls (48); the second found increased levels of including intercellular adhesion molecule-1 and thrombomodulin but not vascular cellular adhesion molecule and E-selectin (28); and the third found elevated levels of plasminogen activator inhibitor-1 and E-selectin (49). Why ITP would be associated with endothelial dysfunction is unknown. The most common hypothesis is that crossreactivity of platelet autoantibodies affects the endothelial cells (50-52). A recent canine ITP model found that autoantibody-induced thrombocytopenia led to alterations in endothelial function. In this study, the VWF:Ag levels correlated with the observed changes in the endothelium (53). Another previously suggested theory is that activated platelets, microvesicles and/or neutrophil extracellular traps can lead to endothelial damage (28). Furthermore, it has been suggested that thrombocytopenia itself can lead to endothelial dysfunction because platelets produce many vasoactive mediators that help maintain endothelial integrity (53). More research is needed to clarify why elevated FVIII and VWF levels and/or endothelial dysfunction may occur in patients with ITP and which role this plays in the occurrence of thrombosis.

Platelet function in patients with ITP

At baseline, we found reduced expression of platelet glycoproteins in patients with ITP compared with that of controls. Reduced expression of GPVI has been reported before and may be due to circulating antibodies against GPVI that cause shedding of the receptor (54, 55). Our findings that platelets in patients with ITP show signs of preactivation and are less responsive to agonists are largely in line with previous studies (24, 49, 56, 57). On the contrary, plasma markers of systemic platelet activation were reduced. This is likely due to the thrombocytopenia: these markers are secreted by platelets (58-60). A very low platelet count will therefore result in lower plasma markers even if each individual platelet secretes more of the marker than in a healthy person. Furthermore, we found decreased levels of platelet desialylation in patients before and after start eltrombopag compared with those of healthy controls. This is not in line with previous research, which

found similar levels of galactose expression in untreated patients and patients on TPO-RA treatment. Furthermore, these studies showed increased levels in patients nonresponding or refractory to treatment, whereas our data showed no difference between responding and nonresponding patients (61, 62). We have no clear explanation for these differences.

Effect of eltrombopag on platelets

We found no evidence that eltrombopag further altered platelet GP expression, activation and reactivity of platelets, platelet desialylation, or platelet activation in vivo. Thus, the suggestion of our systematic review that TPO-RAs may increase platelet GPVI expression was not confirmed by our study. Our results are in line with previous in vitro and in vivo studies in healthy subjects, which also failed to show an effect of eltrombopag on platelet activation (63-65). Of note, the number of patients in the flow cytometry analyses was very small, because many patients were excluded from these analyses owing to too few events in the platelet gate. Larger sample sizes remain necessary to draw definite conclusions, but overall, there seems little evidence that eltrombopag influences the platelet function.

Effect of eltrombopag on coagulation

We found no effect of eltrombopag on most of the coagulation parameters, including D-dimer, TATc, VWF, FV, and FVIII. This is in line with previous research, which found no changes in D-dimer and prothrombin fragments 1 and 2 during eltrombopag treatment (66-68). However, there are few data available on the effect of TPO-RAs on coagulation.

Eltrombopag did increase plasma TG with and without thrombomodulin in almost all parameters in our study, with the most robust evidence for the changes in lag time (the time until the first traces of thrombin were formed). Importantly, plasma TG markers, particularly ETP (the total amount of active thrombin that was formed during TG) and peak height (the maximal amount of thrombin) with or without thrombomodulin, are known risk factors for both primary and recurrent venous thrombosis (27, 46). In this study, almost all absolute values after starting eltrombopag were significantly different than in healthy controls, suggesting a hypercoagulable state. Furthermore, the change and absolute values of the patient who experienced thrombosis a few months later were among the highest in the patient population.

The effect of TPO-RA on plasma TG (without thrombomodulin) in ITP has been assessed in 2 previous prospective studies; both found no changes in plasma TG parameters (66, 69). Therefore, our results should first be confirmed in future studies. Of note, we used a lower concentration of tissue factor in our assay (0.5 pM rather than 1 or 5 pM), making the assay more sensitive to the intrinsic pathway of the coagulation cascade, particularly factors VIII, IX, and XI (70, 71). Because we found no change in FVIII during eltrombopag treatment, the observed changes could be caused by FIX or FXI. Furthermore, the lag time is mainly dependent on free tissue factor pathway inhibitor and free protein S and on FVII, FIX, and fibrinogen, so it may be worthwhile to assess the effect of TPO-RAs on these

markers in future research (72). Moreover, the TG results showed that eltrombopag may decrease the sensitivity to APC, which is associated with an increased risk of thrombosis (73, 74). High levels of FVIII may partly explain this decreased sensitivity, but it does not explain the change during eltrombopag treatment because FVIII levels remained constant.

Overall, the underlying mechanism for the observed changes in TG is unclear. One possible mechanism is through increased levels of microvesicles, as a previous study found that TPO-RAs increase phospholipid-dependent microvesicle-associated TG (75). Another hypothesis is that eltrombopag or the eltrombopag-induced platelets affect the liver in a way that leads to a prothrombotic profile. It is known that eltrombopag can cause liver dysfunction in a small proportion of patients (76-78). However, this hypothesis is not supported by our finding that eltrombopag did not affect levels of FV. Finally, the TPO-receptor is present on hemangioblasts and hematopoietic stem cells, indicating that TPO-RAs may also affect the hemostasis and/or endothelium through this route (79). For example, stimulation of hemangioblasts by TPO leads to the formation of secondary hematopoietic colonies and endothelial cells (80). Overall, further research is needed to elaborate on the effect of eltrombopag on thrombin-generating potential and the underlying mechanisms.

Strengths and limitations

A strength of this study is that it is prospective and assesses several markers that were not previously assessed in patients with ITP receiving TPO-RAs, including plasma β-thromboglobulin and TATc levels, and plasma TG with thrombomodulin. The second strength of this study is the focus on eltrombopag because simultaneously studying different TPO-RAs, which all act in a slightly different manner, may obscure changes that are specific to eltrombopag. The main limitation of this study is the small sample size. Unfortunately, the rarity of the disease and the logistic challenges of performing hemostatic tests limits the feasibility of larger studies, as shown by the fact that all previous similar studies also had small sample sizes. We measured hemostatic parameters only 2-3 weeks after initiation of eltrombopag, owing to which we might have missed any changes occurring after 2-3 weeks of treatment. However, in previous studies, hemostatic changes were noted within several weeks (24). Another limitation is that for a few patients the time between t0 and the start of eltrombopag was relatively long (>2 weeks). However, because this only applies to a small proportion of patients, we do not believe this biased our results. Finally, the proportion of women in our control group was higher than in the patient group. Sex is known to influence many hemostatic parameters, such as protein S, protein C, fibrinogen, FVII, FVIII, FIX, and TATc. Although there are differences per age group, overall women have a more hypercoagulable profile (81). This could have caused us to potentially miss relevant prothrombotic changes in the ITP group compared with those of the control group.

Clinical implications and future research

It is widely known that patients with ITP have a prothrombotic phenotype, partly because of altered platelet function, which is confirmed in this study (49, 56, 57). In addition, our results suggest endothelial activation in patients with ITP. Future research should confirm this finding and investigate its clinical implications. Furthermore, the results of this study suggest that the effect of eltrombopag on hemostasis is through different pathways than platelet function, but the exact underlying mechanisms for the occurrence of thrombosis remain unclear. Future research should confirm the effect on TG and address the role of microvesicles, tissue factor pathway inhibitor, protein S, endothelial function, and other coagulation factors. All prospective studies in this field are, thus far, limited by small sample sizes, indicating practical difficulties with the execution of these studies. Therefore, we advocate for international collaborations to move forward.

Conclusion

Patients with ITP have a procoagulant state, with preactivated platelets, an increased peak height in plasma TG, and elevated levels of FVIII and VWF. The latter finding may be a sign of endothelial activation. Eltrombopag treatment did not influence platelet function but did increase the plasma TG potential by a yet unraveled mechanism.

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Author contributions

W.E.M.v.D., K.P.M.v.G., R.T.U., and R.E.G.S. designed and performed the research. W.E.M.v.D. and G.C.P. performed the laboratory analyses. W.E.M.v.D. performed the statistical analyses and wrote the manuscript. G.C.P., A.H., H.R.K., R.F., N.T., E.R.v.B., K.P.M.v.G., R.E.G.S., and R.T.U. critically revised the manuscript. All authors read and approved the final paper.

Declaration of competing interests

R.E.G. Schutgens received a grant from Novartis. The other authors have no potential conflicts of interest to declare.

SUPPLEMENTARY TABLES

Supplementary table S1. Hemostatic parameters in ITP patients versus controls and the effect of eltrombopag

Variable	At baseline (t0)			During eltrombopag treatment (t1)			
	Controls	ITP patients		ITP patients		Change from baseline	
	Median (IQR)	Median (IQR) at t0	P-value *t0 vs controls*	Median (IQR) at t1	P-value *t1 vs controls*	β (IQR)	P-value *Mixed model (random intercept and slope)*
Platelet glycoprotein expression (MFI) b							
GPVI	4401 (4002 - 4738)	3349 (2853 - 3700)	0,00 a	3652 (3163 - 4246)	0,05	1127 (-742 - 2995)	0,20
α2β1	735 (598 - 825)	575 (422 - 702)	0,02	625 (417 - 707)	0,10	122 (-159 - 403)	0,35
Platelet reactivity (MFI) b							
Fibrinogen binding							
Without agonist	147 (130 - 175)	244 (204 - 335)	0,00	310 (234 - 588)	0,00 a	170 (-95 - 434)	0,18
After ADP stimulation	1768 (1298 - 2141)	1308 (1056 - 1692)	0,09	1437 (479 - 1458)	0,01	-403 (-1099 - 292)	0,22
After TRAP stimulation	3196 (2668 - 3640)	1613 (1374 - 2847)	0,00	1821 (1002 - 2006)	0,00	-388 (-1701 - 924)	0,51
P-selectin expression							
Without agonist	12 (-17 - 38)	257 (34 - 282)	0,00	228 (132 - 413)	0,00 a	795 (-888 - 2478)	0,31
After ADP stimulation	1424 (1266 - 1801)	1149 (759 - 1495)	0,26	1004 (829 - 1147)	0,00	-400 (-1102 - 301)	0,22
After TRAP stimulation	4720 (4307 - 5376)	3016 (2323 - 4401)	0,00	3203 (2810 - 3505)	0,00 a	-789 (-2942 - 1364)	0,42
Platelet desialylation (MFI) b							
Galactose exposure	711 (504 - 801)	411 (303 - 428)	0,01	394 (369 - 528)	0,03	39 (-69 - 146)	0,43
Systemic platelet activation (ng/mL)							
β-thromboglobulin	28,5 (20,4 - 35,8)	5,3 (3,3 - 9,1)	0,00 a	6,9 (3,3 - 14,5)	0,00 a	8,23 (-2,79 - 19,26)	0,13
(ng/mL)	51,8 (39,7 - 60,6)	41,4 (31,2 - 51,5)	0,05	46,5 (33 - 72,4)	0,90	20,43 (-4,17 -	0,10
sP-selectin (ng/mL)							

Supplementary table S1. Hemostatic parameters in ITP patients versus controls and the effect of eltrombopag

Variable	At baseline (t0)			During eltrombopag treatment (t1)			
	Controls	ITP patients		ITP patients		Change from baseline	
	Median (IQR)	Median (IQR) at t0	P-value *t0 vs controls*	Median (IQR) at t1	P-value *t1 vs controls*	β (IQR)	P-value *Mixed model (random intercept and slope)*
Coagulation and VWF						45,02)	
D-dimer (ng/mL)	295,2 (214 - 441,8)	355,7 (221,5 - 441,4)	0,74	342,8 (288,7 -468,9)	0,32	55,4 (-58,4 - 169,1)	0,31
TATc (pM)	49,2 (15,4 - 103,5)	22,2 (14,0 - 41,6)	0,24	23,3 (16,1 - 53,3)	0,34	31,1 (-1,9 - 64,0)	0,06
VWF:RCo (%)	40-150 c	167 (109 - 198)	n/a	159 (125 - 184)	n/a	-11 (-40 - 18)	0,42
VWF:Ag (%)	60-160 c	170 (130 - 202)	n/a	148 (126 - 179)	n/a	-14 (-35 - 7)	0,18
Factor V (%)	60-150 c	104,5 (90 - 120)	n/a	96 (91 - 110)	n/a	-3 (-9 - 3)	0,27
Factor VIII (%)	60-150 c	217 (174 - 272)	n/a	201 (171 - 244)	n/a	-35 (-72 - 3)	0,07
Thrombin generation							
ETP (nM x min)	1546 (1322 - 1701)	1501 (1395 - 1676)	0,83	1548 (1426 – 1818)	0,31	110,1 (4,5 - 215,6)	0,04
Peak (nM)	234,2 (180,4 - 289,8)	273,7 (224,4 - 367,7)	0,05	322,9 (290,5 – 377,0)	0,00 a	41,8 (0,9 - 82,8)	0,05
Lag time (min)	5,6 (4,8 - 7)	6,4 (5,9 - 7)	0,08	4,5 (4,1 - 5,1)	0,02	-2,4 (-3,5 - -1,2)	0,00 a
Thrombin generation with thrombomodulin							
ETP (nM x min)	679 (428 - 887)	753 (651 - 1129)	0,07	1110 (662 - 1304)	0,01	156,6 (-18,4 -331,6)	0,08
Peak (nM)	115,5 (76,8 - 178,3)	180,8 (127,4 - 288,4)	0,02	280,4 (167,3 -356,2)	0,00	64,0 (12,5 - 115,5)	0,02
Lag time (min)	8,9 (6,3 - 12,5)	9,8 (8,7 - 12,8)	0,26	6,8 (5,8 - 8,2)	0,06	-4,6 (-6,7 - -2,5)	0,00 a

a. Remains statistically significant after correction for multiple testing. b. The analyses with FACS data were performed in a total of 9 patients, the other patients were excluded from the analyses due to too few events in the platelet gate. c. Reference values.

ADP: adenosine diphosphate, ELISA: enzyme-linked immunosorbent assay, ETP: endogenous thrombin potential, GP: glycoprotein, ITP: immune thrombocytopenia, MFI: mean fluorescence intensity, sP-selectin: soluble P-selectin, TATc: thrombin/antithrombin complexes, TG: thrombin generation, TRAP: thrombin receptor activating peptide, VWF:Ag: Von Willebrand factor antigen, VWF:RCo: Von Willebrand factor ristocetine.

Supplementary table S2. The effect of eltrombopag on hemostatic parameters in ITP patients, corrected for platelet count

Variable	At baseline (t0)	During eltrombopag treatment (t1)	Change from baseline (corrected for platelet count)	
	Median (IQR) at t0	Median (IQR) at t1	β (IQR)	P-value *Mixed model*
Thrombin generation				
ETP (nM x min)	1501 (1395 - 1676)	1548 (1426 – 1818)	136,2 (32,48 - 239,91)	0,01
Lag time (min)	6,4 (5,9 - 7)	4,5 (4,1 - 5,1)	-2,35 (-3,55 - -1,14)	0,00
Thrombin generation with thrombomodulin				
Peak (nM)	180,8 (127,4 - 288,4)	280,4 (167,3 - 356,2)	63,89 (7,08 - 120,71)	0,03
Lag time (min)	9,8 (8,7 - 12,8)	6,8 (5,8 - 8,2)	-4,36 (-6,52 - -2,2)	0,00

ETP: endogenous thrombin potential, ITP: immune thrombocytopenia

Supplementary table S3. Hemostatic parameters in ITP patients versus controls and the effect of eltrombopag, mixed model with random intercept only

Variable	Change from baseline during eltrombopag treatment§	
	β (IQR)	P-value *Mixed model (random intercept only)*
Platelet glycoprotein expression (MFI) b GPVI	1127 (-742 - 2995)	0,20
α2β1	122 (-159 - 403)	0,35
Platelet reactivity (MFI) b		
Fibrinogen binding Without agonist	170 (-95 - 434)	0,18
After ADP stimulation	-403 (-1099 - 292)	0,22
After TRAP stimulation	-388 (-1701 - 924)	0,51
P-selectin expression Without agonist	795 (-888 - 2478)	0,31
After ADP stimulation	-400 (-1100 - 299)	0,22
After TRAP stimulation	-789 (-2942 - 1364)	0,42
Platelet desialylation (MFI) b Galactose exposure	39 (-69 - 146)	0,43
Systemic platelet activation (ng/mL)		
β-thromboglobulin (ng/mL)	8,46 (-2,28 - 19,19)	0,11
sP-selectin (ng/mL)	19,95 (-4,22 - 44,13)	0,10
Coagulation and VWF D-dimer (ng/mL)	61,35 (-52,46 - 175,16)	0,27
TATc (pM)	32,8 (-5,86 - 71,46)	0,09
VWF:RCo (%)	-10 (-39 - 19)	0,48
VWF:Ag (%)	-13 (-34 - 9)	0,23
Factor V (%)	-3 (-9 - 3)	0,25
Factor VIII (%)	-32 (-71 - 8)	0,11
Thrombin generation		
ETP (nM x min)	107,42 (3,33 - 211,51)	0,04
Peak (nM)	43,02 (2,02 - 84,03)	0,04
Lag time (min)	-2,35 (-3,54 - -1,16)	0,00
Thrombin generation with thrombomodulin		
ETP (nM x min)	146,34 (-27,23 - 319,91)	0,09
Peak (nM)	61,28 (10,14 - 112,42)	0,02
Lag time (min)	-4,49 (-6,74 - -2,24)	0,00

b. The analyses with FACS data were performed in a total of 9 patients, the other patients were excluded from the analyses due to too few events in the platelet gate. c. Reference values.
ADP: adenosine diphosphate, ELISA: enzyme-linked immunosorbent assay, ETP: endogenous thrombin potential, GP: glycoprotein, ITP: immune thrombocytopenia, MFI: mean fluorescence intensity, sP-selectin: soluble P-selectin, TATc: thrombin/antithrombin complexes, TG: thrombin generation, TRAP: thrombin receptor activating peptide, VWF:Ag: Von Willebrand factor antigen, VWF:RCo: Von Willebrand factor ristocetine.

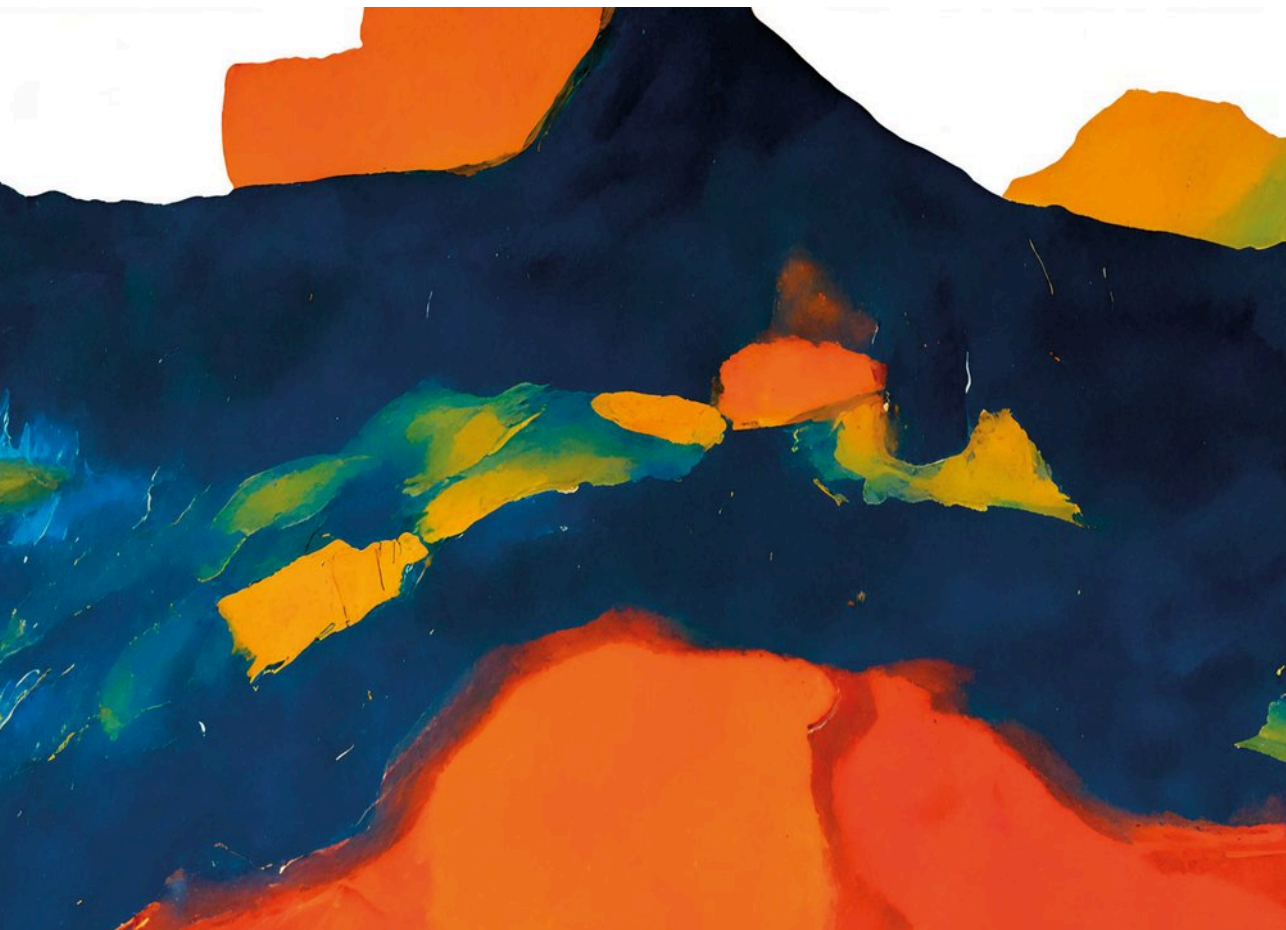
Supplementary tables 4. Change in galactose expression during eltrombopag treatment corrected for response

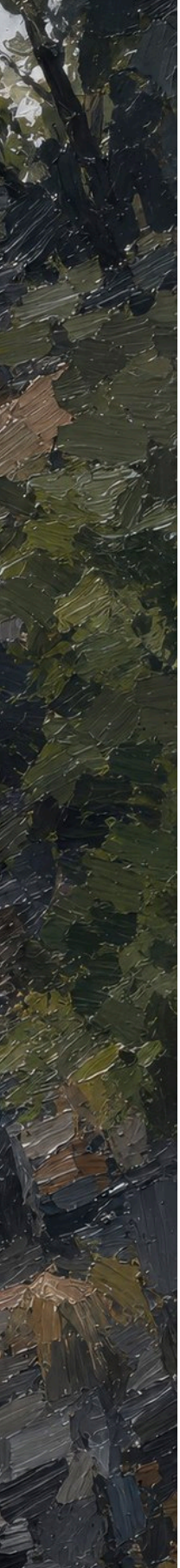
Variable	Change from baseline du ring eltrombopag treatment	
	β (IQR)	P-value *Mixed model (randomintercept only)*
Platelet desialylation (MFI) a		
Galactose exposure	39 (-69 - 146)	0,43
Response to eltrombopag b	-185 (-444 - 75)	0,14

a. The analyses with FACS data were performed in a total of 9 patients, the other patients were excluded from the analyses due to too few events in the platelet gate. b. Response was defined as a platelet count of >30 $*10^9$/L at timepoint 1 or a doubling of the platelet count at baseline.
ITP: immune thrombocytopenia, MFI: mean fluorescence intensity

PART II

Mechanistic modulation of the thrombomodulin pathway





CHAPTER 6

Mimicking the endothelium: liposomal thrombomodulin enhances Protein C activation in whole blood thrombin generation

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Unpublished

ABSTRACT

Background

Thrombomodulin (TM) regulates thrombin generation (TG) by promoting protein C activation on the endothelial surface. Soluble TM is commonly used to assess the protein C pathway in TG assays but exhibits markedly reduced anticoagulant activity in whole blood, limiting its physiological relevance.

Objectives

To determine whether membrane anchoring of a recombinant thrombomodulin fragment (TMi) to phosphatidylserine (PS)-containing liposomes enhances protein C activation and anticoagulant activity in plasma and whole blood TG assays.

Methods

A recombinant TM fragment (TMi, EGF domains 3–6) was expressed, purified, and conjugated to DSPE-PEG2000-DBCO via click chemistry, then post-inserted into liposomes containing 0%, 10%, or 20% PS. Liposomal formulations were characterized for size and protein incorporation. Protein C activation kinetics were assessed using a chromogenic assay. Anticoagulant activity was evaluated by calibrated automated thrombography in normal pooled plasma and in whole blood, comparing liposomal TMi formulations with soluble TMi (sTMi).

Results

Liposomal TMi exhibited enhanced protein C activation compared with sTMi, with increasing PS content improving apparent affinity for protein C (TMi-20%PS Km = 48 nM vs. sTMi Km = 524 nM). In plasma, TMi-20%PS achieved the lowest IC50 for thrombin generation inhibition at both 1 and 5 pM tissue factor. In whole blood, where sTMi activity was markedly reduced, TMi-20%PS retained potent anticoagulant activity (IC50 7 nM vs. 41 nM for sTMi).

Conclusions

Membrane presentation of TM on PS-rich liposomes markedly enhances protein C activation and anticoagulant function, particularly in whole blood, providing a physiologically relevant platform for TG-based assessment of the protein C pathway.

KEYWORDS

Thrombomodulin - Thrombin - Protein C – Liposomes - Blood Coagulation Tests

INTRODUCTION

Thrombin generation (TG) assays are increasingly used to assess the overall hemostatic potential of plasma, primarily in research settings (1). Unlike conventional endpoint assays such as prothrombin time (PT) or activated partial thromboplastin time (aPTT), TG provides dynamic and quantitative insight into the multiple phases of coagulation, including initiation, propagation and inhibition. However, due to limited standardization and unclear clinical utility, TG is not yet widely adopted in routine diagnostics (2, 3). Newer automated platforms such as ST Genesia® (Diagnostica Stago) offer improved reproducibility and ease of use, which may facilitate broader use. In a standard TG assay, coagulation is initiated by adding tissue factor (TF) and phospholipids (PL) to recalcified plasma, followed by kinetic monitoring of thrombin activity via a fluorogenic substrate (4). The assay can be modified using different TF concentrations, alternative initiators such as FXI or celite, or adding anticoagulants to study their effects on thrombin generation (5). TG has also been adapted for use in platelet-rich plasma and whole blood, providing a more physiologically relevant assessment of coagulation.

In vivo, thrombin generation is tightly regulated by the endothelium, which expresses a repertoire of cofactors and receptors that modulate thrombin activity. A key regulator is thrombomodulin (TM), a transmembrane glycoprotein that binds thrombin and shifts its substrate specificity from procoagulant substrates to protein C and thrombin-activatable fibrinolysis inhibitor (TAFI) (6). The thrombin-TM complex enhances protein C activation by more than 1000-fold, generating activated protein C (APC), which inactivates factors Va and VIIIa and thereby downregulates further thrombin generation (7). In TG assays, soluble TM (sTM) is often added to assess the protein C pathway. However, sTM exhibits markedly reduced anticoagulant activity in whole blood compared to plasma; e.g. achieving 50% inhibition of TG requires approximately 20 nmol/L sTM in whole blood, versus only 1.5 nmol/L in plasma—a more than 13-fold difference (8). This diminished activity is likely due to the absence of membrane anchoring, as sTM lacks the spatial orientation, neighboring cofactors and surface-bound presentation of endothelial TM. Additionally, the complex cellular environment of whole blood may further impair its function.

To address this, we developed TM-functionalized liposomes by conjugating a recombinant TM fragment (TMi, comprising EGF domains 3-6) to liposomes using click chemistry. The liposomes were formulated with varying levels of phosphatidylserine (PS), a negatively charged phospholipid that plays a critical role in coagulation by facilitating the assembly of clotting complexes and is exposed on activated platelets and extracellular vesicles (9). We hypothesized that the negatively charged liposomal membrane would promote protein C recruitment and activation, in part by mimicking the electrostatic contribution of endothelial protein C receptor (EPCR) (10). Previous work has shown that the lipid composition of TM-containing liposomes affects both protein C and TAFI activation and may modulate TM's cofactor activity (11).

In this study, we evaluated whether liposomal conjugation of TMi enhances anticoagulant activity in TG assays performed in plasma and whole blood. We further examined how the PS content of the liposomes influences TMi function and protein C activation. By presenting TM in a membrane-mimetic context, we aim to improve the physiological relevance of TG and overcome the limitations associated with using sTM in whole blood.

METHODS

Reagents

All lipids, including 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and DSPE-PEG2000-DBCO, were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (3β-hydroxy-5-cholestene and 5-Cholesten-3β-ol) was obtained from Sigma St. Louis, MO, USA). Recombinant sortase A (7M variant, calcium-independent, pET30b-7M SrtA, Addgene plasmid #51141, gift from Hidde Ploegh, ordered via Addgene, Watertown, MA, USA) and tobacco etch virus (TEV) protease were produced in-house. Chromogenic substrate S-2366 was obtained from Chromogenix (lot.N1237485), and the fluorogenic thrombin substrate I-1140 (Z-Gly-Gly-Arg-AMC) from Bachem (lot.1000066678). Hirudin was obtained from Sigma. Mouse anti-Myc primary antibody (clone 9E10) was produced in-house, and donkey anti-mouse IgG IRDye 800 secondary antibody and Odyssey Blocking Buffer were purchased from LI-COR Biosciences. Gly_3-azide was from IRIS Biotech GmbH (Marktredwitz, Germany). Thrombin calibrator was purchased from Diagnostica Stago S.A.S. Normal pooled plasma (NPP) was obtained from six healthy donors via the Sanquin Blood Bank (UMC Utrecht, The Netherlands). All buffer components and general chemicals were of analytical grade and obtained from Sigma-Aldrich unless stated otherwise.

Expression and purification of recombinant thrombomodulin fragment

A recombinant fragment of human thrombomodulin including both the protein C and TAFI binding sites (TMi), spanning the C-terminal portion of epidermal growth factor–like domain 3 through domain 6 (residues Cys351 to Gly491, UniProt canonical numbering) and incorporating an oxidation-resistant M406L substitution, was expressed in *E. coli* One Shot™ BL21 Star™ (DE3) pLysS chemically competent cells (ThermoFisher Scientific) as a periplasmic fusion protein with an N-terminal DsbA tag to promote disulfide bond formation. The construct also contained a C-terminal His-tag for purification, a Myc-tag for detection, a TEV protease cleavage site for DsbA removal, and an LPETG motif for sortase-mediated conjugation.

Cells were grown in 5 L auto-induction medium, harvested, and lysed by three-freeze thaw cycles. TMi was purified by immobilized metal affinity chromatography (IMAC) using a 5 mL HiTrap™ TALON® crude column (Cytiva, Marlborough, USA, lot.10340906) prepacked with cobalt-based Superflow Sepharose resin. The DsbA tag was cleaved by incubation

with TEV protease at 4 °C overnight in redox buffer (50 mM Tris-HCl, 200 mM NaCl, 3 mM reduced glutathione, 0.3 mM oxidized glutathione, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8). EDTA was neutralized by addition of 20 mM $MgCl_2$ and a second round of IMAC was performed to remove cleaved DsbA.

Sortase A–mediated transpeptidation was then performed to attach a Gly_3-Lys-azide group to the LPETG motif, enabling copper-free click chemistry and removing the C-terminal His-tag, as previously described (12). A third IMAC step removed His-tagged enzymes (sortase A and TEV protease). Final purification was carried out by size-exclusion chromatography (SEC) on a HiLoad 26/600 Superdex 75 pg column (Cytiva). Protein purity was assessed by SDS-PAGE, and concentrations were determined by UV absorbance at 280 nm.

Liposome preparation

Liposomes were prepared with molar ratios of PE/PS/PC/cholesterol of 0.1/0/0.6/0.3 (0%PS), 0.1/0.1/0.5/0.3 (10%PS), and 0.1/0.2/0.4/0.3 (20%PS). Lipids (DOPC, DOPS, DOPE, and cholesterol) were dissolved in chloroform, dried under reduced pressure using a rotary evaporator (Büchi® Rotavapor® R-210), and further dried under a nitrogen stream. Lipid films were rehydrated in HEPES-buffered saline (HBS), bath-sonicated, and extruded four times through 200 nm polycarbonate membranes (Liposofast LF-50, Avestin) to produce unilamellar liposomes.

Conjugation of TMi to liposomes

Azide-labeled, purified TMi was conjugated to DSPE-PEG2000-DBCO by incubation at a 1:3 molar ratio for 72 hours at 4°C. Conjugation efficiency was assessed by SDS-PAGE. The resulting TMi–DSPE-PEG conjugate was post-inserted into liposomes at a TMi-to-total-lipid molar ratio of 1:100 by incubation at 37°C for 2 hours. Unconjugated TMi was removed by dialysis (Spectra/Por Float-A-Lyzer G2, 100 kDa, 1 mL) against 1× HBS overnight at 4 °C. Liposomes were recovered and stored at 4 °C until use.

Characterization of TMi-functionalized liposomes

Protein incorporation was assessed by SDS-PAGE followed by Coomassie staining (Bio-Safe™ G-250, Bio-Rad), and protein concentration was estimated via a standard curve of clicked TMi. Surface display and removal of non-inserted conjugate were evaluated by dot blot. Both liposome samples and flow-through obtained after filtration using 100 kDa centrifugal filters (Amicon Ultra, Sigma-Aldrich) were applied to nitrocellulose membrane, blocked with Odyssey Blocking Buffer (LI-COR), and probed with a mouse anti-Myc primary antibody followed by donkey anti-mouse IgG IRDye 800 secondary antibody.

Total lipid content was determined using a modified Rouser phosphate assay, with phosphate standards, perchloric acid digestion, and subsequent colorimetric development using ammonium molybdate and ascorbic acid. Absorbance was measured at 797 nm.

Liposome size distribution was assessed by dynamic light scattering (DLS) using a Zetasizer Nano ZSP (Malvern Panalytical), samples were diluted 1:30 in PBS.

Protein C activation kinetics

Kinetics of protein C activation were assessed for soluble TMi (sTMi) and all liposome-conjugated TMi formulations. Reactions were performed at 37 °C in HEPES buffer (10 mM HEPES, 150 mM NaCl, 3 mM $CaCl_2$, 0.1% bovine serum albumin (BSA), pH 7.4) with varying concentrations of protein C (0–500 nM, kindly provided by J. Meijers) and fixed concentrations of TMi (1.0–4.0 nM). Thrombin (Enzyme Research Laboratories) was added at half the molar concentration of TMi.

Reactions were stopped after 2 or 5 minutes by addition of hirudin (1 U/mL). Generated activated protein C (APC) was quantified using the chromogenic substrate S-2366 (final concentration 333 µM). Absorbance at 405 nm was measured kinetically at 37 °C. Reaction rates were converted to APC concentrations using a standard curve generated from purified APC (Prolytix).

Thrombin generation in plasma

Thrombin generation was measured in NPP using the calibrated automated thrombogram (CAT) method (4). Plasma was spiked with 5uM of the 20%PS empty liposome and varying concentrations of liposomal TMi or sTMi (0-50nM) and triggered with 1 or 5 pM tissue factor (TF, Dade Innovin, Siemens) in HBS containing 0.1% BSA. Reactions were initiated by adding activator mix (final concentrations: 16.7 mM $CaCl_2$, 0.42 mM fluorogenic thrombin substrate I-1140). Final phospholipid (PL) concentrations were equalized across conditions by addition of empty 20%PS liposomes. Measurements were performed in duplicated in 96-well plates (ThermoFisher, lot.12565500) using a Fluoroskan Ascent FL reader. Thrombin calibrator was used for calibration. Data were analyzed using CAT software, and dose–response curves of endogenous thrombin potential (ETP) versus TMi concentration were fitted.

Thrombin generation in whole blood

Whole blood TG was assessed as previously described, with minor modifications (13, 14). Citrated whole blood from healthy volunteers was obtained via the MiniDonor biobank facility of the University Medical Center Utrecht (Biobank number 18-774). Blood was processed within 4 h of collection.

Trigger mixes contained 1 pM tissue factor, 9 mM $CaCl_2$, 16.6 µM empty 20%PS liposomes, and titrated concentrations of liposomal TMi or sTMi (0–100 nM) in BSA buffer. After pre-incubation for 10 minutes at 37 °C, reactions were initiated by mixing the trigger, blood, and fluorogenic substrate (417 µM in 6% BSA buffer; I-1140 Z-Gly-Gly-Arg-AMC, Bachem). Fluorescence was measured for 60 minutes (excitation 340 nm, emission 450 nm) at 30-

second intervals using a SpectraMax iD3 plate reader (Molecular Devices) with intermittent orbital shaking (5 s).

Reactions were performed in duplicate in clear, round-bottom 96-well plates (ThermoFisher, lot 12565500). Fluorescence data were analyzed using a validated preprogrammed spreadsheet template in Microsoft Excel to calculate the WB-thrombogram parameters (13). Endogenous thrombin potential (ETP) values were normalized to the no-TMi control.

Statistics

All data are reported as mean ± SD. IC_{50} values were derived by four-parameter nonlinear regression. Comparisons between groups were performed using one-way ANOVA with Tukey's post hoc test. Analysis was performed in GraphPad Prism (version 10.6.1). Plasma thrombin generation parameters were derived from CAT software, and whole blood thrombin generation parameters were calculated using a validated Microsoft Excel template. Experiments were performed in three to six independent replicates, depending on the assay: protein C activation kinetics (n = 3), plasma thrombin generation (n = 4–6), and whole blood thrombin generation (n = 4–5).

RESULTS

Production, characterization, and conjugation of TMi to liposomes

Recombinant TMi, comprising EGF domains 3–6, was successfully expressed in *E. coli* and purified using immobilized metal affinity chromatography followed by size-exclusion chromatography. Purified TMi was conjugated to DSPE-PEG_{2000}-DBCO via click chemistry (**Figure 1A**). SDS-PAGE analysis showed an increase in apparent molecular weight after conjugation, with disappearance of the unconjugated TMi band, indicating efficient and complete conjugation (**Figure 1B**).

The clicked TMi was post-inserted into three liposomal formulations containing increasing amounts of phosphatidylserine (PS): TMi-0%PS, TMi-10%PS, and TMi-20%PS (**Figure 2A**). To maintain overall lipid balance, phosphatidylcholine (PC) was adjusted accordingly, while phosphatidylethanolamine (PE) and cholesterol were kept constant. The resulting molar ratios of PE/PS/PC/cholesterol were 0.1/0/0.6/0.3 (TMi-0%PS), 0.1/0.1/0.5/0.3 (TMi-10%PS), and 0.1/0.2/0.4/0.3 (TMi-20%PS). Dynamic light scattering confirmed similar particle diameters across all formulations, with mean diameters ranging from 180 to 210 nm. Protein-to-lipid ratios were also similar among formulations (0.65-0.86%).

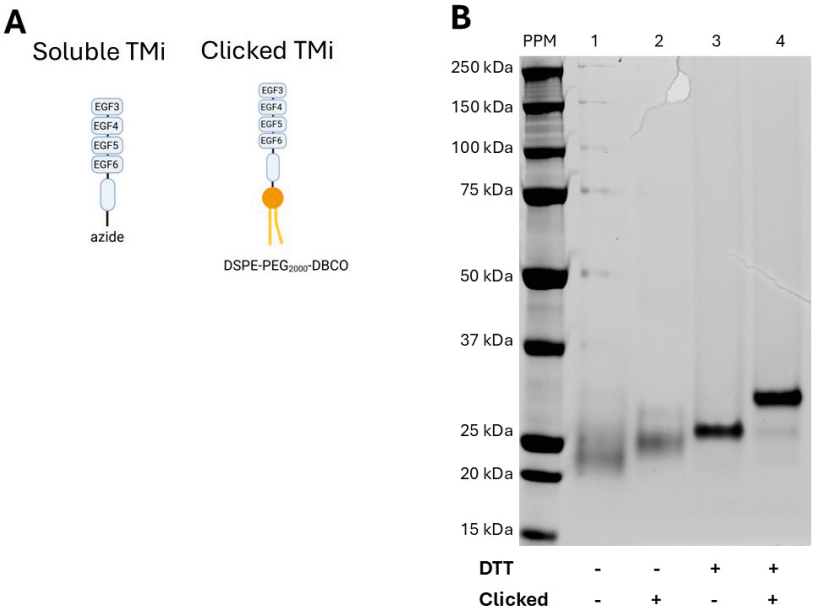

Figure 1. Production, purification and conjugation of TMi

A) Schematic representation of the TMi fragment (EGF domains 3-6 of thrombomodulin), showing both the soluble form and lipid-conjugated (clicked) TMi. B) SDS-PAGE analysis of purified soluble and clicked TMi, stained with Coomassie Blue. Samples were run under reducing (+DTT) and non-reducing (–DTT) conditions to assess molecular weight and disulfide bond status.

Protein C activation kinetics

The functional activity of TMi formulations was assessed by measuring thrombin-mediated protein C activation, with soluble TMi (sTMi) as a control (**Figure 2B**). Michaelis–Menten constants (Km) were derived from these curves.

Liposomal TMi formulations showed markedly reduced Km values compared with sTMi, indicating enhanced apparent affinity for protein C. In particular, TMi-10%PS (Km = 124 ± 47 nM) and TMi-20%PS (Km = 48 ± 2 nM) showed significantly improved kinetic parameters relative to sTMi (Km = 524 ± 138 nM) (**Figure 2C**). The 0%PS formulation also exhibited a lower Km (323 ± 61nM), although this difference did not reach statistical significance (p = 0.06). These data indicate that membrane incorporation of thrombomodulin enhances thrombin-mediated protein C activation, with increasing PS content further improving enzymatic efficiency.

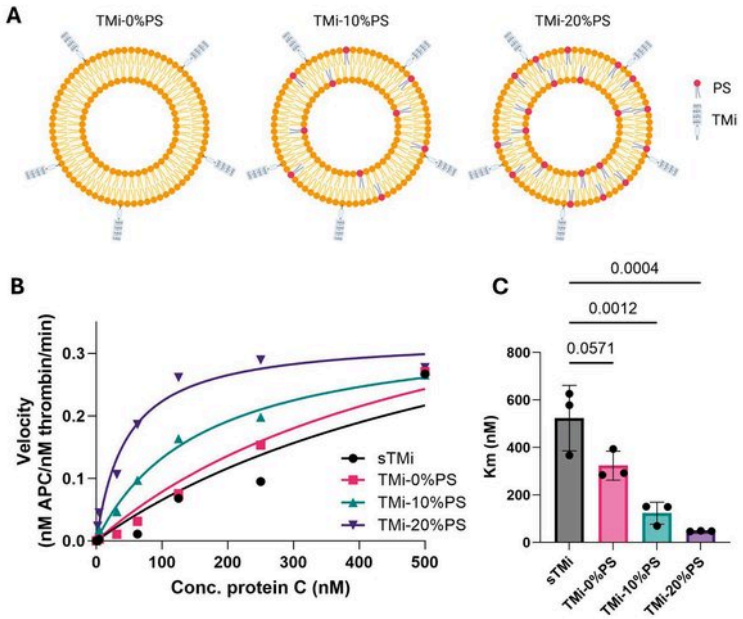

Figure 2. Enzyme kinetics of protein C activation by different liposomal TMi formulations
A) Schematic representation of the three liposomal TMi formulations: 0%PS-TMi, 10%PS-TMi, and 20%PS-TMi. B) Representative Michaelis-Menten curves from a single experiment, showing reaction velocity (nM activated protein C [APC] per nM thrombin per minute) at varying protein C concentrations. C) Boxplot of Km values for each formulation from three independent experiments (n=3).

Liposomal TMi enhances anticoagulant activity in plasma TG assays

Addition of sTMi to normal pooled plasma inhibited thrombin generation in a dose-dependent manner (**Figure 3A, C**). All liposomal TMi formulations reduced thrombin generation more effectively than sTMi. At 1 pM TF, the IC_{50} for TMi-20%PS was 0.6 ± 0.3 nM, compared with 3.1 ± 0.6 nM for sTMi ($p < 0.0001$). TMi-0%PS and TMi-10%PS also significantly inhibited thrombin generation, with IC_{50} of 1.1 ± 0.2 nM and 1.4 ± 0.2 nM, respectively (**Figure 3B**).

Similar trends were observed at 5 pM TF. TMi-20%PS again showed the strongest inhibitory effect, with an IC_{50} of 1.3 ± 0.3 nM, compared with 7.1 ± 2.6 nM for sTMi ($p < 0.001$). TMi-0%PS and TMi-10%PS displayed intermediate activity, with IC_{50} values of 2.4 ± 1.0 nM and 2.8 ± 0.7 nM, respectively (**Figure 3D**).

Overall, TMi-20%PS consistently exhibited the greatest anticoagulant activity. At 1 pM TF, its IC_{50} was significantly lower than that of TMi-10%PS ($p = 0.007$), indicating that increasing PS content enhances anticoagulant efficacy, although differences between 0% and 10% PS were modest.

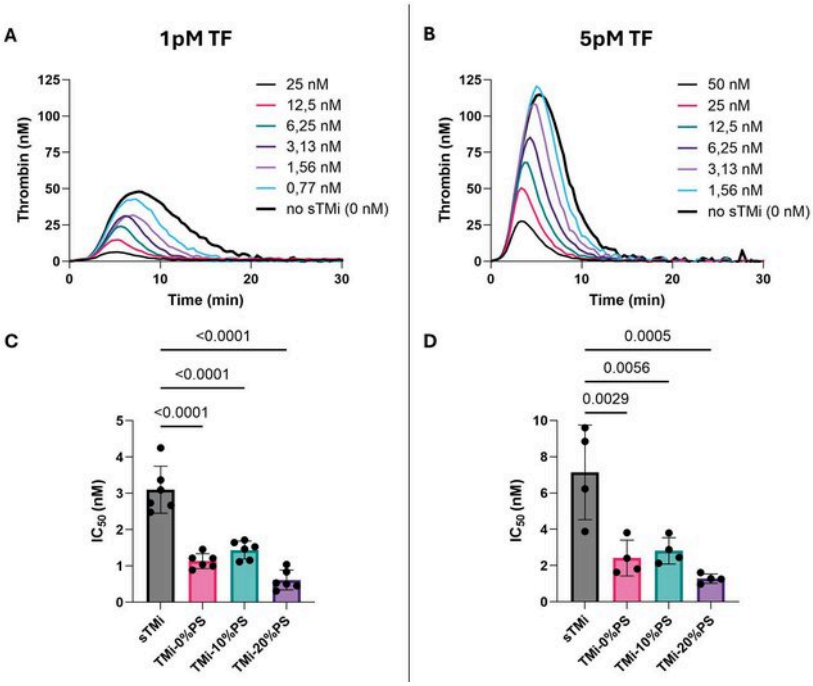

Figure 3. Thrombin generation in plasma with different liposomal TMi formulations
(A, B) Representative thrombograms from a single experiment showing thrombin generation in normal pooled plasma at increasing concentrations of sTMi, triggered with 1 pM tissue factor (TF) (A) and 5 pM TF (B). All conditions contained 5 µM of empty %20PS liposomes to maintain a consistent phospholipid (PL) background. (C, D) IC_{50} values of the different liposomal TMi formulations and sTMi, representing the concentration required to achieve 50% inhibition of the endogenous thrombin potential (ETP) at 1 pM (C) and 5 pM TF (D).

Liposomal TMi overcomes reduced TM sensitivity in whole blood

Addition of sTMi to whole blood inhibited thrombin generation in a dose-dependent manner (**Figure 4A**). However, sTMi demonstrated markedly reduced anticoagulant activity in whole blood compared with plasma, requiring substantially higher concentrations to achieve inhibition. Specifically, IC_{50} values increased from 3-7 nM in plasma (1-5 pM TF) to 41 nM in whole blood. In contrast, liposomal TMi formulations needed much lower concentrations, particularly TMi-20%PS. This formulation achieved approximately 70% reduction in ETP at concentrations where sTMi had minimal effect. The IC_{50} for TMi-20%PS was significantly lower than that of sTMi (7 ± 4 nM vs. 41 ± 29 nM, $p < 0.01$). TMi-0%PS and TMi-10%PS also showed enhanced activity relative to sTMi, with IC_{50} of 13 ± 11 nM and 11 ± 9 nM, respectively, although these differences did not reach statistical significance (**Figure 4B**).

Collectively, these results demonstrate that liposomal presentation substantially enhanced TMi anticoagulant efficacy in whole blood. Among the liposomal formulations tested, increasing PS content was associated with greater inhibition, while differences between 0% and 10% PS remained relatively small.

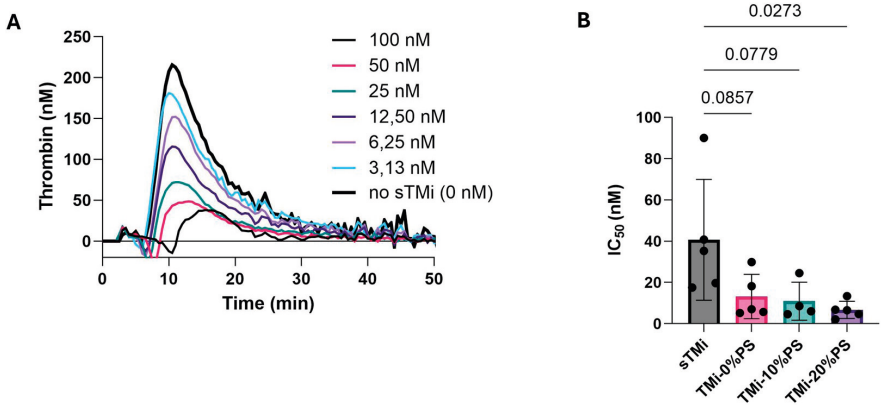

Figure 4. Thrombin generation in whole blood with different liposomal TMi formulations
(A) Representative thrombograms from a single experiment showing thrombin generation in whole blood at increasing concentrations of sTMi, triggered with 1 pM tissue factor (TF) and 16uM of empty 20%PS liposomes. (B) IC_{50} values of the different liposomal TMi formulations and sTMi, representing the concentration required to achieve 50% inhibition of the endogenous thrombin potential (ETP).

DISCUSSION

In this study, we developed three liposomal formulations of thrombomodulin (TMi) and demonstrated that membrane anchoring significantly enhances its anticoagulant activity in TG assays in both plasma and whole blood. Among the tested formulations, liposomes enriched with 20% phosphatidylserine (TMi-20%PS) were most potent, showing a 6-fold lower IC_{50} compared to sTMi. Higher PS content enhanced TMi activity, also reflected by a lower Km for protein C activation. These findings underscore the importance of membrane presentation and lipid composition in mimicking physiological TM function. Overall, our approach provides a novel strategy to study protein C–mediated anticoagulant function in TG assays under physiologically relevant conditions.

Our findings build on and extend previous studies on membrane-anchored thrombomodulin. Similar to earlier reports (15, 16), we observed a marked 10-fold reduction in Km after liposome conjugation of thrombomodulin, with similar Km values for liposomes containing 20% PS (15) and liposomes without PS (16). Our approach indicates this reduction in Km translates into substantially increased efficacy in protein C activation in complex biological matrices such as plasma or whole blood, whereas both previous studies assessed the constructs only in purified systems. Collectively, these results highlight the importance of both membrane presentation and lipid composition in optimizing thrombomodulin activity. These findings also aligns with animal studies showing that TM targeted to red blood cells or endothelium exhibits greater anticoagulant efficacy with soluble TM (17, 18).

We found that protein C activation increased with rising PS content in the liposomes, indicating a PS-dependent enhancement of TM activity. This observation is consistent with previous studies showing that PS-rich membranes support TM-mediated protein C activation (11, 15). Mechanistically, the negative charge of PS facilitates protein C binding via its Gla-domain. Galvin et al. showed membrane incorporation of TM does not enhance activation of protein C of Gla-domainless protein C, highlighting the importance of the Gla domain for PS-mediated enhancement. In the absence of endothelial protein C receptor (EPCR), which generates high-affinity protein C binding sites (Km ~20 nM) and enhances activation rates by up to ~100-fold on endothelial cells (19), PS-rich liposomes may partially compensate by concentrating protein C near membrane-bound TM. Our data suggest that TM anchored on PS-containing liposomes can therefore partially mimic this anticoagulant surface. In addition, phosphatidylethanolamine (PE), which is abundant in physiological membranes and present in our liposomes, may further enhance this effect. Previous studies have shown that PE-containing TM liposomes increase protein C activation relative to PC alone, while also modulating cofactor selectivity, favoring protein C over TAFI activation (11). In addition, biophysical studies demonstrate strong synergy between PS and PE in promoting binding of Gla-domain–containing coagulation factors, particularly at low PS levels characteristic of physiological membranes (20). Together, these findings indicate

that PS/PE-containing liposomes recapitulate key structural and functional features of endothelial and microparticle-associated anticoagulant surfaces.

Our results suggest that liposomal TM formulations hold promise for diagnostic applications in TG assays by enabling sensitive and robust assessment of protein C pathway function in both plasma and whole blood. The enhanced potency of liposomal TM reduces the amount of protein required, which may lower assay costs and improve scalability. In addition, controlled modulation of lipid composition provides a mechanistic tool to dissect lipid-dependent regulation of protein C activation. Applied to patient samples, this platform could support functional evaluation of the protein C pathway and offer a standardized, physiologically relevant complement to existing coagulation diagnostics. Our findings highlight how membrane presentation and lipid composition govern thrombomodulin activity, principles that may also inform future translational approaches beyond diagnostic applications (17, 18).

While this study demonstrates the utility of liposomal TM in plasma and whole blood, several factors warrant further investigation to support broader application. Experiments were performed using samples from healthy donors, which do not capture the variability of clinical conditions in which the protein C pathway may be dysregulated. The stability and batch-to-batch reproducibility of the liposomal TM formulations also require evaluation to ensure robustness for diagnostic use. Incorporating additional components, such as EPCR, or optimizing lipid composition and protein-to-lipid ratios may further enhance physiological relevance and assay performance. Testing TM variants lacking the EGF3 domain could help isolate protein C-specific effects, and the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) with these liposomal formulations should also be assessed. Finally, clinical validation in disease-specific samples will be essential to establish the diagnostic potential of this platform.

In conclusion, anchoring thrombomodulin on PS-rich liposomes significantly enhances its anticoagulant activity in TG assays, particularly in whole blood, where soluble TM is less effective. This strategy improves assay sensitivity and better mimics physiological cofactor presentation, providing a promising platform for functional assessment of the protein C pathway. Optimizing lipid composition and validating the approach in clinical samples will be essential to fully realize the diagnostic potential of this approach.

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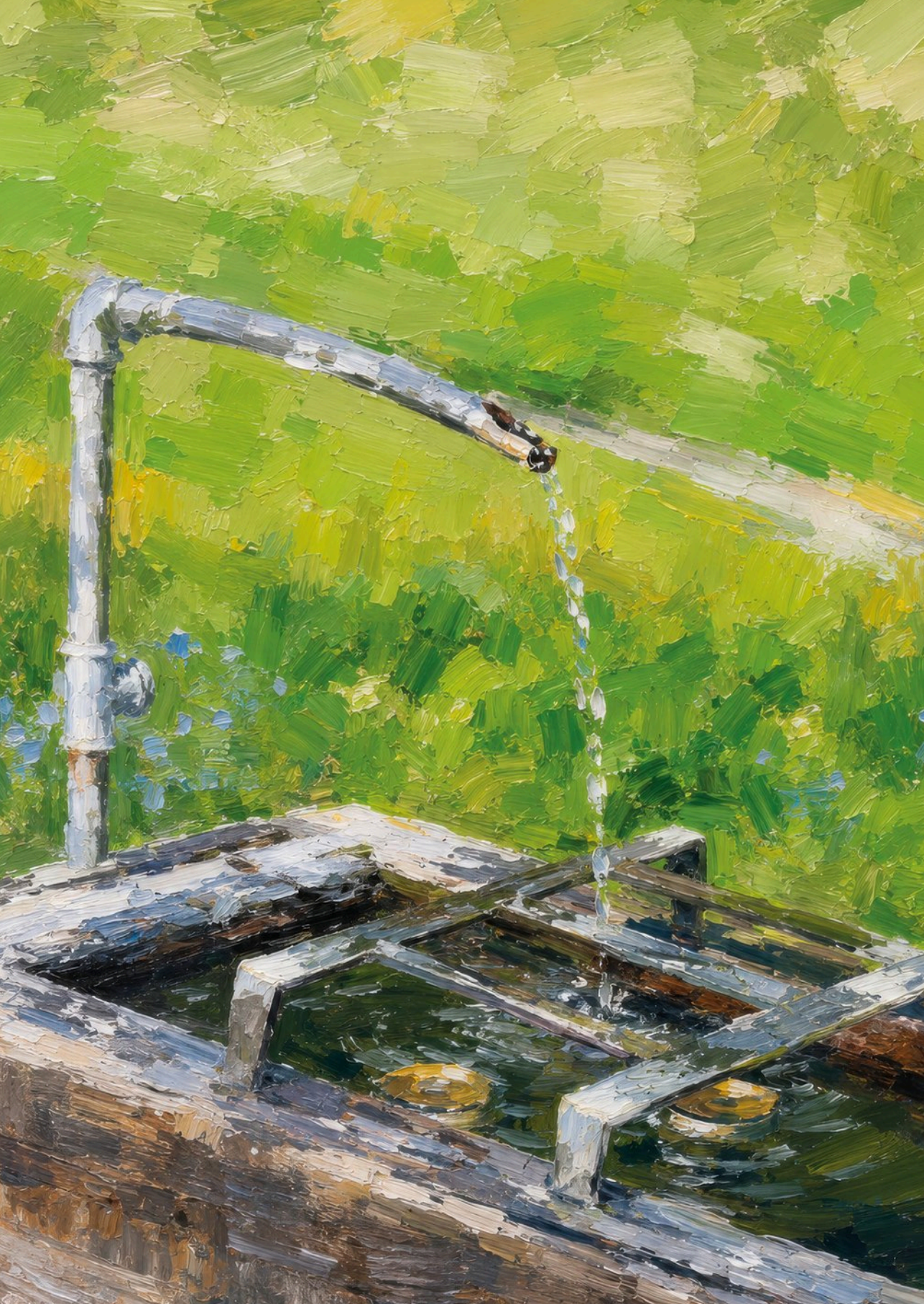
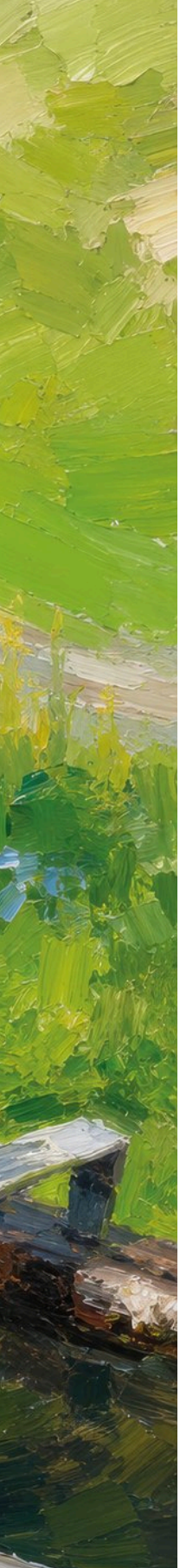
Authorship contribution

Geke C. Poolen – Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original Draft, Writing – Review & Editing, Project Administration, Supervision
Lennart de Vries – Methodology, Investigation, Data curation, Visualization
Jente Schoenaker – Methodology, Investigation
Jasper Verhagen – Methodology, Investigation
Rolf T. Urbanus – Conceptualization, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition





CHAPTER 7

VhH anti-thrombomodulin clone 1 inhibits TAFI activation and enhances fibrinolysis in human whole blood under flow

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J Thromb Haemost. 2022 May;20(5):1213-1222.

ABSTRACT

Background

Thrombomodulin on endothelial cells can form a complex with thrombin. This complex has both anticoagulant properties, by activating protein C, and clot-protective properties, by activating thrombin-activatable fibrinolysis inhibitor (TAFI). Activated TAFI (TAFIa) inhibits plasmin-mediated fibrinolysis.

Objectives

TAFIa inhibition is considered a potential antithrombotic strategy. So far, this goal has been pursued by developing compounds that directly inhibit TAFIa. In contrast, we here describe variable domain of heavy-chain-only antibody (VhH) clone 1 that inhibits TAFI activation by targeting human thrombomodulin.

Methods

Two llamas (*Lama Glama*) were immunized and phage display was used to select VhH anti-thrombomodulin clone 1. Affinity was determined with Surface Plasmon Resonance (SPR) and binding to native thrombomodulin was confirmed with flow cytometry. Clone 1 was functionally assessed by competition-, clot lysis- and thrombin generation assays. Lastly, the effect of clone 1 on tPA-mediated fibrinolysis in human whole blood was investigated in a microfluidic fibrinolysis model.

Results

VhH anti-TM clone 1 bound recombinant thrombomodulin with a binding affinity (K_D) of 1.70.4±nM and showed binding to native thrombomodulin. Clone 1 competed with thrombin for binding to thrombomodulin and attenuated TAFI activation in clot lysis assays and protein C activation in thrombin generation experiments. In a microfluidic fibrinolysis model, inhibition of thrombomodulin with clone 1 fully prevented TAFI activation.

Discussion

We have developed VhH anti-TM clone 1 which inhibits TAFI activation and enhances tPA-mediated fibrinolysis under flow. Different from agents that directly target TAFIa, our strategy should preserve direct TAFI activation via thrombin.

KEYWORDS

Activated protein C - Fibrinolysis - Thrombin activatable fibrinolysis inhibitor - Thrombomodulin - Variable domain of the heavy chain of heavy-chain-only antibodies

INTRODUCTION

To prevent excessive fibrin accumulation, thrombin formation is controlled by several anticoagulant pathways. The transmembrane receptor thrombomodulin plays an essential role in maintaining the haemostatic balance. Thrombomodulin is expressed by vascular endothelial cells and is continuously exposed to circulating blood. Thrombomodulin is able to capture circulating thrombin, forming the thrombomodulin-thrombin complex, thereby altering thrombin's substrate specificity (1). When in complex with thrombomodulin, thrombin loses its ability to convert fibrinogen into fibrin. Instead, it activates Protein C, which then inhibits the formation of thrombin by inactivating the coagulation cofactors Va and VIIIa (2). From this perspective, the thrombomodulin-thrombin complex has an anticoagulant function. However, the complex also has clot-protective properties, as it can activate TAFI (3). TAFI is produced in the liver as a zymogen and acts as an inhibitor of fibrinolysis upon activation (4). Fibrinolysis is mediated by the enzyme plasmin. During fibrinolysis, plasmin exposes carboxy-terminal lysine residues in fibrin fibers through limited proteolysis (5). These lysine residues act as binding scaffolds for plasminogen and enhance plasmin generation up to 1000-fold. TAFIa removes these lysine residues and consequently abrogates this feedback loop (6). From this perspective, the thrombomodulin-thrombin complex slows down fibrinolysis and has a procoagulant function. Whether the anti- or procoagulant properties of thrombomodulin prevail, is directly related to its concentration (7).

Thrombomodulin consists of various domains: a C-type lectin like domain, six epidermal growth factor (EGF)-like domains, a serine-threonine-rich region, a transmembrane region and a short cytoplasmic tail. The divergent properties of thrombomodulin involve distinct structural regions on the EGF-domains. Thrombin binds to EGF-like domains 5 and 6 (8); Protein C and TAFI both bind regions on EGF-domain 4 (9); and TAFI also binds EGF-domain 3 (10–12). Inhibiting TAFI activation is considered a potential antithrombotic strategy as it improves physiological fibrinolysis (5, 13). First, because elevated TAFIa levels have been associated with an increased risk for deep vein thrombosis, angina pectoris and coronary artery disease (14, 15). Second, because inhibiting TAFI activation could reduce dosing of the conventional therapeutic plasminogen activator tPA, which bears an inevitable risk for therapy-related bleeding (16).

On a mechanistic level, TAFI inhibition can be performed either by directly blocking TAFIa or by blocking thrombomodulin-mediated TAFI activation (5). Several strategies have been reported for direct inhibition of TAFIa, involving antibodies (17, 18), small molecules (19) and VhHs (13). VhHs are specifically interesting as they have high affinity for conformational epitopes, their small size allows for improved penetration of target tissue (5) and they lack the Fc-domain of conventional antibodies, limiting the risk for triggering an endogenous immune response (20). Earlier, a VhH anti-rat-TAFI has been described that inhibits fibrin deposition in murine lungs in a thromboembolism model and, in addition, enhances fibrinolytic therapy with tPA (16). To our knowledge, however, there

have not been strategies that inhibit TAFI activation by targeting the thrombomodulin-thrombin complex. It can be argued that interfering early in the activation cascade increases therapeutic effectivity (21, 22). However, it might simultaneously also interfere with protein C activation (23).

We here describe the development of a VhH targeting human thrombomodulin that prevents TM-mediated TAFI activation, thereby enhancing fibrinolysis in human whole blood.

MATERIALS AND METHODS

Subjects

Citrated human pooled normal plasma (PNP) was prepared from fresh venous blood of 170 healthy volunteers. All donors provided written informed consent in accordance with the declaration of Helsinki. Approval was obtained from the local ethics committee. Fresh whole blood for flow studies was obtained from healthy volunteers through venepuncture, collected in BD vacutainer trisodium citrate (105mM) tubes and used within 4 hours after collection.

Biochemicals

The entire extracellular region of thrombomodulin, spanning from ala19 to his516 and containing the C-type lectin domain, 6 EGF-like domains and serine-threonine–rich (S-T) region), was expressed with a N-terminal tobacco etch virus (TEV)-cleavable His_6-tag and purified by U-Protein Express BV (*Utrecht, the Netherlands*). VhHs targeting GP1b (clone 17), fibrin (clone B12), and azo-dye RR6 (clone R2, negative control) (24) were produced in *Escherichia coli* (*E. coli*) and purified through sequential immobilized metal affinity chromatography and size exclusion chromatography as described (25). According to instructions of the manufacturers, VhH anti-GP1bα clone 17 was labelled with AlexaFluor488-NHS-ester (*Thermo Fisher, Roskilde, Denmark*). VhH anti-fibrin clone B12 was labelled with Gly_3-azide (*IRIS Biotech GmbH, Marktredwitz, Germany*) through sortagging and clicked to DBCO-AlexaFluor-647 as described (20).

VhH selection

Two llamas (*Lama Glama*) received four rounds of subcutaneous immunizations with the recombinant extracellular domain of human thrombomodulin with weekly intervals. A bacteriophage library containing the heavy chain only-antibody repertoire of these llamas was created as described (25). A transformation efficiency of 10^7 was achieved. Subsequently, phage-display was used to select VhH anti-TM clones. Hereto, thrombomodulin was biotinylated with EZ-Link Sulfo-NHS-LC-Biotin according to the instructions of the manufacturer (*Life Technologies, Carlsbad, USA*) and immobilized on a neutravidin-coated polysorp flat bottom plate (0.5µg/mL) in PBS (137mM NaCl, 2.7mM KCl, 9.2mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.4) at 4°C overnight. Phages were then panned

on thrombomodulin, eluted with 0.1M triethanolamine, pH 11.5 and allowed to infect *E. coli* (TG1), as described (25). TG1 were plated on Yeast Tryptone-agar plates containing glucose (2% w/v) and ampicillin (100µg/mL) and incubated overnight at 37°C. The following day, colonies representing individual VhHs were picked and grown. VhH production was initiated with 0.1M Isopropyl β-d-1-thiogalactopyranoside (IPTG) in 2xYT medium with 0.2% glucose and 100µg/mL ampicillin and culture supernatants were used for subsequent selection of VhH anti-TM clones. Hereto, thrombomodulin was immobilized on Nunc MaxiSorp 96-well microtiter plates (0.5µg/mL) in PBS at 4°C overnight, after which plates were blocked with 2% skimmed milk (*Sigma Aldrich, St. Louis, MO, USA*). Culture supernatants were incubated on immobilized thrombomodulin. After thorough washing with PBS containing 0.1% tween-20, plates were incubated with mouse anti-c-myc antibody (clone 9E10, 1µg/mL) in PBS containing 2% skimmed milk for 1 hour at room temperature (RT), washed thoroughly again and incubated with HRP-conjugated rabbit anti-mouse IgG (*Dako Cytomation, Glostrup, Denmark*) for 1 hour at RT. Plates were washed a final time and incubated with 100µL/well TMB (*Tebu-bio, Heerhugowaard, the Netherlands*) per well. Colorimetric reactions were stopped with 50µL 0.1M H_2SO_4 and plates were read in a SpectraMax iD3 at 450nm. VhH clones binding thrombomodulin were sequenced.

VhH production and purification

VhHs anti-TM with a unique complementarity-determining region 3 (CDR3) were codon optimized and ordered as gBlocks at Integrated DNA Technologies (*Coralville, USA*). gBlocks were ligated in pJET1.2 vector, cut with restriction enzymes BamHI and NotI (*New England Biolabs; Leiden, The Netherlands*) and ligated into the pTH4.Click production vector (20). One-shot Bl21 pLysS (*E. coli*) were transformed with VhHs and grown in 2xYT media with 0.2% glucose, 100µg/mL ampicillin and 34µg/mL chloramphenicol. VhH-production was initiated with 1mM IPTG, followed by overnight production at 37°C. The following day, bacteria were collected through centrifugation at 5000xG for 15 min, resuspended in 25mM HEPES, 500mM NaCl, pH 7.8, 10mM MgCl2, and 1µg/ml DNaseI, after which they were lysed in 3 sequential freeze-thaw cycles. VhHs were purified from supernatant with immobilized metal affinity chromatography with Talon Superflow Sepharose (*Cytiva, Marlborough, USA*). Purified VhHs were then subjected to size exclusion chromatography on a Superdex 200 pg column (*Cytiva, Marlborough, USA*). VhH preparations were >95% pure as confirmed with SDS-Page and Coomassie blue staining. Extinction coefficients were calculated for each VhH with the online ProtParam Tool (http://web.expasy.org/protparam/) and concentrations were determined by measuring absorbance at 280nm.

Surface Plasmon Resonance (SPR) experiments

SPR experiments were performed on a Biacore T100 system (*Cytiva, Marlborough, USA*). Hereto, 400RU of thrombomodulin was immobilized on a CM5 Series S sensor chip with standard amine-coupling chemistry according to the instructions of the manufacturer. Flow channel 1 received a blank immobilization and was used as a reference. VhH anti-TM clones were injected at various concentrations as indicated at a flowrate of 30uL/

min in 10mM HEPES, 150mM NaCl, 0.01% Tween-20, pH 7.4 for 2 min, followed by a 10 min dissociation phase. Thrombomodulin surfaces were regenerated with 10mM glycine, pH 2.0 for 1 min at a flowrate of 30uL/min. Signals obtained in the reference channel were subtracted from the sensorgrams of the channel that contained thrombomodulin. Reference-corrected sensorgrams were fitted with a 1:1 Langmuir binding model with Biacore T100 evaluation software to determine association (K_a), dissociation (K_d) and affinity (K_D) constants. Experiments were repeated three times.

Binding of VhHs to endothelial cells

100µL VhH anti-TM clone 1 and VhH R2 (functioning as isotype-control) were incubated with 900µL of Alexa Fluor™ 647 NHS Ester (*Thermo Fisher Scientific, Waltham, USA*) and 100µL $NaHCO_3$ (0.1M, pH 8.3) for 1 hour at RT in dark (molar ratio VhH:Dye of 1:40). Free label was removed by ZebaSpin (*Thermo Scientific, Waltham, Massachusetts, USA*). HUVECs were isolated from umbilical cords by trypsinization as described (26) and cultured in Endothelial Cell Growth Medium-2, supplemented with Supplement Mix (C-39216) and penicillin-streptomycin, in a T-75 cell culture flask at 37°C, 5% CO_2. Medium was refreshed every 2-3 days. Flow cytometry experiments were performed when cells were in passage 2-4. Hereto, cells were washed with PBS (2x) and detached with 20mM EDTA. Subsequently, cells were centrifuged at 300xG for 5 min and resuspended in fresh medium. 1µg, 3µg or 5µg of VhH anti-TM clone 1 or VhH R2 was added to 10^5 cells in 100µL and incubated for 45 min at RT. Unbound VhHs were removed by spinning down cells, removing the supernatant and resuspension of cells in Dulbecco PBS (Sigma Aldrich). Cells were diluted (1:6) with fixative solution (137mM NaCl, 2.7mM KCl, 1.12mM NaH_2PO_4, 1.15mM KH_2PO_4, 10.2mM Na_2HPO_4, 4mM EDTA, 1.11% formaldehyde, pH 6.8). Flow cytometry experiments were performed on a BD FACS Canto II (*BD Biosciences, San Jose, CA,USA*). Cells were gated based on forward and side scatter. Binding of VhHs was expressed as median fluorescent intensity (MFI) measured in the APC-channel. Signals obtained with VhH R2 isotype control were subtracted to correct for background fluorescence.

Competition assay

Thrombomodulin (50µL of 0.5µg/mL) was coated on a MaxiSorp microtiter plate in PBS at 4°C overnight. Plates were blocked with 1% BSA (w/v) in PBS for 1 hour at RT and then washed three times with 0.1% Tween-20 in PBS. To test competition with thrombin for binding to TM, human α-thrombin (15nM; *ERL, South Bend, IN, USA*) was incubated with unlabelled VhH anti-TM clone 1 or VhH R2 (control) at indicated concentrations for 1 hour at RT. Plates were washed and incubated with goat anti-human thrombin (1:1000; *Affinity Biologicals, Lancaster, Canada*) for 1 hour at RT, washed again and incubated with HRP-conjugated rabbit anti-sheep IgG (1:2000; *Dako Cytomation, Glostrup, Denmark*) for 1 hour at RT. Plates received a final washing step and were incubated with TMB and H_2SO_4 as indicated earlier. Absorbance was measured in a SpectraMax iD3 at 450nm. Data were presented as residual binding of either thrombin, VhH anti-TM clones 1 or 3, or VhH R2 (control). Inhibition data were analysed with non-linear regression in GraphPad prism 8.0.1.

Clot lysis assay

Clot lysis assays were performed in PNP in a microtiter plate with or without thrombomodulin. A titration range of VhH anti-TM clone 1 or VhH R2 (control) was added at final concentrations as indicated. To induce coagulation 50µL PNP was incubated with 50µL coagulation buffer (25mM HEPES, 137mM NaCl, 3.5mM KCl, 20mM $CaCl_2$, pH 7.4) with 0.1% BSA, containing 10nM thrombomodulin, 0.5pM Tissue Factor (TF, Dade® Innovin®; *Siemens, Marburg, Germany*), 4µM PS:PC:PE phospholipids vesicles in 30:20:50 ratio (Coag reagent I; *Avanti polar lipids, Alabaster, USA*), 2nM tPA (Actilyse*; Boehringer Ingelheim, Alkmaar, the Netherlands*), and VhHs (at indicated concentrations) or 25µg/mL PCPI (*Sigma Aldrich, Zwijndrecht, the Netherlands*) where indicated. Changes in absorbance at 405nm were measured in a SpectraMax iD3 at 37°C for 2hrs with 20s intervals. Clot lysis times were determined from the clot-lysis turbidity profile and defined as the time from the midpoint of the clear to maximum turbid transition, representing clot formation, to the midpoint of the maximum turbid to clear transition, representing the lysis of the clot (27). Inhibition data were analysed with non-linear regression in GraphPad prism 8.0.1. Where indicated, data were presented as relative (%) inhibition of TAFI activation, in which 0% inhibition was equal to CLT in the presence of thrombomodulin and 100% inhibition was equal to the CLT in the absence of thrombomodulin in control samples without VhHs.

Thrombin generation assay

Thrombin generation was measured with the Calibrated Automated Thrombogram (CAT) method as described (28), with a few modifications. In short, 60µL of PNP with or without 25nM thrombomodulin was incubated with 38µL of VhH anti-TM clones or VhH R2 (control) at indicated concentrations in Immulon 2 HB microtiter plates with 7µL of reagent mix containing 0.5pM TF and 4µM phospholipids in HBS with 0,1% BSA for 10 min at 37°C. Next, thrombin generation was initiated with 15µL of FluCa (100mM $CaCl_2$, 2.5mM z-Gly-Gly-Arg-AMC (*Bachem, Bubendorf, Switzerland*) in HBS with 0.1% BSA. Thrombin-α2-macroglobulin (20µL) complexes (*Thrombinoscope BV, Maastricht, the Netherlands*) and 25µL HBS were added to calibrator wells. Fluorescence was measured in a SpectraMax iD3 with excitation at 390nm, emission at 475nm at 15s intervals for 1 hour. Thrombin generation parameters were deduced from fluorescence data as described (29) and adjusted for calibrator activity. Inhibition data were analysed with non-linear regression in GraphPad prism 8.0.1.

Flow studies in human whole blood

Coverslips (20x50 mm) were functionalized with aminopropyltriethoxysilane (*Sigma Aldrich, Zwijndrecht, the Netherlands*) as described (30), thoroughly washed and dried, coated with a mixture of horm collagen (100µg/mL; *Takeda, Linz, Austria*), recombinant human TF (10pM) and thrombomodulin (10nM) in HT buffer (145mM NaCl, 5mM KCl, 500nM Na_2HPO_4 , 1mM $MgSO_4$, 10mM HEPES, pH 7.4) for 1.5 hours at RT and subsequently blocked with 1% HSA (w/v) in HT-buffer at 4°C overnight. Next, coverslips were attached to a parallel plate flow chamber with vacuum as described (31) and mounted in a Zeiss Observer Z1 microscope, equipped with Zeiss Fluar 40x/1.30 objective and Zeiss Filtersets 10 and 50 in combination

with LED 470nm and LED 625nm, respectively. A DIC filter block in combination with a halogen light bulb was used to take brightfield images. Prior to the experiment, citrated whole blood was supplemented with tPA (5.7nM), AlexaFluor488-conjugated VhH anti-GPIbα (clone 17; 190ng/mL), AlexaFluor647-conjugated VhH anti-fibrin (clone B12; 830ng/mL) and either VhH anti-TM clone 1 (514nM), VhH R2 (negative control; 514nM) or TAFIa inhibitor PCPI (25µg/mL) and was recalcified with 3.1mM $MgCl_2$ and 6.6mM $CaCl_2$. Then, blood was pulled through the flow chamber with a Harvard apparatus syringe pump at a shear rate of 800 s^{-1}. Snap shots were taken at a 40 times magnification with an interval of 20 seconds to monitor platelet adhesion and fibrin deposition in real time. After 7 min, non-calcified blood samples with same supplements entered the flow chamber, allowing tPA-mediated fibrinolysis to predominate and, consequently, to investigate fibrinolysis without continuous clotting. Fluorescent intensities were analysed with ZEN Pro software (version 2.0). To correct for inter-donor variability, the maximal absolute fibrin signal from VhH R2 runs (control) for each donor was set at 100% and the maximal signals from VhH anti-TM clone 1 and PCPI (positive control) were presented as percentage thereof.

Statistical analysis

Differences between runs in flow studies after supplementing whole blood with VhH anti-TM clone 1, PCPI or VhH R2 were analysed with Kruskal-Wallis analysis with multiple comparisons from mean values with GraphPad Prism software 8.0.1. Results were considered statistically significant at $p \leq 0.05$.

RESULTS

Selection and characterization of two high affinity VhHs targeting TM

Phage display yielded forty-nine clones with affinity for thrombomodulin, derived from eight distinct families based on sequence variation. VhH anti-TM clone 1 showed high binding in solid-phase binding assays during the panning procedure (not shown).

Table 1. Biophysical characteristics

	VhH anti-TM clone 1
Target	Extracellular thrombomodulin domains
Full sequence amino acids	155
CDR1	GFTFSSYWMY
CDR2	VSKISNGGRDTYYADS
CDR3	SKDSNGLER
Mw (DA)	16523.06
Isoelectric point	6.77
Ext. coeff. (280nm)	1.630
Thrombomodulin binding KD	1.68±0.40nM
Ka (1/Ms)	$6.11E^{5}$±$3.25E^{5}$
Kd (1/s)	$9.76E^{-4}$±$3.78E^{-4}$

Subsequently, clone 1 was codon-optimized for production in *E. coli*, produced and purified. The biophysical properties of clone 1 are listed in **Table 1**. Binding characteristics were analysed with SPR **(Fig. 1A),** which indicated that clone 1 has an affinity of 1.70.4±nM (K_D) for recombinant thrombomodulin. To confirm reactivity towards native human thrombomodulin, binding of fluorophore-labelled clone 1 to quiescent HUVECs was assessed with FACS, showing specific and dose-dependent binding **(Fig. 1B).** In addition, clone 1 fully attenuated thrombin binding to thrombomodulin (IC_{50} 148.6nM) **(Fig. 1C**).

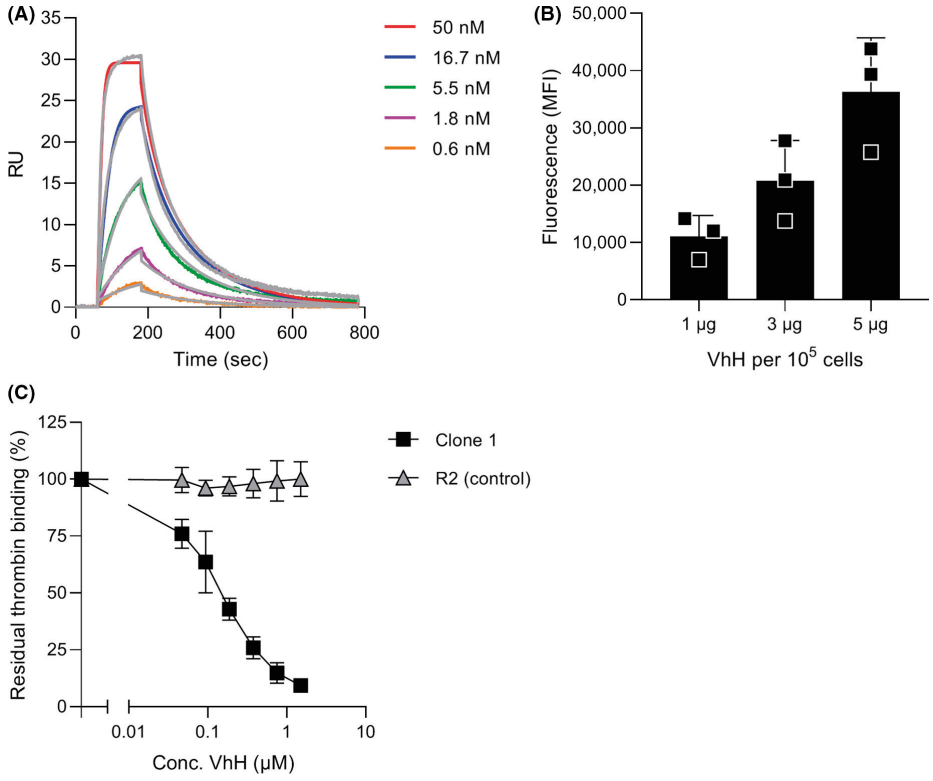

Figure 1. Selection and characterization of VhH anti-TM clone 1

(A) Clone 1 was injected over a thrombomodulin-immobilized surface at the indicated concentrations for SPR analysis. Association was measured for 2 min, followed by 10 min of dissociation and full regeneration of the surface. Reference-corrected sensorgrams are shown in colour and the fit on the data is shown in grey. (B) Quiescent HUVECs (10^5 cells in 100µL) were incubated with fluorescently labelled VhH anti-TM clone 1 or VhH R2 isotype control (1-, 3- or 5µg). Fluorescence was measured with FACS. Mean fluorescent intensity (MFI) was corrected for VhH R2 isotype control for each concentration separately. (C) 15nM human α-thrombin was incubated on thrombomodulin-coated wells in the presence of indicated concentrations of unlabelled clone 1 or VhH R2 (control). Thrombin binding in the absence of VhH was set at 100%. All experiments were performed in triplicate.

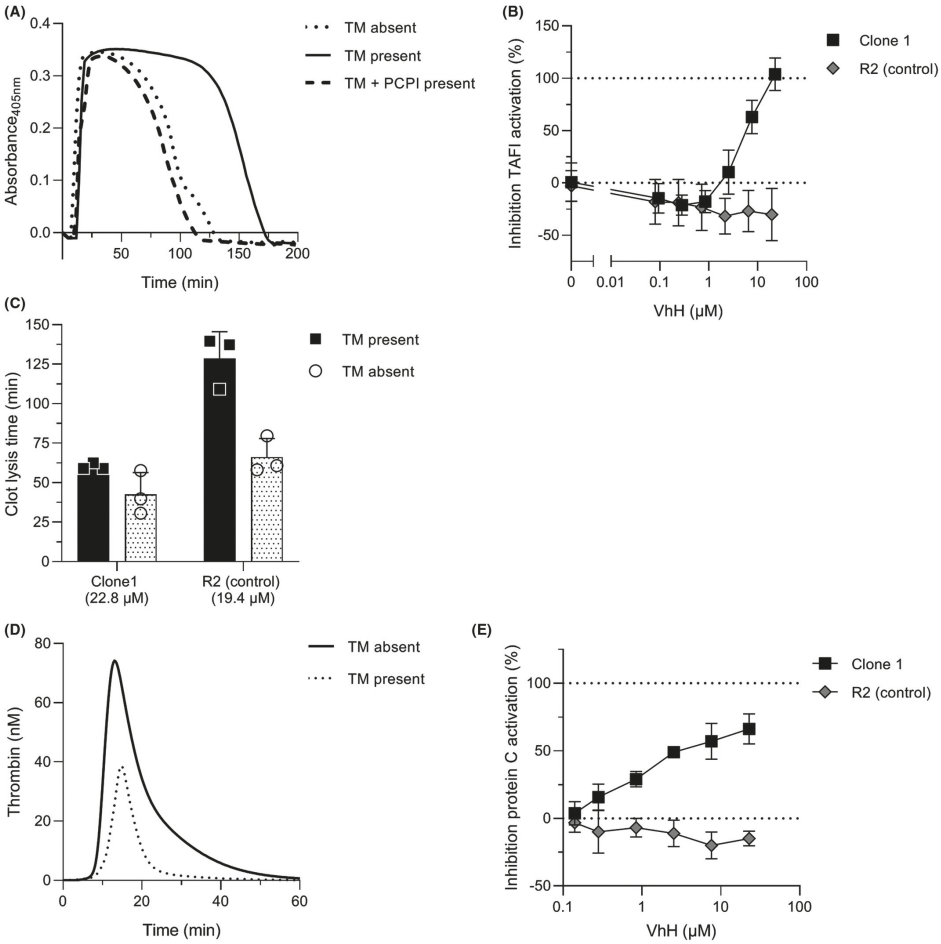

Figure 2. Inhibition of TAFI and Protein C activation by VhH anti-TM clone 1

(A) To measure TAFI activation clot lysis experiments were performed. Plasma was spiked with tPA and coagulation was initiated with 0.5pM TF and 4μM phospholipids (30:20:50 ratio PS:PC:PE), in the presence or absence of 10nM thrombomodulin and PCPI (5μg/mL) as indicated. Clot formation and clot lysis were measured as an increase and decrease in absorbance at 405nm. (B) Clot lysis times (CLT) were determined in the presence of thrombomodulin and clone 1 or negative control VhH R2 at the indicated concentrations. Data are presented as relative (%) inhibition of TAFI activation, in which 0% inhibition was equal to CLT in the presence of thrombomodulin and 100% inhibition was equal to the CLT in the absence of thrombomodulin in control samples without VhHs. (C) CLTs in the absence of thrombomodulin were determined at highest concentrations of clone 1 or negative control VhH R2 and measured in a SpectraMax iD3 (absorbance at 405nm). (D) Representative thrombograms obtained in plasma after initiation of coagulation with 0.5pM TF and 4μM phospholipids (30:20:50 ratio PS:PC:PE) in the presence or absence of 25nM thrombomodulin. (E) Relative inhibition of protein C activation in PNP in the presence of clone 1 or VhH R2 (control) at indicated concentrations. Data are presented as relative (%) inhibition of protein C, in which 0% inhibition was equal to the ETP in the presence of thrombomodulin and 100% inhibition was equal to the ETP in the absence of thrombomodulin in control samples without VhHs. All experiments were performed in triplicate. (B, C, E) Data represent normalized means and SDs of each individual run.

Clone 1 interferes with thrombomodulin-activity and inhibits both TAFI activation and protein C activation

To determine whether clone 1 influences the procoagulant properties of thrombomodulin, clot lysis experiments were performed in the presence and absence of 10nM thrombomodulin. Addition of thrombomodulin caused an approximately two-fold lengthening of the CLT. The effect of thrombomodulin on CLT was completely attenuated in the presence of TAFIa inhibitor PCPI, confirming that the thrombomodulin-induced lengthening of the clot lysis time was TAFI-dependent (**Fig. 2A).** Clone 1 induced a dose-dependent shortening of the CLT in the presence of thrombomodulin, leading to full inhibition of TAFI activation by targeting thrombomodulin (IC_{50}: 6.1μM) (**Fig. 2B**). In contrast, isotype control VhH R2 had no effect on the CLT. Clot lysis times in the absence of thrombomodulin at the highest concentration of clone 1 showed no significant reduction of the clot lysis time (**Fig. 2C),** indicating that inhibition of TAFI activation was thrombomodulin-dependent.

However, the observation that clone 1 interferes with binding of thrombin to recombinant thrombomodulin suggest that it potentially interferes with protein C activation. This hypothesis was assessed with TF initiated thrombin generation assays. Addition of 25nM thrombomodulin to plasma caused a 2-fold reduction in endogenous thrombin potential (ETP), consistent with protein C activation (**Fig. 2D).** Indeed, addition of clone 1 also inhibited activation of protein C (IC_{50}: 4.7μM) **(Fig. 2E).**

Clone 1 enhances fibrinolysis under flow

To investigate whether clone 1 had fibrinolytic properties in flowing blood, we developed a microfluidic fibrinolysis model. Hereto, sodium citrate anticoagulated whole blood was recalcified with $CaCl_2$, spiked with tPA and perfused over a surface coated with collagen, TF and thrombomodulin at an arterial shear rate. To prevent clogging of the flow chamber, we allowed non-recalcified (but for the remainder identical) whole blood to perfuse after 7 min and we stopped each run after 15 min. Under these conditions, platelet adhesion starts immediately, while fibrin formation starts with a delay of approximately 2 min (**Fig. 3A**). Both platelet adhesion and fibrin formation increased over time. Fibrin formation reached a maximum at 9.6±0.9 min, which was followed by a rapid disintegration of the fibrin network (**Fig. 3B, 3D).** In contrast, platelet adhesion remained stable despite fibrinolysis **(Fig. 3B, 3C**).

To confirm TAFI activation in this fibrinolysis model, whole blood was spiked with TAFIa inhibitor PCPI. Whereas kinetics of platelet aggregation was similar (**Fig. 3C),** fibrin deposition was reduced **(Fig. 3D)**. Spiking with PCPI resulted in a 40.2±19.0% reduction in fibrin deposition at t=7 min (p=0.01) compared with perfusions in the presence of VhH R2 (control) (**Fig. 3E).** Like inhibition of TAFIa with PCPI, blocking thrombomodulin-mediated TAFI activation with clone 1 had no effects on platelet aggregation kinetics **(Fig. 3C**) and similarly reduced fibrin deposition compared with VhH R2 (control) (33.6±13.0% reduction, p=0.015) at t=7 min (**Fig. 3E).** Combined, these data suggest that blocking thrombomodulin with clone 1 effectively prevents TAFI activation.

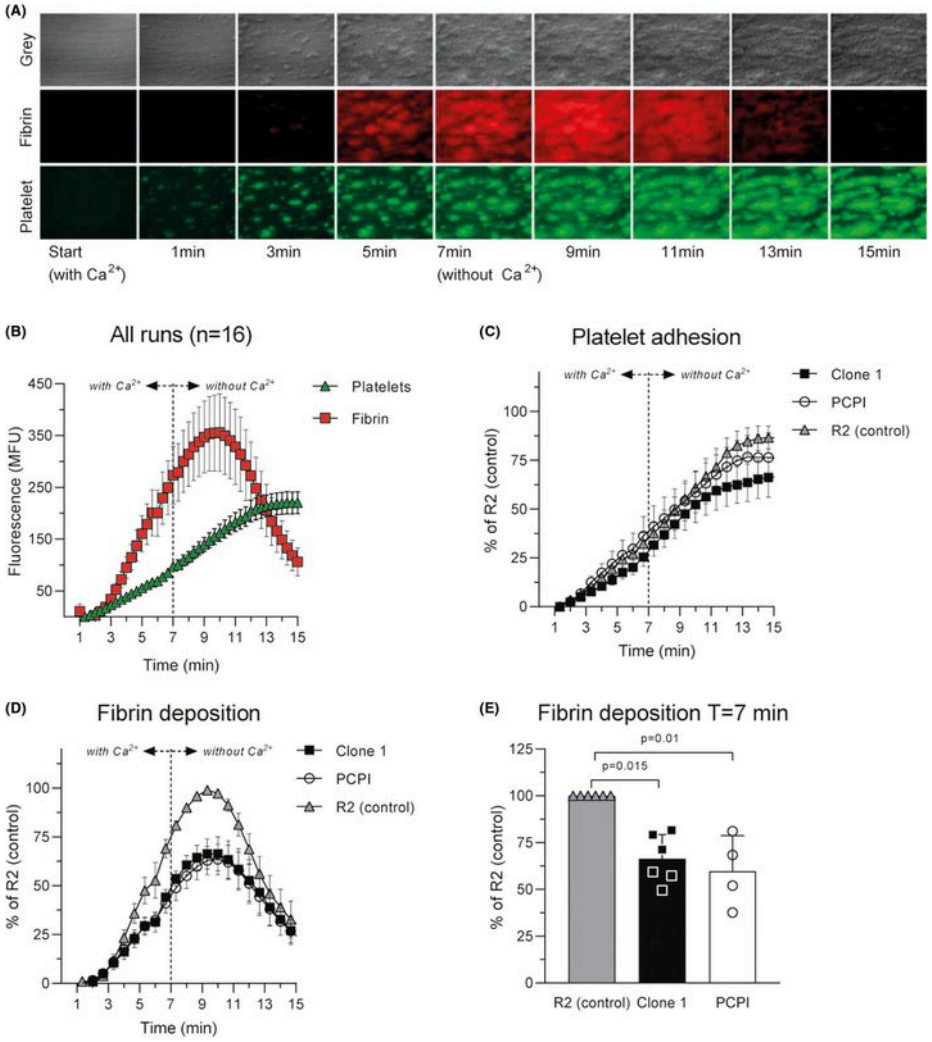

Figure 3. Blocking thrombomodulin-dependent TAFI activation with clone 1 enhances fibrinolysis in a microfluidic flow model

A total of 16 runs were performed with blood from 6 healty donors. From 4 of these donors, separate runs were performed with subsequently PCPI (25µg/mL), clone 1 (514nM) or VhH R2 (control) (514nM). From 2 of these donors, separate runs were performed with either clone 1 (514nM) or VhH R2 (control) (514nM). (A) Citrated whole blood samples were recalcified and spiked with tPA and perfused at a shear rate of 800 s^{-1} over a coverslip coated with human TF, horm collagen and thrombomodulin. After 7 min, non-calcified blood samples with same supplements entered the flow chamber, allowing tPA-mediated fibrinolysis to predominate. Platelets were stained with AlexaFluor488-conjugated anti-GPIbα VhH clone 17 (shown in green) and fibrin was stained with AlexaFluor647-conjugated anti-fibrin VhH clone B12 (shown in red). Images were captured every 20 seconds at 40x magnification. Images represent a single run. (B) Platelet adhesion and fibrin deposition were quantified with ZEN Pro software and plotted over time. Graphs represent mean±SEM values of all runs (N=16). (C-D) Whole blood was supplemented with tPA and either VhH R2 (control, N=6), clone 1 (N=6) or PCPI (N=4), followed by perfusion in the microfluidic fibrinolysis model. Platelet adhesion and fibrinolysis were evaluated over time. Data represent mean±SEM values. (E) Maximum fibrin deposition in the presence of calcium (at t=7 min) was derived from biological replicates, and is expressed as percentages of the run suppelemented with VhH R2 (control) within the same donor. Kruskal-Wallis analysis with multiple comparisons was performed with GraphPad Prism 8.0.1.

DISCUSSION

We developed a VhH targeting human thrombomodulin (VhH anti-TM clone 1) that interferes with thrombin binding to thrombomodulin and, consequently, activation of protein C and TAFI by the thrombin-thrombomodulin complex. In addition, clone 1 completely attenuated TAFI activation in an *in vitro* fibrinolysis model in flowing human whole blood and enhanced tPA-induced fibrinolysis.

The thrombomodulin-thrombin complex has both antifibrinolytic effects by activating TAFI and anticoagulant effects by activating protein C. Whether thrombomodulin has net antifibrinolytic or anticoagulant effects depends on its concentration: low concentrations favor TAFI activation and high concentrations favor protein C activation (7). Clone 1 interferes with thrombin binding to thrombomodulin and, consequently, activation of protein C and TAFI by the thrombin-thrombomodulin complex. We therefore cannot exclude dual effects of the VhH on protein C and TAFI activation in our fibrinolytic flow model. However, it is unlikely that inhibition of protein C activation was of significant influence on fibrin formation in the flow model, as this would have resulted in increased fibrin formation. Instead, our data clearly demonstrate reduced fibrin deposition in the presence of clone 1 and to a similar extent as TAFI inhibition with PCPI. This corresponds with a low concentration of immobilized thrombomodulin on the glass surface, generating a net antifibrinolytic environment.

TAFI can be activated by plasmin (32) and both the thrombin-thrombomodulin complex or by thrombin itself. Direct thrombin dependent TAFI activation requires a high local thrombin concentration, which is thought to be achieved by feedback amplification of coagulation through thrombin mediated FXI activation under low TF conditions (33–35). While high thrombin concentrations are likely to occur under static conditions, rheological conditions in a growing thrombus favour flushing out coagulation factors that lack surface binding capacity, such as FXI (33, 36). While thrombin itself is retained in the fibrin network, it loses substrate specificity due to the involvement of both its exosites in fibrin binding (37). This makes the thrombomodulin-thrombin complex the most likely source of TAFI activation under high shear stress conditions in flowing blood.

Data obtained in our fibrinolysis model support a dominant role for this complex in TAFI activation in flowing blood. Nevertheless, the bleeding pattern in patients with congenital FXI deficiency indicate a predilection for tissue with high fibrinolytic activity (38), offering strong indirect evidence of a role for FXI in inhibition of fibrinolysis (35). It should be noted that our fibrinolysis model incorporated arterial flow conditions (shear rate 800 s^{-1}), favouring the flush-out of FXIa. We cannot exclude that FXI contributes to inhibition of fibrinolysis under venous conditions.

In addition, our model incorporates exogenous tPA to induce fibrinolysis, while no exogenous tPA was added in the *in vivo* models described by others (16, 17, 19, 39).

Together with presumable low concentration of thrombomodulin, this tips the balance towards profibrinolytic conditions in our model, which might explain the difference in these observations. Patients with acute ischemic stroke will often be treated with tPA to restore blood flow to ischemic tissue, resulting in a similar profibrinolytic environment. It has been suggested to target TAFI itself to enhance fibrinolysis under these conditions (16), potentially leading to enhanced thrombus resolution and preservation of brain tissue. However, as a failure to properly activate TAFI is thought to be the main determinant in the bleeding tendency observed in FXI deficient patients, this strategy is not without bleeding risk (38). By targeting TAFI activation by the thrombomodulin-thrombin complex only, direct TAFI activation by thrombin can be preserved, potentially reducing bleeding risk. It should be noted that this strategy only holds clinical potential when protein C activation remains largely unaffected. Whether inhibition of TAFI activation by targeting thrombomodulin will enhance fibrinolysis in the setting of a pre-existent thrombus remains to be determined.

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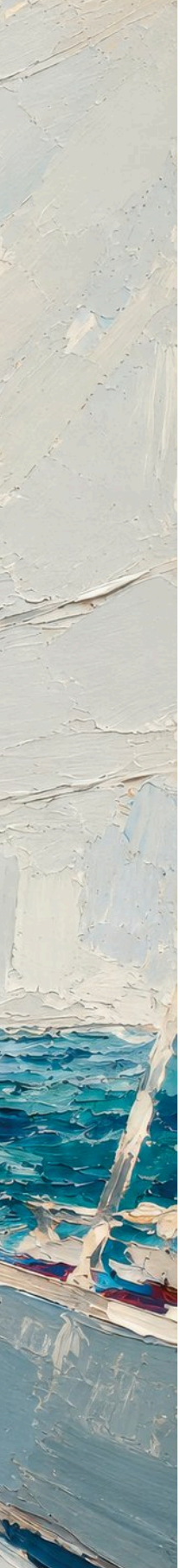
Authorship contributions

J.C.M. Meijers, R. Schiffelers, C. Maas and R.T. Urbanus conceived/designed the study. M.V.A. van Moorsel, G. Poolen, C.A. Koekman, S. Verhoef, S. de Maat, A. Barendrecht and N.D. van Kleef performed experiments. Marc V.A. van Moorsel, Geke Poolen (equal contribution) and R.T. Urbanus wrote the paper.

Conflict of interest

M.V.A. van Moorsel, S. de Maat and C. Maas are co-founders of TargED Biopharmaceuticals B.V., a biotech spinout company of University Medical Centre Utrecht. However, TargED Biopharmaceuticals B.V. has no rights concerning compounds nor techniques presented in this manuscript. G. Poolen, C.A. Koekman, S. Verhoef, A. Barendrecht, N.D. van Kleef, J.C.M. Meijers, R. Schiffelers and R.T. Urbanus have no relevant disclosures.



CHAPTER 8

Summary and general discussion

SUMMARY

Venous thromboembolism is a common thrombotic disorder presenting complex clinical challenges, particularly regarding the optimal duration of anticoagulation therapy. Extending anticoagulation reduces the risk of recurrence but increases bleeding risk. Accurate prediction of recurrence risk could help guide decisions on whether to continue or stop anticoagulation.

In **Chapter 2**, we investigated platelet and endothelial biomarkers and found that elevated levels of von Willebrand Factor during anticoagulation were associated with early recurrence, especially in men. These findings suggest VWF might be a useful biomarker for personalized risk prediction, enabling more tailored anticoagulation strategies if validated in further studies.

Chapter 3 employed unbiased mass spectrometry to discover new biomarkers but did not find proteins that significantly differed between patients with and without recurrence, either during or after anticoagulation.

Chapter 4 evaluated global coagulation assays focusing on thrombin generation and thrombin dynamics. We found that reduced endogenous thrombin potential (ETP) and reduced thrombin inhibition by antithrombin were linked to recurrence, particularly when excluding hormone-related VTE cases. Additionally, impaired thrombomodulin-mediated inhibition of thrombin generation was associated with recurrence risk.

Chapter 5 shifted focus to patients with immune thrombocytopenia (ITP), a patient group with heightened thrombotic risk. These patients showed elevated ETP, which increased further following eltrombopag treatment, highlighting a prothrombotic state.

After establishing the importance of thrombin generation in predicting recurrence and assessing thrombotic state, Part II of the thesis examined thrombomodulin's ability to modulate thrombin generation for diagnostic and therapeutic purposes, focusing on strategies to enhance or inhibit its activity.

In **Chapter 6**, we developed liposomes conjugated with thrombomodulin (TM-liposomes) that more effectively inhibited thrombin generation in plasma and whole blood compared to soluble thrombomodulin. The lipid composition of these liposomes influenced their efficacy, with higher phosphatidylserine content enhancing protein C activation. These TM-liposomes may improve diagnostics by more closely mimicking the natural endothelial membrane environment and enabling better assessment of the activated protein C pathway in thrombin generation.

Chapter 7 describes the design of a nanobody (VhH) that inhibits thrombin-activatable fibrinolysis inhibitor (TAFI) activation by thrombomodulin. We demonstrated that this VhH

enhanced fibrinolysis in whole blood under flow conditions, offering a new strategy to enhance clot breakdown and possibly reduce thrombotic complications.

GENERAL DISCUSSION

Introduction: why prediction matters

Predicting VTE recurrence remains highly challenging in clinical practice. This is probably partly due to the heterogeneity of patient risk factors, the variable natural history of the disease and limitations in currently available biomarkers. Even the most recent risk models, such as VTE-PREDICT, trained on one of the largest datasets to date, demonstrate only modest discrimination, with C-statistics ranging from 0.48–0.71 for recurrence and 0.61–0.68 for bleeding in external validation (1). Such performance limits reliable individual risk stratification in clinical practice. Consequently, many patients continue to experience recurrent events, often accompanied by significant physical complications, such as post thrombotic syndrome, and psychological burdens including anxiety and reduced quality of life (2-5). These life-altering consequences profoundly impact patients' health and daily functioning (6, 7).

In response to this uncertainty, indefinite anticoagulation after a first thrombotic event is increasingly prescribed and is supported by several clinical guidelines (8-10). However, the true benefit of this approach is difficult to evaluate. A recent meta-analysis found no significant mortality advantage of extended anticoagulation compared to short-term therapy (11). Prolonged anticoagulation carries increased bleeding risk and also imposes a growing burden on healthcare resources (12). Ideally, only patients with high risk of recurrence and a low risk of bleeding should receive extended treatment, but accurately identifying these patients remains challenging.

Another crucial factor in determining the duration of anticoagulation is the patient's preferences. Central to this treatment decision is a difficult dilemma: which is more feared—a recurrent thrombosis or a major bleeding event? Since individual patients may weigh these risks differently, shared decision making is essential in managing VTE, as emphasized in current guidelines. Risk prediction alone is not enough; therapeutic choices must also reflect patients' individual values, concerns, and preferences (13).

True shared decision making involves more than simply asking patients for their preferences; it depends on providing clear, personalized information about their actual risks and benefits (13). Without tailored communication tools, patients often remain unaware of their individualized risk profiles, hindering meaningful participation in treatment decisions for both patients and clinicians. There is significant room for improvement. For example, a recent study in pediatric VTE revealed that many adolescents and their caregivers lack adequate understanding of the condition and its treatment (14), underscoring the need for enhanced education and communication strategies—such as interactive educational apps (15). Similarly, a large cohort study of adult VTE patients on oral anticoagulants found that nearly one in five had limited health literacy (16).

Current literature on effective clinical shared decision making in VTE is limited (17). To address this gap, the recently launched ETHER study (NCT06731244) is evaluating a novel shared decision-making intervention that combines time-dependent, multicomponent risk prediction scores with socio-anthropological assessments (TDMI) (18). The goal is to enhance patient understanding and engagement, ultimately improving clinical outcomes. It will be exciting to see whether this approach achieves it goals.

More broadly, outcome assessment in VTE should extend beyond traditional clinical endpoints such as recurrence and bleeding to also encompass quality of life and other patient-centered outcomes (6). Patients' lived experiences, including physical functioning, psychological well-being, and social participation, profoundly affect their overall health and satisfaction with treatment. Incorporating these broader measures into research and clinical practice can provide a more holistic understanding of treatment impact, guiding therapies that align better with patients' values and priorities. Ultimately, this approach supports more comprehensive care that addresses not only survival but also the quality and meaning of patients' lives.

Heterogeneity in VTE risk: one size does not fit all

VTE patients represent a highly heterogeneous population, consisting of distinct subgroups with varying clinical characteristics, baseline risks, and prognostic trajectories. However, in recurrence prediction and biomarker research, these differences are often ignored or overlooked. Most existing prediction tools, such as the Vienna Prediction Model, DASH, and VTE-PREDICT, operate under the assumption of a single, homogeneous population. They do not account for subgroup-specific effects or variations, which limits their accuracy and generalizability. As a result, external validation studies have consistently demonstrated only moderate discriminatory performance and variable calibration across different patient populations (19, 20).

When these differences are ignored and all patients are modeled together, risk estimates become distorted (21). From a statistical perspective, if both baseline hazards and predictor effects differ between subgroups, pooled models yield coefficients that are weighted averages across the entire population. Consequently, these coefficients are often inaccurate for any single subgroup, resulting in miscalibration and diminished clinical utility (22, 23). In practical terms, predictors that are highly predictive in one subgroup may appear weak or insignificant when averaged across all patients, while predictors with limited relevance in specific subgroups may be overemphasized, further complicating individualized risk assessment. A Canadian study on mortality prediction showed that while a model can have good performance overall, it may preform much worse in specific subgroups, highlighting the limitation of a one-size-fits-all approach (24).

Some studies have specifically investigated subgroups within VTE patients, providing strong evidence of heterogeneity. For instance, the COMMAND VTE Registry-2 utilized

latent class analysis to identify three distinct phenotypes among patients with unprovoked VTE, primarily differentiated by age and comorbidity burden. While the risk of recurrence was comparable across these groups, the older and more comorbid phenotype exhibited a substantially higher bleeding risk and a greater tendency to discontinue anticoagulation prematurely (25). Similarly, the PREFER in VTE registry applied clustering techniques to reveal five patient profiles with widely differing recurrence hazards, ranging from the lowest risk observed in young women to the highest in patients with multiple comorbidities (26). A hierarchical clustering study in deep vein thrombosis (DVT) patients also identified four phenotypes with differing recurrence risks and distinct rates of other complications such as post-thrombotic syndrome and arterial thrombotic events (27, 28). Beyond clinical characteristics, proteomic-based phenotyping suggests that these clinical subgroups may correspond to underlying biological differences (29).

This structural limitation cannot be overcome simply by adding more predictors or by training on larger datasets to fine-tune coefficients; instead, it requires models that explicitly incorporate subgroup variation. One promising approach is latent-class survival models, which can estimate subgroup-specific hazard functions and predictor effects (30). Future models should leverage large, diverse datasets, including both traditional and non-traditional risk factors, and integrate multiple outcome domains. Not only recurrence, but also bleeding, mortality, and patient-reported outcomes—into personalized decision frameworks (31). Here, advances in artificial intelligence and machine learning hold significant potential to enhance subgroup detection, model complex interactions, and improve prediction accuracy (32). However, ensuring transparency, interpretability, and clinical trust remains crucial for these tools to be effectively adopted in practice (33, 34).

Biomarkers: potential, pitfalls, and the role of context

Building on the need for models that account for subgroup variation, biomarkers also hold substantial promise for improving recurrent VTE prediction but require similarly nuanced interpretation (35). Our findings highlight both their potential and the critical importance of context. Across this thesis, we evaluated biomarkers from different biological layers: single circulating proteins, global plasma proteomics, and functional coagulation assays, each highlighting both the potential and the limitations of biomarker-based risk prediction.

In **Chapter 2**, elevated von Willebrand factor was associated with an increased risk of VTE recurrence, but this association depended strongly on three key contextual factors: (1) timing of recurrence (early versus late events), (2) timing of biomarker assessment (during versus after anticoagulation), and (3) patient sex. These findings emphasize that the prognostic value of a biomarker cannot be interpreted independently of *when*, *in whom*, and *for which outcome horizon* it is measured. Such contextual factors are often overlooked in biomarker research, where pooled estimates are commonly reported without stratification by sex, differentiation between early and late recurrences, or precise reporting of sampling time. As a result, clinically relevant patterns may be obscured,

potentially also contributing to the limited external validity of many biomarkers despite statistically significant associations in discovery studies.

Most recurrences occur within the first year after discontinuation of anticoagulation (36), and biomarkers measured closer to the event are more likely to capture relevant pathophysiological signals than those measured months or years before the second event (37). In our study, biomarkers were more strongly associated with early recurrences than with later events. This distinction is rarely examined in biomarker research but recognizing it may reveal clinically relevant patterns that are otherwise overlooked.

The timing of biomarker measurement relative to anticoagulation is critical. Biomarkers measured during anticoagulation may be influenced by treatment effects, as observed for D-dimer and TG; nevertheless, in our study, VWF showed stronger associations with recurrence when measured during therapy than after cessation. These observations suggest that risk prediction during anticoagulation may capture high-risk periods before early recurrences occur, and our results indicate that some biomarkers may be predictive within this interval. The importance of considering these contextual factors was also illustrated by studies of D-dimer, in which hazard ratios for recurrence differed substantially according to the timing of measurement and varied between men and women (38).

Building on the temporal context discussed above, sex represents another key factor that can influence biomarker associations and recurrence risk. Sex differences deserve particular emphasis. Across cardiovascular research, sex-specific data are often underreported (39-41). In VTE, distinct sex differences are well established: women often present with identifiable transient risk factors such as hormonal contraception or pregnancy, whereas men consistently experience higher recurrence rates, even after adjusting for these factors (42). This pattern suggests that differing underlying mechanisms may drive disease processes in each sex, potentially impacting both the behavior and predictive value of certain biomarkers. It is plausible that a biomarker with strong prognostic value in men may have minimal predictive relevance in women, and vice versa. Without stratifying analyses by sex, these differential effects risk being masked, limiting the development and application of biomarker-driven precision medicine.

Lessons from thrombin generation, thrombin dynamics and proteomics

While VWF illustrates how a single biomarker can show context-dependent prognostic value, the analyses of thrombin generation (TG) and thrombin dynamics (TD) in **Chapter 4** provide a complementary perspective. Unlike static protein concentrations, TG and TD capture the functional capacity of the coagulation system, integrating the net effects of multiple pro- and anticoagulant pathways.

In our study, most TG and TD parameters showed limited or inconsistent associations with recurrence in the overall cohort. However, when analyses were restricted to patients

with unprovoked VTE, reduced thrombin generation, impaired thrombomodulin (TM) sensitivity, and decreased thrombin inactivation were associated with recurrence. These findings underscore that functional biomarkers may be informative primarily within biologically more homogeneous subgroups. At the same time, the observed effect sizes were modest, and none of the TG or TD parameters alone achieved sufficient discriminative performance for clinical decision-making, highlighting that even functional assays remain highly context dependent. This suggests that, while functional assays may better reflect underlying pathophysiology than isolated protein levels, they are unlikely to serve as stand-alone predictors of recurrence.

Optimizing the measurement of the APC pathway in thrombin generation assays was the focus of the work in **Chapter 6**. Thrombin generation in the presence of TM provides additional predictive information for VTE recurrence beyond standard TG parameters. Our optimization approach might reduce the amount of TM required while still capturing subtle differences in APC pathway activity. These TM-modulated parameters integrate both pro- and anticoagulant influences, potentially reflecting individual variation in recurrence risk and improving the precision of risk prediction.

The proteomics study described in **Chapter 3** further tempers expectations regarding biomarker discovery. Despite using unbiased mass spectrometry and detecting clear anticoagulation-related protein changes, no plasma proteins or protein patterns discriminated patients with recurrent VTE from those without recurrence. These negative findings suggest that recurrence risk is unlikely to be driven by large, stable differences in circulating protein abundance alone. Relevant biological processes may be transient, localized to the vessel wall, or distributed across multiple pathways, thereby escaping detection in systemic plasma measurements. Functional protein activity may differ substantially from protein abundance. Future research should explore activity-based measures, such as phosphorylation or glycosylation, which may better reflect clinically relevant hypercoagulability.

Functional activity-based assays, including thrombin generation as well as studying protein phosphorylation and glycosylation, are technically demanding, sensitive to pre-analytical variation, and lack full standardization across laboratories. While feasible in research settings, their implementation in routine clinical practice will remain challenging. Future developments in assay standardization and automation will be essential if such functional biomarkers are to be translated into clinical use.

An additional challenge lies in methodological differences between research fields. In proteomics, stringent correction for multiple testing is essential and standard practice. However, if recurrence risk is associated with subtle and distributed protein changes rather than pronounced differences in individual proteins, such signals are unlikely to survive strict multiple-testing correction. From this perspective, the absence of strong

proteomic signals is informative in itself and argues against overly optimistic expectations of identifying a single, highly predictive plasma biomarker for VTE recurrence.

Taken together, these findings illustrate that the predictive value of biomarkers, such as VWF, depends on multiple contextual factors, including timing of recurrence, timing of measurement relative to therapy, and patient sex. Failing to account for these factors can mask clinically meaningful associations and limit both research and clinical application. Explicit consideration of these dimensions is therefore essential for accurate risk stratification. Future biomarker studies in VTE should stratify analyses by sex, standardize and transparently report the timing of biomarker assessment, and differentiate between early and late recurrences in predictive modeling to capture temporal heterogeneity. At the same time, the findings across this thesis suggest that biomarkers are unlikely to fully meet early expectations of identifying a single, robust predictor of VTE recurrence with high discriminative accuracy. Given the multifactorial nature of recurrence risk, the modest effect sizes observed, and the strong influence of clinical context, biomarkers are likely most useful as modifiers of risk within multivariable, context-aware prediction models, refining estimates for specific subgroups, time horizons, and clinical decisions rather than serving as stand-alone predictors.

Dual-purpose biomarkers: clinical implications of new findings

A recent study presented at the latest ISTH meeting revealed a challenging new insight: elevated on-treatment D-dimer levels predict not only increased risk of VTE recurrence but also higher risk of major bleeding during extended anticoagulation (43). This dual association complicates clinical decision-making regarding whether to continue or discontinue anticoagulant therapy. In this case, the biomarker provides no clear guidance; it signals elevated risks on both sides. Patients with high D-dimer levels may face substantial danger if anticoagulation is discontinued, whereas continued therapy carries a significant risk of bleeding.

Therefore, the need for novel biomarkers that can more clearly discriminate between these competing risks remains critical. Importantly, biomarker evaluation must always consider multiple relevant clinical outcomes to ensure balanced and effective patient management.

These findings challenge the assumption that biomarkers will always simplify treatment decisions. Instead, they highlight the need for more sophisticated, context-aware models that incorporate patient-specific factors (e.g., age, sex, renal function, history of bleeding) alongside biomarker levels. Moreover, it reinforces the importance of shared decision making, where clinical judgment and patient preferences play a central role in navigating such uncertainty.

Future therapies: toward safer anticoagulation

An alternative approach to addressing the challenges of risk prediction and clinical decision-making, is to focus on making anticoagulation itself safer. Lower bleeding risk can be achieved with low-dose DOACs, which have been shown to be non-inferior to standard doses while reducing bleeding complications (44-46). Beyond this, next generation anticoagulants hold promise for further improving the balance between efficacy and bleeding risk, potentially transforming long-term VTE management.

Factor XI inhibitors: safer by design?

Among the most promising next-generation anticoagulants are agents that inhibit factor XI or its activated form, factor XIa. These target the intrinsic pathway of coagulation, which plays a central role in pathological thrombosis but is comparatively less important for normal hemostasis (47). This mechanistic selectivity raises the possibility of preventing thrombosis while minimizing bleeding risk.

Phase 2 and early phase 3 trials, including PACIFIC-AF, AXIOMATIC-TKR, have demonstrated promising results. They either maintained efficacy in preventing thrombotic events while reducing bleeding risk, or showed no significant increase in bleeding compared to standard anticoagulant therapy (48-51). These findings have generated considerable enthusiasm for factor XI inhibition as a potential paradigm shift in long-term anticoagulation especially for patients who are currently undertreated due to concerns about bleeding.

However, optimism has been tempered by the recent premature termination of the OCEANIC-AF trial investigating asundexian, which was halted due to a higher stroke rate in the experimental arm (52). While subtherapeutic dosing has been suggested as a possible explanation, the result also raises the possibility that factor XI inhibition may not be universally effective across all patient populations or clinical settings. These findings highlight the critical importance of careful dose selection, patient stratification, and rigorous trial design to accurately assess the risk–benefit balance of this emerging drug class.

If ongoing phase 3 studies confirm the promising bleeding safety profile of factor XI inhibitors without compromising their efficacy, these agents could fundamentally transform the management of extended anticoagulation. By enabling safer long-term therapy, these agents might reduce the need for intensive risk stratification and enable broader protection against recurrent VTE and other thrombotic events. Until then, cautious optimism is warranted, and robust evidence from large, well-designed trials will be essential before these drugs can be integrated into routine practice.

Thrombomodulin and the APC pathway

Another potential class of novel anticoagulants includes agents that enhance the protein C pathway, such as recombinant thrombomodulin (rhTM). In Japan, rhTM is approved

for treating disseminated intravascular coagulation (DIC), particularly in sepsis-induced coagulopathy (53). A meta-analysis suggested that rhTM may reduce mortality and promote DIC resolution without significantly increasing bleeding risk (54). Although the international SCARLET trial showed neutral overall results, post-hoc analyses of the French cohort indicated potential benefit in patients with sustained coagulopathy who were not receiving concomitant heparin (55). Trials indicated that rhTM significantly reduced coagulation activation markers, including D-dimer, prothrombin fragment 1.2, and thrombin–antithrombin complex levels, consistent with suppression of thrombin generation.

While primarily studied in coagulopathy and sepsis, rhTM has also been explored for VTE prophylaxis after orthopedic surgery (56). Although these studies were small and not powered to demonstrate definitive clinical benefit compared with standard anticoagulants, they support the mechanistic plausibility of rhTM as an antithrombotic agent. Thrombomodulin binds thrombin, altering its activity to activate protein C, which then inactivates factors Va and VIIIa, producing both anticoagulant and anti-inflammatory effects.

However, unlike factor XI inhibitors, activation of the protein C pathway is more directly linked to bleeding risk due to its broad downregulation of thrombin generation. Trials conducted outside Japan have often been underpowered or yielded inconclusive efficacy signals, limiting enthusiasm for large-scale global development. Future directions may focus on enhancing efficacy such as coupling thrombomodulin to liposomes (57), as we explored in **Chapter 6**. Another promising approach is inhibiting TAFI activation by targeting thrombomodulin; as shown in **Chapter 7**, this strategy resulted in faster clot resolution. Inhibiting activation of this fibrinolysis inhibitor may also promote clot breakdown in vivo. Further research is needed to determine these approaches can deliver clinically meaningful VTE prevention, while maintaining an acceptable safety profile.

The development of safer anticoagulants holds promise to eventually lessen the reliance on strict risk stratification, particularly if long-term therapy proves to be safer and more tolerable. Until such therapies become widely available and firmly established, however, personalized decision making and refined prediction models will remain critical components of optimal VTE management.

Conclusion and personal reflection

This thesis contributed to advancing VTE recurrence prediction by identifying new biomarkers associated with recurrence and demonstrating the critical influence of contextual factors such as timing and sex on their predictive utility. We also developed two novel approaches to modulate thrombomodulin function, which may hold promise for diagnostics and therapy.

Looking ahead, improving precision and clinical relevance in prediction tools will require incorporating subgroup heterogeneity, dynamic risk factors, and integration with patient-centered decision-making frameworks. These models should be designed to support, not replace, clinical judgment, ensuring that statistical predictions are balanced with the lived realities, preferences, and values of individual patients.

At a deeper level, the use of prediction models reflects a broader human desire to reduce uncertainty in an inherently unpredictable future (58). Numbers and algorithms can inform and guide decisions, but they are abstractions; simplified representations of complex realities. Over-reliance on them risks obscuring the individual experiences and moral considerations that must shape treatment choices. Sanne Blauw's *The Number Bias* warns against overvaluing numbers as unquestionable truths, cautioning that such "number bias" can obscure the nuanced realities of patient care (59). As Philip Tetlock's work on *Superforecasting* reminds us, the best decisions come from those who embrace uncertainty, remain adaptable, and think probabilistically (60).

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APPENDICES

NEDERLANDSE SAMENVATTING

Veneuze trombo-embolie is een veelvoorkomende trombotische aandoening die complexe klinische uitdagingen met zich meebrengt. Een van die uitdagingen is de optimale duur van antistollingsmedicatie; het verlengen van antistolling vermindert het risico op een tweede trombose, maar verhoogt het bloedingsrisico. Een nauwkeurige voorspelling van het recidiefrisico kan helpen bij de beslissing om antistollingsmedicatie voort te zetten of te staken.

In **Hoofdstuk 2** onderzochten we trombocyt- en endotheelbiomarkers en vonden we dat verhoogde spiegels van von Willebrand Factor (VWF) tijdens gebruik van antistolling geassocieerd waren met vroeg recidief van trombose, met name bij mannen. Deze bevindingen suggereren dat VWF een nuttige biomarker kan zijn voor gepersonaliseerde risicobepaling, waardoor een meer op maat gesneden antistollingsstrategie mogelijk wordt, mits gevalideerd in vervolgstudies.

Hoofdstuk 3 maakte gebruik van massaspectrometrie om nieuwe biomarkers voor recidief trombose te identificeren. Hoewel de data robuust waren en de effecten van antistollingsmedicatie goed meetbaar waren, vonden we geen eiwitten die geassocieerd waren met een recidief, zowel tijdens de behandeling met antistolling als na het staken ervan.

Hoofdstuk 4 evalueerde globale stollingstesten met de nadruk op trombinegeneratie en trombinedynamiek. We vonden dat een verminderd endogeen trombinepotentieel (ETP) en verminderde trombineremming door antitrombine geassocieerd waren met recidief, met name wanneer hormoon-gerelateerde VTE-gevallen werden uitgesloten. Daarnaast was een verminderde trombomoduline-gemedieerde remming van trombinegeneratie geassocieerd met het recidiefrisico.

Hoofdstuk 5 richtte zich op patiënten met immuuntrombocytopenie (ITP), een patiëntengroep met een verhoogd tromboserisico. Deze patiënten toonden een verhoogd ETP, dat verder toenam na behandeling met eltrombopag, wat wijst op een protrombotische toestand.

Na het vaststellen van het belang van trombinegeneratie bij het voorspellen van recidief en het beoordelen van de trombotische toestand, richtten we in deel II van het proefschrift onze aandacht op het vermogen van trombomoduline om trombinegeneratie te moduleren voor diagnostische en therapeutische doeleinden, met de nadruk op strategieën om de activiteit ervan te versterken of te remmen.

In **Hoofdstuk 6** ontwikkelden we liposomen geconjugeerd met trombomoduline (TM-liposomen) die trombinegeneratie in plasma en volbloed effectiever remden dan oplosbaar trombomoduline. De lipidesamenstelling van deze liposomen beïnvloedde

hun werkzaamheid, waarbij een hoger gehalte aan fosfatidylserine de activering van proteïne C versterkte. Deze TM-liposomen kunnen de diagnostiek verbeteren doordat ze de natuurlijke endotheel-membraanomgeving nauwkeuriger nabootsen en een betere beoordeling van de geactiveerde proteïne C-route in trombinegeneratie mogelijk maken.

Hoofdstuk 7 beschrijft het ontwerp van een nanobody (VhH) dat de activering van de door trombomoduline geactiveerde fibrinolyseremmer, TAFI, remt. We toonden aan dat dit VhH de fibrinolyse in volbloed onder stroomcondities versterkte, wat een nieuwe strategie biedt om stolselafbraak te bevorderen en mogelijk trombotische complicaties te verminderen.

LIST OF PUBLICATIONS

Publications included in this thesis

Poolen GC, Urbanus RT, Roest M, Geersing GJ, de Laat B, Schutgens REG. Elevated levels of (active) von Willebrand factor during anticoagulation are associated with early recurrence of venous thromboembolism. J Thromb Haemost. 2025 Aug;23(8):2558-2567.

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van Moorsel MVA*, **Poolen GC***, Koekman CA, Verhoef S, de Maat S, Barendrecht A, van Kleef ND, Meijers JCM, Schiffelers RM, Maas C, Urbanus RT. VhH anti-thrombomodulin clone 1 inhibits TAFI activation and enhances fibrinolysis in human whole blood under flow. J Thromb Haemost. 2022 May;20(5):1213-1222.

**These authors contributed equally and share first authorship.*

Other publications

Li S, **Poolen GC**, van Vliet LC, Schipper JG, Broekhuizen R, Monnikhof M, Van Hecke W, Vermeulen JF, Bovenschen N. Pediatric medulloblastoma express immune checkpoint B7-H3. Clin Transl Oncol. 2022 Jun;24(6):1204-1208.

Oral & poster presentations

2025 Poster presentation, ISTH, Washington, United States.
Title: Elevated (active) von Willebrand Factor levels during anticoagulation predict early recurrence

2025 Oral presentation, GTH, Lausanne, Switzerland.
Science Slam title: To stop or not to stop anticoagulants after a first VTE

2025 Poster presentation, BSTH/NVTH, Newcastle, United Kingdom.
Title: Elevated (active) von Willebrand Factor levels during anticoagulation predict early recurrence

2024 Poster presentation, EHA, Madrid, Spain.
Title: Elevated levels of (active) von Willebrand Factor during anticoagulation are associated with early recurrence of venous thromboembolism in men

2024 Oral presentation, NVTH, Koudekerke, the Netherlands.
Title: Elevated levels of (active) von Willebrand Factor are associated with early recurrence of VTE in men

2023 Oral presentation, ISTH, Montréal, Canada.
Title: Pioneering in prediction: A neural network approach to predict recurrent VTE using coagulation parameters

2023 Poster Presentation, ISTH Montreal, Canada.
Title: A decreased endogenous thrombin potential is associated with recurrent venous thromboembolism

2023 Poster presentation, NVTH, Koudekerke, the Netherlands.
Title: Neural Network Approach to Predict Recurrent VTE Based on Coagulation Parameters

2022 Poster presentation, ISTH, Londen, United Kingdom.
Title: VhH anti-thrombomodulin clone 1 inhibits TAFI activation and enhances fibrinolysis in human whole blood under flow

Grants

2023 Van Leerzem Fonds. *PREVENTING: Predictie van REcidief VENeuze Trombose met kunstmatige INtelliGentie* (Prediction of recurrent venous thrombosis using artificial intelligence). Personal grant awarded: €50,000.

EEN WOORD VAN DANK

Een woord van dank aan iedereen die heeft bijgedragen aan de totstandkoming van dit proefschrift. Ik ben ontzettend dankbaar voor alle mensen die, ieder op hun eigen manier, een rol hebben gespeeld tijdens deze bijzondere jaren. Dit proefschrift draagt mijn naam, maar het is onmogelijk om zo'n traject alleen af te leggen.

Rolf, aan het eind van mijn bachelor Biomedische Wetenschappen liep ik, na een tip van Kristin Denzer, voor het eerst jouw werkkamer binnen. Het bachelorvak '*Trombose, Hemostase en Rode bloedcellen*' had ik helaas gemist, maar zo leerde ik jou en het CDL alsnog kennen. Dat bleek het begin van een lange samenwerking. Eerst kwam ik terug voor een masterstage (waarbij ik je mastervak opnieuw wist mis te lopen) en uiteindelijk zelfs voor een promotietraject. Achteraf heb ik er af en toe best spijt van gehad dat ik die vakken nooit gevolgd heb. Met de jaren werd de drempel van jouw kamerdeur steeds lager. Ik ontdekte hoe betrokken je bent en hoe oprecht geïnteresseerd. Jij vroeg altijd ook even naar hoe het met míj ging en hoe het thuis was. Ik denk dat ik dat uiteindelijk nog wel meer gewaardeerd heb, dan al je inhoudelijke kennis en scherpe ideeën. Slim ben je zonder twijfel. Hoewel je al lang niet meer dagelijks op het lab staat, wist je altijd weer nieuwe suggesties te bedenken voor experimenten of analyses. Wel schrok jij soms van de R-scripts die op mijn scherm openstonden en verdwaalden we wel eens in de statistische, epidemiologische en methodologische uitdagingen van dit proefschrift, maar het is gelukt. Zonder jou was ik nooit zo ver gekomen. Dank voor je vertrouwen, je begeleiding en vooral voor wie je bent en je oprechte interesse.

Roger, in september 2021 dacht ik dat ik bij jou op sollicitatiegesprek kwam. Jij was echter al enthousiast aan het vertellen dat we in juni 2022 met z'n allen naar de ISTH in Londen zouden gaan. Blijkbaar was het al wel redelijk zeker dat ik een maand later zou starten aan dit uitdagende promotietraject. In de kelder van jouw VCK stond een vriezer vol samples van de VISTA-studie op me te wachten. Daarmee mocht ik aan de slag en dat vormde de basis van een groot deel van mijn onderzoek. Natuurlijk was het niet altijd eenvoudig. Regelmatig zaten we met elkaar te puzzelen hoe we ingewikkelde vraagstukken het beste konden aanpakken. Juist die gezamenlijke zoektocht heb ik erg gewaardeerd. Dank dat je mij deze kans hebt gegeven en het vertrouwen had om mij dit project toe te vertrouwen, ik heb er ontzettend veel van geleerd!

Bas, wat fijn dat jij ook betrokken was als copromotor bij dit promotietraject. Ik vond het heerlijk om af en toe de trein in te stappen richting het zuiden. Wat heb je een prachtige plek gecreëerd daar in Maastricht. Ik heb genoten van de bezoeken aan Maastricht en van de weken waarin ik met jullie team experimenten mocht uitvoeren. Jullie stonden altijd klaar om mee te denken en kennis te delen. Zonder jullie hulp had dit onderzoek ongetwijfeld veel langer geduurd. Dank voor je betrokkenheid, je expertise en de gastvrijheid waarmee ik altijd werd ontvangen.

Ook wil ik graag de leden van mijn beoordelingscommissie bedanken: prof. **de Kleijn**, prof. **Henskens**, dr. **Kruip**, prof. **Schiffelers** en prof. **Visseren**. Hartelijk dank voor de tijd en aandacht die jullie hebben besteed aan het lezen en beoordelen van dit proefschrift. Ik kijk uit naar onze ontmoeting tijdens de verdediging en ben benieuwd naar jullie vragen, inzichten en de discussie die daaruit zal ontstaan.

Mijn dank gaat ook uit naar de patiënten en onderzoekers die betrokken waren bij de VISTA-studie. De plasma samples en de database die zijn opgebouwd, vormden de basis van dit onderzoek. **Geert-Jan**, dank voor het meedenken, je suggesties en het soms meelezen.

Team; bedankt! Zonder jullie om mij heen was dit traject zoveel saaier en eenzamer geweest. **Minka**, vanaf mijn eerste tot laatste dag was jij erbij; altijd net een stapje voor. Fijn om bij je af te kunnen kijken. We zijn verschillend, maar lijken soms ook best wel op elkaar. Allebei een beetje eigenwijs en we willen het allebei ook wel graag net beter weten! Ik ben heel blij dat je deze dag naast mij wilt staan als mijn paranimf! Vele reisjes hebben we samen gemaakt; natuurlijk Koudekerke, maar ook Londen, Montreal, Newcastle en ik denk toch wel het hoogtepuntje: Washington & New York. Leuk om elkaar steeds weer beter te leren kennen. Bijvoorbeeld dat je niet zo'n held bent in bergwandelen, al vind ik je zeker een held op de piste. Echt stoer dat je dat bent gaan leren en nu steeds soepeler de berg afkomt! Ik hoop dat dr. Zivkovic nog vele beurzen toegekend krijgt, super leuk dat je in de wetenschap blijft werken en we blijven elkaar voorlopig nog wel tegenkomen in het UMC; daar ben ik blij mee! **Jente**, mijn eerste student. Zo leuk om je door de jaren heen te zien groeien. Tijdens je stage vond je het soms best even spannend, bijvoorbeeld toen ik je alleen liet werken toen ik een tijdje op vakantie & congres was, maar wat ben jij zelfstandig! Jij gaat lekker gestructureerd en gepland te werk, daar kan ik wat van leren. En je pakt ook enthousiast allerlei andere taken op; NVTH bestuur, wetenschapsdagen en natuurlijk het onderzoeksvoorstel begeleiden bij Onderzoeksmethoden; leuk dat je die onderwijstaak hebt overgenomen. Samen gingen we met de ferry naar Engeland; jij kon heerlijk slapen, dwars door de storm heen. Ik moet nog wel eens terugdenken aan onze gesprekken in de trein van Edinburgh naar Newcastle; je bent open, betrokken en geïnteresseerd. Heel veel succes met het afronden van je onderzoek; ik ben ontzettend benieuwd wat jouw volgende stap gaat zijn! **Vossa**, we hebben niet heel veel direct samengewerkt, maar ik ben je dankbaar voor jouw bijdrage aan de groep. Bijvoorbeeld dat mijn studenten altijd bij jou terecht konden met vragen en dat je altijd bereid bent om even mee te helpen of te zoeken! Dat is echt super fijn en zegt ook iets over wie jij bent! **Tessa**, het is alweer een tijdje geleden dat jij promoveerde. Je was een enorm voorbeeld voor mij! Super fijn dat ik tijdens jouw promotie als jouw paranimf, al een keer kon meekijken naar hoe dat allemaal zou gaan. Ik vind het mooi en inspirerend om te zien hoe jij je ambitie combineert met je gezin en alle andere rollen en taken die je hebt; familiemens, sporter, vriendin, noem maar op. Jij blijft jezelf uitdagen en ontwikkelen en zit nooit stil; ook nu weer in je opleiding tot klinisch chemicus. Blijf lekker jezelf! **Femke,** we hebben niet samen gewerkt; maar ik

heb je nu al een paar keer ontmoet; wat ben je een leuk mens! Heel veel succes met je onderzoek; je bent echt een leuke toevoeging aan de groep. **Rowan**, jij hoort er voor mij gewoon helemaal bij! Als student zijn we ooit al samen begonnen en daarna volgde nog veel meer. Jouw trouw, loyaliteit en interesse waardeer ik enorm. De vele tripjes die ook wij samen gemaakt hebben zijn enorm vrolijke, fijne herinneringen. Je leeft mee met anderen en neemt altijd de tijd; blijf wie je bent en blijf trouw aan jezelf! Ik ben benieuwd waar de toekomst jou brengt; heel veel liefs.

Lieve studenten; Jente, jou heb ik al genoemd, na jou volgden **Jasper** en **Lennart**; the golden boys. Ik heb jullie beide al een keer mogen toespreken; maar het was heerlijk om met jullie samen te werken. Het gaf afwisseling en plezier, ook al ging er soms weleens iets mis. Het plezier dat ik ontdekte in begeleiden, uitleggen en samen nadenken over onderzoek heeft er mede voor gezorgd dat ik uiteindelijk voor het onderwijs heb gekozen. Dank voor jullie harde werken; hoofdstuk 6 was er niet geweest zonder jullie. Ook **Janoek**, dank voor je harde werken aan de review die helaas niet helemaal af is gekomen, ik hoop dat je er ondanks dat veel van hebt mogen leren. Ik heb onze samenwerking gewaardeerd en weet dat het niet voor niks is geweest, veel van de bronnen heb ik uiteindelijk in andere artikelen gebruikt! **Jasper**, **Floor** en **Eva,** jullie heb ik mogen begeleiden met schrijfopdrachten; ook dat waren leerzame processen. Dank voor de samenwerking!

Inmiddels heb ik heel wat jaren op het CDL doorgebracht, en dat is niet voor niets. De koffie op de gang, lunchen in de Brink om half twaalf, borrels in Café Koekman, labfeestjes, taart(competities) en alle spontane gesprekjes tussendoor maakten dat ik me hier thuis voelde. Iedereen draagt hier zijn/haar steentje aan bij; het lab draaiende houden en goed organiseren, de spontane en geïnteresseerde gesprekjes door de dag heen; bedankt allemaal! De PI's: **Ray,** dank voor leiden van de hele bende en je altijd aanwezige enthousiasme en gezelligheid. **Pieter**, we komen elkaar vaker tegen; maar ik herinner me zeker ook geïnteresseerde gesprekjes tijdens lunch in de Brink of momenten op de gang dat je ineens vroeg of ik nog iemand voor de liturgiecommissie wist te vinden. Dank voor die vertrouwdheid. **Richard**, we hebben iets prachtigs gemeen: NIJKERK! Bedankt dat mijn vader een keer mijn sleutels bij je af mocht geven toen ik ze daar had laten liggen.

Nog veel belangrijker zijn de analisten, jullie zijn de bindende factor op het CDL en zonder jullie zou het een puinhoop worden; ontzettend veel dank voor al jullie inzet! **Arnold**, tijdens mijn masterstage werkten we veel samen, dat was super fijn. Daarna kwam ik vooral bij je met vragen over de ÄKTA die weer eens een te hoge druk aangaf. Hoe jij de eindeloze en eeuwige vragenstroom blijft opvangen is werkelijk bewonderenswaardig, maar ook jouw enthousiasme, interesse, plezier en stabiliteit zijn dingen waar we allemaal op bouwen. **Simone**, sorry als ik soms zeurde over het VMT cleaning schedule. Dank voor hoe je van alles regelt en organiseert! Heerlijk om met jou lekker te kletsen over het weekend of vakanties. Dank voor je interesse en meeleven! **Arjan**, lab 3!, al was ik er niet zo vaak ik heb het erg gewaardeerd vlak naast je te zitten. Jij brengt een hoop expertise

op het lab, maar ook interessante gesprekken of discussiepunten. **Jerney**, door jou zal ik nooit meer het woord 'papadag' durven gebruiken, ik had er nooit zo over nagedacht, maar je hebt helemaal gelijk. Heerlijk hoe jij als je zoiets vind dat lekker ongezouten neer kan zetten en je daar ook echt voor staat. Recht voor z'n raap, dank voor je eerlijkheid. **Annet**, met jou kun je alles bespreken of het nou serieus of minder serieus is. Bedankt dat je naar anderen luistert! Mooi dat je al zo lang lief en leed deelt op het CDL, dat er nog maar veel mooie jaren op het lab voor je mogen volgen. **Noortje**, tijdens mijn stage was ik van je onder de indruk, ik was nog een beetje een jong onschuldig meisje. Ik kon erg genieten van je openheid en je nieuwsgierigheid, we hebben mooie gesprekken gehad, dank daarvoor! **Brigitte**, een stabiele factor. Net als veel van de analisten heb je al veel jaren meegemaakt op het CDL, misschien wel het meest? Jouw ervarenheid is onmisbaar, toch komt een moment van afscheid misschien wel een keer dichterbij.. Dank voor de leuke gesprekken over meubels opknappen, tuineren en je interesse en meeleven. **Jennifer**, met jou kun je heerlijk kletsen; jij vraagt door en luistert echt. Dank voor wie je bent en voor al je inzet. **Cor**, bedankt dat je mij en Lennart een keer meegenomen hebt naar de elektronenmicroscoop; fascinerende apparaten en leuk om een keer de deeltjes die we hadden gemaakt echt te kunnen zien. Mooi ook dat je je inzet voor het groene beleid op het lab; ga daar mee door! Er valt vast nog een hoop te winnen.

PhD's; we waren een flinke club en wat fijn om samen lief en leed te kunnen delen deze jaren. **Jonathan**, onze dokter, maar vooral rasonderzoeker. Jij houdt van onderzoek en bent een echte denker. Heerlijk om af en toe wat diepzinnige, filosofische gespreken te voeren. Maar wat konden we ook heerlijk samen lachen. Super mooi dat we je ook mochten opzoeken in New York, dank! **Marissa**, nu ook een getrouwde vrouw, welkom bij de club! Mooi om samen in Madrid te zijn geweest, dat we Titine konden overhalen om de trein met ons uit te proberen en toen ook nog bijna UMC-famous zijn geworden op Connect. Wat hebben we heerlijk gelachen in het appartement en op de terrasjes met sangria. Ik hoop je vaak te zien, gelukkig is Zeist lekker dichtbij. Als ik straks weer een fiets heb kunnen we vast samen weleens een tochtje over de vliegbasis maken! **Myrthe**, mijn vaste buurvrouw, heerlijk om naast elkaar te zitten en met elkaar mee te leven, in de PhD, maar ook daarbuiten. Toen je weg was miste ik je, gelukkig zaten ook **Caren** en **Bart** vaak genoeg naast me en deden die bijna niet voor je onder. **Luuk**, de musicus, heerlijk om met jou te kletsen, mijn tegenover buurman op het lab. Als ik er was, was ik altijd blij dat jij tegenover mij zat. Stay strong, bijnnnaaa klaar! **Pol**, I am very happy we finally got to ski together last year. Daring the Harakiri! I always loved hearing about all your adventures and travels and wish you many more. **Diego**, you eat more meat then I do, but that's okay. We can compensate for each other. **Willemijn**, je woont gewoon in Zwitserland, super cool! Dat is iets waar ik misschien soms ook weleens een beetje van droom. Ook jouw sportiviteit is aanstekelijk en heeft mij overgehaald om de UMC estafette te rennen en zelfs de kwartmarathon van Utrecht. Bedankt voor de aansporing en aanmoediging! **Alejandra**, crossing the finish line of the ¼ marathon together is something I will never forget. Thanks for encouraging me that day, but also on the normal everydays in the office!

Mariona, your laughter always filled the hallway, thanks for sharing it with us. It always made me smile as well. **Tanja,** das wir zusammen im Ankerlift auf den Berg waren wurde ich nie vergessen. Du hast es geschafft! Jetzt musst du noch die PhD schaffen, das wurdest du sicher auch gut machen! **Titine,** samen begonnen in de bachelor, leuk om al zolang een beetje zijdelings samen op te trekken. Hoogtepuntje was natuurlijk wel de trein naar Madrid! Dank voor al je gezelligheid. **Maryse,** kamergenootje van het laatste uur, dank voor die uren samen op de kamer met je interesse en betrokkenheid! Ik hoop dat je het goed hebt (gehad) in Toronto. Ook alle anderen; **Caro, Clara, Federica, Gaspard, Hinde, Ludovica, Naomi** en **Nadine,** many thanks; together we make the CDL the nice place that it is! I wish you all the very best.

Ook zonder **Martijn, Leida** en **Joukje** zouden een hoop zaken minder soepel lopen. Jullie zorgen dat alles soepel blijft draaien en lopen. Heel veel dank daarvoor! Daarnaast zeker veel dank aan alle oudere(n) en wijzere(n): **Dan, Martijn, Sander, Olivier, Omnia, Tom, Maria-Laura,** and **Shaquib.** Jullie kennis maar ook aandacht en betrokkenheid bij de afdeling nu of in het verleden zijn mega waardevol! **Danielle,** ook jij hebt al heel wat jaren op het CDL doorgebracht; ik heb jou interesse in mij (en anderen) altijd super gewaardeerd. Je bent heel betrokken en leeft echt met mensen mee! Dat zijn super mooie eigenschappen; ook ben je trouw en werk je hard! **Marc** bij jou begon het allemaal, jij liet zien hoe leuk het lab kon zijn! Met jou was het nooit saai om onderzoek te doen en ik heb de tijd dat we samenwerkten echt enorm gewaardeerd. **Sandra**, jij was in die tijd ook een bron van raad! Leuk om in die tijd samen op te trekken. In die tijd werkten we ook samen met **Tim,** ik denk met plezier terug aan die stagetijd. We hebben veel lol gehad met allerlei experimenten, de pipetboys en nog veel meer, dank daarvoor!

VCK collega's: ook al was ik niet vaak in de kliniek, ik vond het altijd mooi om te zien en te horen hoe jullie je inzetten voor onze patiënten. Dat is natuurlijk waar we het uiteindelijk allemaal voor doen! Jullie hebben hart voor de zaak en wanneer ik jullie zag of sprak, waardeerde ik de interesse en voelde het toch verbonden. **Ottelien**, we hebben elkaar inmiddels best goed leren kennen. Het begon allemaal met dat jij (samen met Anna) een Green Team wilde starten, dat is gelukt! Inmiddels zijn we ook soort van buren en vriendinnen, dank dat je me zo ontzettend welkom heet in Soesterberg, dat waardeer ik enorm! **Karin**, dank voor het altijd meedenken en inplannen van de afspraken; we spraken elkaar vooral via de mail, maar weet dat het superfijn is dat je hierbij ondersteunt. Ook alle PhD's: mooi hoe we met elkaar een clubje vormde deze jaren; met onze formele en informele PhD meetings. We hebben elkaar steeds beter leren kennen! Inmiddels een hele lijst: **Floor, Konrad, Johan, Anne, Sigrid, Karlijn, Amber, Annick, Geoff, Thomas** en **Gijs**. Voor degene die al klaar zijn; heerlijk, fijn dat jullie een voorbeeld waren! Voor degene die nog volgen; heel veel succes! Sigrid, we hebben twee keer samen opgetrokken in Papendal, daar heb ik goede herinneringen aan. We deelden een hotelkamer en het enthousiasme voor het Green Team, veel dank!

Ook dank aan alle collega's bij Synapse Research Institute die mij hebben geholpen met experimenten; **Veronique** en **Harmen** en ook **Dana** en **Joke**. En natuurlijk **Romy** voor de thrombin dynamics en allerlei analyses; de formules in dit boekje heb ik aan jou te danken! Ook dank aan **Mark** voor het inhoudelijk meedenken zo nu en dan. En natuurlijk dank voor de gezelligheid op congressen zoals in Zwitserland (GTH) en Washington, waarvoor ook dank aan **Frouke**. Ook de PhD collega's: **Rachel** en **Ruth**, fijn dat jullie me allemaal welkom heetten in Maastricht. Ook **Janneke** bedankt; ik heb leuke herinneringen aan onze dag samen in Montreal met hottub en museumbezoek, en ook in Koudekerke een kamer delen was erg gezellig!

Daarnaast dank aan **Maartje** en **Eva** bij Sanquin**,** fijn om met jullie samen te werken aan het proteomics stuk. Soms best een beetje puzzelen om onze werelden bij elkaar te krijgen, maar het is gelukt en heeft tot een mooie publicatie geleid! Dank voor de prettige samenwerking!

Er zijn nog een heleboel anderen die ik wil bedanken: mijn mede-Green Team Research leden - leuk om samen voor iets te gaan wat ons aan het hart ging & gaat, of het nou ging om minder printen in klinische studies, data cleaning challenges of duurzaam drukken van proefschriften; we hebben hopelijk anderen soms een beetje kunnen aanmoedigen om ook op werk / in onderzoek na te denken over duurzaamheid. Mijn medecommissieleden van het PhD programma Cardiovascular Research, met elkaar hebben we het programma weer een beetje op de kaart kunnen zetten met mooie, leerzame en interessante PhD-dagen en lunches. Leuk om op deze manier samen te werken, organiseren en nieuwe skills te leren.

Ook denk ik terug aan al mijn docenten: van mijn basisschooljuffen en meesters, middelbare school docenten tot aan docenten bij BMW en BMS; jullie hebben allemaal bijgedragen aan wie ik nu ben, tijdens jullie lessen ontwikkelde mijn nieuwgierigheid en voedden jullie mij op. Het wordt niet altijd genoemd of bewust erkend; maar jullie investeerden al in mij zonder dat ik dat zelf echt besefte; heel veel dank!

Daarnaast ben ik dankbaar voor mijn nieuwe collega's, dank voor het vertrouwen! Ik heb me snel welkom gevoeld bij de Medische Fysiologie, maar ook tijdelijk bij CMM. Dank dat jullie mij deze kans(en) hebben gegeven en ik me nu verder kan ontwikkelen binnen het onderwijs!

Ergens moet je natuurlijk ook een grens stellen want zo kunnen we iedereen wel bedanken, maar misschien is dat juist de kunst: inzien dat je eigenlijk iedereen dankbaar mag zijn. We doen het tenslotte samen. Als laatste wil ik heel graag nog wat mensen uit mijn 'privé'-leven bedanken!

Elselijn, allebei werkende in het academische wereldje, hebben we vaak veel uit te wisselen. Zeker nu in onze onderwijs(gerelateerde) functies. Heerlijk om daar met je over te sparren. Maar vooral heerlijk om over zoveel andere dingen te praten en het leven te delen. Op nog vele wandelvakanties, zeilweekendjes, bijkletsavondjes, uitjes naar musea, uitproberen van restaurantjes en ontelbaar veel andere mooie momenten. Een deel van mijn promotietijd zijn we zelfs huisgenootjes geweest, ik ben zo blij dat je me vroeg of ik bij jou wilde komen wonen, weg uit dat muizenhol. Els, ik ben je ontzettend dankbaar voor onze vriendschap, je trouw en je altijd luisterend oor! En omdat je misschien ook ooit wel had gehoopt een PhD te kunnen doen (wie weet komt het ooit nog op je pad..), vind ik het super leuk dat je vandaag naast mij wilt staan als paranimf! Dankjewel! **Johanne** & **Daan,** het is inmiddels wat verder reizen naar jullie, maar ik hoop die rit nog ontzettend vaak te maken. Jo, al zolang samen op school en daarna samen studeren, wel jammer dat jij deze laatste mijlpaal van een PhD doen geskipt hebt. Wat ik ben super trots op jou dat je nu in opleiding bent tot kinderarts! Er valt nog zoveel over onze lange vriendschap te zeggen, maar vooral; ik hou heel veel van jou! Dank aan jullie beide voor jullie support en interesse in mijn/onze levens, ook als PhD'ers. **Iris** & **Joost,** ook naar jullie is het inmiddels wat verder rijden, maar dat geeft niet hoor, dat doen we met liefde. Dank voor jullie interesse en meeleven altijd weer. Ier, er was een tijd dat we nog twee deuren in onze kamer nodig hadden, maar inmiddels kunnen we gelukkig heel erg goed samen door een deur. Ik ben heel dankbaar voor onze vriendschap, de logeerpartijtjes, drankjes, etentjes, stadsbezoekjes, wandelingen en nog veel meer! **Lize** & **Stijn,** we zien elkaar niet meer zo vaak als eerst, maar ik ben altijd blij om jullie te spreken. Ook jullie leven al lang met mij mee en daar ben ik heel blij mee. Lies, jij stelt altijd goede vragen die me weer aan het denken zetten, dank daarvoor! **Sarah** & **Jouke,** jullie wonen gelukkig lekker in de buurt, ik kan zelfs op de fiets. Saar, wat fijn dat jij al zolang betrokken bent op mij. Bijzonder dat we elkaar in zoveel fases van het leven meemaken, soms intensiever, soms wat meer op afstand, maar altijd vol liefde. Ik waardeer onze vriendschap echt ontzettend. Leden van **Dispuut Maïs Cena**; jullie waren mijn studententijd en hebben mij allemaal op je eigen manier gevormd, ik kijk met zoveel plezier en dankbaarheid terug op die tijd; *'als ik terug kon in de tijd dan wel met jou'*! **Corline** en **Kees** (en eigenlijk ook **Punt**); de PhD-medestanders; onze PhD-memes brachten vaak een lach op m'n gezicht als ze ineens in mijn mailbox belanden! Als laatste van de drie heb ik het traject nu ook kunnen afronden. **Chiel, Gerlinde** & **Maurits**, dat er nog maar veel oudjaarsavonden, (wandel)vakanties, spontane spelletjesavonden en andere gezelligheid mag volgen!

Ook voor mijn familie ben ik dankbaar; **opa** & **oma Poolen,** vroeger kreeg ik altijd lekker dikke pannenkoeken bij jullie, ook heb ik bij jullie zalm en brie leren eten en herinner ik me BBQ's op de Colenbranderstraat. Ik was jullie grootste kleindochter; ook al vond ik het niet leuk om kleinkind genoemd te worden. Jullie leerden mij om altijd dankbaar te zijn voor wat me gegeven was, als ik een goed rapport kreeg was het niet de bedoeling dat ik een te hoge pet van mezelf op kreeg. Ik denk dat dat een wijze les was; ook mijn kennis en inzicht zijn dingen die ik heb ontvangen. Datzelfde leerde ik van **opa** & **oma van den**

Brink; helaas hebben we voor dit leven afscheid genomen tijdens mijn promotietijd. Ik ben dankbaar voor jullie voorbeeld in trouw zijn aan de Heer. Ook al mijn **ooms** & **tantes,** hebben mij gevormd tot wie ik ben. Dank voor jullie interesse en meeleven. Ik besef me vaak dat er een rijke kring van mensen om mij heen staat, heel bijzonder!

Lieve schoonfamilie, **Alwin** & **Simone**, dank dat we altijd welkom zijn bij jullie. Dat jullie betrokken zijn en geïnteresseerd en dat jullie ook vaak even vroegen hoe het ging met het onderzoek. Fijn dat jullie ook daarin zo meeleefden! Schoonbroertjes en zusjes; **Rianne** & **Tim, Julian, Ko** en **Lysanne**; ook jullie leefden mee en vroegen altijd geïnteresseerd naar mijn onderzoek. Dank daarvoor! Ook dank voor de afleiding, verjaardagen, gezellige etentjes en andere uitjes. Het is ook heerlijk af en toe even helemaal niet met werk bezig te zijn.

Lieve **Lianne** & **Ruben, Tom** & **Tessa,** jullie weten niet half hoe belangrijk jullie voor me zijn. Zo fijn dat we het over alles kunnen hebben thuis. Lianne, misschien ga je me wel achterna, leuk dat we nu als collega's in het UMC af en toe samen een koffietje of ijsje kunnen halen! Tom, wie had het geraden; maar ook jij ging de medische kant op, benieuwd waar je zult belanden als je BMT hebt afgerond! Lieve **papa** & **mama**, jullie snapten het niet altijd; '*Waarom wil je dat nou gaan studeren en wat wil je daarmee worden dan?', 'Is het lab niet best wel saai?'* en *'Wat doe je dan nu precies?'*. Maar jullie hebben me altijd gesteund in mijn nieuwsgierigheid. Behalve dan als ik voor de 100ste keer '*Waarom?*' vroeg en mama zei; '*Geke, er staat geen bordje bij..*', wat zoveel wilde zeggen als houd nu maar even op, ik weet het ook niet. Dank dat ik altijd een veilige haven heb bij jullie, mezelf kan zijn en ook altijd weer even kan opladen bij jullie! Dank dat jullie mij liefhebben, al mijn hele leven lang.

Lieve **Marek**, lieve man van mij, het is nog een beetje gek om te zeggen/schrijven, maar je bent het wel: mijn man! We zaten afgelopen jaren samen in hetzelfde schuitje. Mooi om elkaar ook in deze PhD-trajecten te steunen en motiveren; als het even tegen zat of aan moed en plezier ontbrak, dank daarvoor. Jij ook veel succes met de laatste loodjes; you can do it <3. Gelukkig is het leven meer dan werk & PhD alleen, ik kijk uit naar de rest van ons leven samen en nog veel mooie avonturen. De echt sentimentele dingen heb ik natuurlijk in mijn geloftes al tegen je gezegd maar ik hou van jou!

Boven alles wil ik **God**, mijn hemelse Vader bedanken. Alles bestaat in en door Hem en Hij is degene die mij bovenstaande tienduizend redenen tot dankbaarheid heeft geschonken!

ABOUT THE AUTHOR

Geke Poolen was born on 9 July 1997 in Nijkerk, the Netherlands, where she also grew up. After obtaining her bilingual VWO diploma from Christelijk College Groevenbeek in Ermelo in 2015, she studied Biomedical Sciences at Utrecht University, graduating in 2018. She subsequently completed the Master's programme *Biology of Disease* in 2021. During her master's, Geke conducted research at the Department of Clinical Chemistry and Haematology of the University Medical Center Utrecht, where she developed and characterised nanobodies targeting several endothelial proteins. She subsequently joined the German Cancer Research Center (DKFZ) in Heidelberg, Germany, where she investigated the role of LRG1 in tumour-associated vascular processes.

In October 2021, Geke started her PhD at the Van Creveldkliniek, University Medical Center Utrecht, under the supervision of Dr. Rolf Urbanus, Prof. Dr. Roger Schutgens, and Dr. Bas de Laat. Her research combined clinical and fundamental studies in thrombosis and coagulation, focusing on improving the prediction of recurrent venous thromboembolism using laboratory biomarkers and global coagulation assays, while also investigating the anticoagulant protein thrombomodulin.

After completing her PhD, Geke joined the Department of Medical Physiology at University Medical Center Utrecht, where she now works in (bio)medical education. She teaches in several courses within the Faculty of Medical Sciences and serves as coordinator of the Master's programme *Cardiovascular Health and Disease*. Outside work, Geke enjoys hiking, skiing, sailing, reading, and spending time with family and friends.