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THE ARCTIC UNIVERSITY OF NORWAY

Biomarkers of major bleeding after incident venous thromboembolism

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December 2020

A dissertation for the degree of Philosophiae Doctor

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Acknowledgements

The work presented in this thesis was carried out at the K.G Jebsen Thrombosis Research and Expertise Center (TREC), Department of Clinical Medicine, Faculty of Health Sciences at the UiT – The arctic university of Norway. I started out as an MD/PhD student at the research program for medical students at the UiT in 2015, and graduated as a medical doctor in the summer of 2019. I received an independent research grant from the Northern Norway regional health authorities (Helse-Nord) which funded my PhD position until March 2020 when I started as a medical intern at the University hospital in Tromsø.

First, I wish to express my deepest gratitude and many thanks to my main supervisor John-Bjarne Hansen. It is hard to imagine my PhD-journey without your solid leadership and guidance. I feel lucky to have been a part of the TREC team these past years with you as my main supervisor. From the very beginning, you have challenged me, encouraged me and facilitated an ideal arena to thrive in, both scientifically and socially, and I am truly grateful for that. Your hard work, vast knowledge and dedication to science is impressive and inspiring, and I have learned a great deal from you. “Takk for alt”.

I would like to thank my co-supervisors Sigrid K. Brækkan and Vania M. Morelli. Sigrid, you are a true inspiration and a role model both on and off work, and I am utterly thankful for all your help and teachings in epidemiology and Stata. Other than being amazing at your job I find your work as a singer/songwriter particularly impressive. If I were to describe you with a song it would have to be a mash-up with ABBA – Dancing queen, Daft Punk – Harder, better, faster, stronger, and a little bit of Coolio – Gangsta’s paradise. Vania, I was truly lucky to have you to co-supervise my PhD-project. You are extremely dedicated, thorough and have an eye for details that I admire. I am grateful for your patience and the many constructive discussions we have had. The contributions from you both to writing all three manuscripts as well as this thesis have been invaluable.

To my close friends Esben Bjøri and Christian B. Arkteg – thank you for all the great times we have shared so far. Moments from everyday life at the office, scientific events in Amsterdam, Berlin and The Hague, non-scientific adventures, to brewing beer and climbing, have all produced memories that I will cherish for life. I am profoundly glad for the friendship we share, and owe you many thanks for always keeping my beer supply topped off throughout these years.

I would like to thank my co-authors Kristian Hindberg, Esben Bjøri and Ellen E. Brodin for their contributions. Kristian, thank you for sharing your statistical expertise with me and for challenging discussions in many different topics. We do not always agree, but I often walk away with a new perspective or angle. I will always remember to plot my data!

A special thanks to the former and current members of TREC. Whether it be TREC ski-day, Holmenkollen relay, seminars, congresses abroad, parties, TRECxercise, CLOTtery or Friday cake, it has all been a delight because of you. Thank you for contributing and creating a constructive scientific and social environment. Many thanks to Trond Isaksen and Trond Børvik for collecting much of the data for this work and many laughs. Helle Jørgensen, thank you for helping me with practical issues and making sure that everything just works. My fellow "research-liners" Birgit Småbrekke, Ludvig B. Rinde, Trygve S. Ellingsen, Jostein Lappegård, Caroline Lind, Gunhild Lerstad, Hanne Skille, Joakim Sejrup, Carl Arne Løchen Arnesen, Fridtjof B. Rinde, Magnus Edvardsen, Ellen-Sofie Hansen, and of course TREC all-stars Erin M. Hald, Line Evensen, Robin Liang, Birgitte B. Tøndel, Cathrine Ramberg, Bjarne Østerud, Gro Grimnes, Lars Horvei, Olga V. Gran, Ina I. Høiland, Timofey Sovershaev, Søren B. Jensen, Nadia Arshad, Nadezhda Latysheva, Omri Snir, Jacob Odeberg, Lynn Butler, Line Wilsgård, Marthe N. Thorsen, Tobias Frischmuth, Linda Cronen, Clèment Naudin, Eike E. Struck, Sofia Öling, Casper Ullsten-Wahlund, thank you all.

My dear family Inger, Roald and Anne-Marte, thank you for your endless love and support. Thank you mom for always worrying about my basic needs, reminding me to take brakes and inviting my family and me to dinner in stressful times. To my childhood friends Aleksander, Stian and Stein Jr, thank you for always being able to take my mind of work.

Tonje, my dearest friend, I love you. Thank you for bearing with me. I feel lucky every day with you and little Sondre who came in to our lives in March. If you ever read this son, finish your homework and clean your room. Second, know that I am proud of you and that I love you. Finally, if you at some point consider pursuing a PhD, think long and hard, it is not often that you come across such good people as your father has encountered.

Håkon
December 2020

Summary

Major bleeding (MB) events are feared and potentially fatal complications of anticoagulant treatment in patients with venous thromboembolism (VTE). Even though several clinical risk factors are associated with MB risk, the predictive power of currently available risk assessment models remain limited for VTE. The aim of this thesis was to identify biomarkers of MB risk during the first year after an incident VTE.

In all three papers included in this thesis, the study population consisted of subjects with incident VTE derived from the Tromsø study, a population-based cohort study with repeated measurements and blood sampling of the inhabitants in Tromsø. Participants were symptomatic VTE cases requiring treatment, who were diagnosed with objective criteria from 1994 to 2012/16. Information on VTE characteristics, including blood sample measurements, was collected from the medical records at the University Hospital of North Norway (UNN). In the first paper, subjects were genotyped for prothrombotic single nucleotide polymorphisms (SNPs) in candidate biomarker genes, while D-dimer and platelet count, measured at VTE diagnosis, were assessed in the second and third papers. MB events were classified according to the International Society on Thrombosis and Haemostasis (ISTH) criteria and identified by review of medical records during the first year after VTE diagnosis.

In the first paper, we found no association between individual SNPs (rs6025 [Factor V Leiden], rs1799963 [Prothrombin G20210A], rs8176719 [ABO blood type], rs2066865 in *FGG*, and rs2036914 in *F11)* or number of risk alleles and MB risk. The results were similar after excluding patients with active cancer, and when restricting the analysis to the first three months after VTE. In the second paper, we excluded subjects with cancer and those who developed an in-hospital VTE prior to the analyses and found that a high D-dimer (upper 20th percentile) was associated with increased risk of MB. The risk was particularly pronounced in patients with deep vein thrombosis (DVT), provoked VTE and during the first three months after diagnosis. In the third paper, we found that platelet count measured at VTE diagnosis was associated with higher risk of MB in a dose-response manner. Furthermore, when platelet count was measured years prior to developing a VTE (at the Tromsø study inclusion), an increasing platelet count was also associated with a higher risk of MB.

Our findings suggest that D-dimer and platelet count, which are already widely available for most of the VTE patients after the initial diagnostic investigation, may aid the identification of VTE patients at risk of MB during anticoagulant treatment.

Sammendrag

Alvorlig blødning er en fryktet og potensielt fatal komplikasjon ved antikoagulasjonsbehandling av pasienter med venøs tromboembolisme (VTE). Selv om flere kliniske risikofaktorer er assosiert med blødningsrisiko er evnen til å predikere alvorlige blødninger begrenset med tilgjengelige risikomodeller for VTE. Målet med avhandlingen var å identifisere biomarkører for alvorlig blødningsrisiko etter førstegangs VTE.

I denne avhandlingen besto studiepopulasjonen i alle tre artiklene av individer med førstegangs VTE blant deltakere av Tromsøundersøkelsen, en populasjonsbasert kohortstudie med repeterte målinger og blodprøver av innbyggerne i Tromsø. Deltakerne var symptomatiske og behandlingskrevende VTE pasienter som fikk diagnosen bekreftet med objektive undersøkelser fra 1994 til 2012/16. Kliniske variabler og blodprøvesvar rundt innleggelsen for VTE ble innhentet fra medisinske journaler ved Universitetssykehuset Nord-Norge (UNN). I den første artikkelen ble studiedeltakerne gensekvensert for protrombotiske genvarianter (single nucleotide polymorphisms [SNPs]) i mulige biomarkørgener, mens D-dimer og blodplatetall målt ved VTE diagnose ble undersøkt i artikkel to og tre. Alvorlige blødninger ble klassifisert i henhold til International Society on Thrombosis and Haemostasis (ISTH) kriterier og identifisert ved gjennomgang av medisinske journaler det første året etter VTE diagnose.

I artikkel I fant vi ingen assosiasjon mellom individuelle SNPs (rs6025 [Factor V Leiden], rs1799963 [Prothrombin G20210A], rs8176719 [ABO blod type], rs2066865 i *FGG*, og rs2036914 i *F11)* eller antall risikoalleler og risiko for alvorlig blødning. Vi gjorde liknende funn etter at pasienter med aktiv kreft ble ekskludert fra analysen og når vi begrenset analysen til de første tre månedene. Pasienter med aktiv kreft og pasienter som utviklet VTE under sykehusinnleggelse ble utelatt fra analysene i artikkel II, hvor vi fant at høy D-dimer (øverste 20%) var assosiert med økt risiko for alvorlig blødning. Risikoen var spesielt uttalt hos pasienter med dyp venetrombose (DVT), pasienter med provosert VTE og i løpet av de først tre månedene etter diagnose. I artikkel III fant vi at blodplatetall målt ved VTE diagnose var assosiert med økt risiko for alvorlig blødning i et dose-respons forhold. Videre fant vi at økende blodplatetall målt flere år i forkant av VTE (ved inklusjon i Tromsøundersøkelsen), også var assosiert med økt risiko for alvorlig blødning.

Våre funn taler for at D-dimer og blodplatetall, som allerede er tilgjengelig hos de fleste VTE pasienter etter innledende diagnostikk, kan bidra til identifikasjon av VTE pasienter med forhøyet risiko for alvorlig blødningskomplikasjon av antikoagulasjonsbehandling.

List of papers

I. **Prothrombotic genotypes and risk of major bleeding in patients with incident venous thromboembolism**
Håkon Sandbukt Johnsen, Esben Bjøri, Kristian Hindberg, Sigrid K. Brækkan, Vania M. Morelli, John-Bjarne Hansen
Thrombosis Research 2020; 191: 82-9

II. **D-dimer measured at diagnosis of venous thromboembolism is associated with risk of major bleeding**
Håkon Sandbukt Johnsen, Kristian Hindberg, Esben Bjøri, Ellen E. Brodin, Sigrid K. Brækkan, Vania M. Morelli, John-Bjarne Hansen
Thrombosis and Haemostasis Open 2019;3:e77-e84

III. **Platelet count and risk of major bleeding in venous thromboembolism**
Håkon Sandbukt Johnsen, Sigrid K. Brækkan , Vania M. Morelli, John-Bjarne Hansen
Platelets 2020:1-9

Abbreviations

ACCP – American College of Chest Physicians

AF – Atrial fibrillation

APC – Activated protein C

APTT – Activated partial thromboplastin time

AT – Antithrombin

AUC – Area under the curve

BMI – Body mass index

CAT – Cancer-associated venous thromboembolism

CI – Confidence interval

COCs – Combined oral contraceptives

DOAC – Direct oral anticoagulant

DVT – Deep vein thrombosis

F – Coagulation factor

FDA – Food and Drug Administration

GWAS – Genome-wide-association studies

HR – Hazard ratio

ICD – International classification of diseases

IR – Incidence rate

ISTH – International Society on Thrombosis and Haemostasis

LMWH – Low molecular weight heparin

MB – Major bleeding

MPV – Mean platelet volume

NETs – Neutrophil extracellular traps

NSAIDS – Nonsteroidal anti-inflammatory drugs

CRNMB – clinically relevant non-major bleeding

PE – Pulmonary embolism

PAI-1 – Plasminogen activator inhibitor 1

RAM – Risk assessment model

RCT – Randomized controlled trial

SHR – Sub-distribution hazard ratio

SNP – Single nucleotide polymorphism

TF – Tissue factor

TFPI – Tissue factor pathway inhibitor

TTR – Time in therapeutic range

UFH – Unfractionated heparin

UNN – University hospital of North Norway

VKA – Vitamin K antagonist

VTE – Venous thromboembolism

vWF – von Willebrand factor

1. Introduction

Venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), is a severe and frequently recurring disease associated with great suffering and mortality. While treatment with anticoagulants efficiently lower the risk of a second thrombotic event and recurrence-related death, the risk of bleeding is concurrently increased, inflicting major bleeding (MB) events in 3-6% of VTE patients per year with modern treatment.[1-3] If anticoagulants are withheld, a deadly or non-deadly recurrent thrombotic event can occur in up to 50% of patients within two weeks.[4] Although a minimum of three months of anticoagulation is recommended for most cases of VTE, the ideal treatment duration is uncertain in patients at high risk of recurrence and depends on the risk of MB.[5] It is therefore essential to obtain accurate assessment of MB risk in order to guide decisions regarding treatment duration in this situation. In addition, patients at high risk of MB may benefit from targeted preventive measures during the initial active treatment period, when the absolute risk of MB is highest.[6,7]

Several clinical factors, such as advancing age and cancer, are associated with increased risk of MB and may identify high-risk VTE patients when combined in risk assessment models (RAMs).[8] The HAS-BLED score, originally derived in patients with atrial fibrillation (AF), has recently been assessed in VTE patients with promising results.[9] However, most validation studies demonstrate that the HAS-BLED score and other currently available bleeding RAMs generally have inconsistent and modest to low ability to distinguish VTE patients at high risk of MB from those at low risk.[10-12] Hence, improving risk prediction of MB in VTE patients has been identified as a major aim of future research.[13]

Biomarkers play a key role in the diagnostic work-up in subjects suspected of VTE[14] and recent studies indicate that biomarkers may also have a role in predicting risk of primary, recurrent and cancer-associated VTE.[15,16] A biomarker is a measured characteristic of an individual indicative of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention.[17] A model combining age, biomarkers and clinical history (ABC-model) has recently outperformed the HAS-BLED score in patients with AF.[18] The potential of biomarkers to identify VTE patients at higher bleeding risk prior to anticoagulation has been scarcely investigated. The focus of this thesis is on the identification of biomarkers of MB risk during anticoagulant treatment in the first year after an incident VTE.

1.1 Venous thromboembolism

DVT arises typically in the valve pockets of deep veins in the lower extremities, from where the thrombus may extend and cause obstruction of venous flow. A PE might occur if the thrombus dislodges or partly breaks off, travels through the bloodstream, and lodges in the narrow vasculature of the lungs.[19] However, DVTs cannot be detected in up to half of all PE patients investigated with ultrasonography or magnetic resonance imaging.[20] Alternative explanations on the origin of PE include *de novo* thrombus formation in the lung or embolization of a thrombus of cardiac origin. In support of the latter notion, AF has been associated with higher risk of PE than DVT, and explained 20% of PE events in the Tromsø study.[21] In VTE patients, the residing venous thrombus may cause symptoms dependent on the extent of thrombosis, adequacy of collaterals and the severity of vascular occlusion and inflammation.[19] Classical signs and symptoms of DVT include redness, swelling, heat, swollen superficial veins and pain of the affected extremity. The clinical features of PE are often less specific compared to DVT and may range from asymptomatic to circulatory collapse and death, but also include dyspnea, tachypnea, coughing and pleuritic chest pain.

1.1.1 Epidemiology

VTE is considered the third most common cardiovascular disease after coronary heart disease and stroke, with an annual incidence of 1 to 2 per 1000 in the general population.[22-24] Long-term observational studies suggest that the overall incidence of VTE might be slightly increasing.[25-27] Although the more extensive use of better diagnostic tools may explain the increase in PE rates, the prevalence of strong VTE risk factors, such as advanced age, cancer and obesity, are increasing and likely to impact VTE incidence rates.[28] The annual incidence of VTE increases exponentially with age and is around 100 times more common in the elderly compared to young adolescents.[29] Women at reproductive age have a higher incidence of VTE than men at the same age.[29] However, when women-specific reproductive risk factors (e.g. pregnancy and oral contraceptives) are taken into account, the risk of a first VTE is higher in men compared to women across all ages.[30] VTE incidence further varies according to ethnicity (e.g. relatively low in Asians and higher in Caucasians)[31], which in part can be explained by an uneven distribution of both inherited (e.g. Factor V Leiden) and acquired (e.g. obesity) VTE risk factors observed across different regions.[32,33]

After an incident VTE, short- and long-term complications frequently occur. The cumulative incidence of a recurrent VTE is 8-13% in the first year, and up to 30-36% within 10 years after the index event.[24,34-36] A second VTE tends to present at the same site as the

first, i.e. a PE is more likely to recur as a PE rather than a DVT.[37,38] Although a recurrent VTE may be regarded as a complication of the first event, a second thrombotic event can be interpreted as an acute exacerbation of an underlying prothrombotic state.[34] The risk of VTE recurrence declines rapidly within the first 6-12 months, then more modestly the following years but never declines to the baseline risk prior to the first event.[34,36,39,40] The index VTE event can be classified as provoked if a transient (e.g. surgery) or persistent (e.g. cancer) major or minor risk factor is present in the 3 months prior to the event, and unprovoked if no such environmental factor is identifiable.[41] However, risk factors may fluctuate, interact and display different magnitude of reversibility in individuals, which makes the classification somewhat arbitrary.[41] Still, the risk of recurrent VTE has been shown to be around 8-fold higher in patients with unprovoked VTE compared to those with surgery-provoked VTE, affecting 1.0% and 7.9% within the first year after discontinuing anticoagulation, respectively, as demonstrated in a systematic review.[42] Although there is considerable variation across cancer sites, cancer-associated VTE (CAT) is associated with a high risk of recurrence. In general, recurrence occurs in around 20% of cancer patients within the first year after an incident VTE, even after taking competing risk of death into account.[43-45]

Post-thrombotic syndrome (PTS) is the most common long-term complication of VTE. Within two years after a DVT of the lower extremities, 20-50% of patients will develop PTS and 5-10% will have severe PTS.[46,47] The clinical manifestation of PTS is typically characterized by chronic pain, heaviness, swelling, new varicose veins and venous ulcers in severe cases of the affected extremity.[48] A proximal DVT, such as involvement of the iliac or common femoral vein, is associated with a 2 to 3-fold increased risk of PTS, compared to distal (calf) DVT.[47] Other risk factors include a previous ipsilateral DVT, obesity, advanced age and inadequate anticoagulation. The presence and severity of PTS may be determined by the Villalta scale.[49] The scale consists of five symptoms and six clinical signs that correlate well with patient-perceived quality of life and clinical measurements considered to be related to the underlying pathophysiology of PTS.[50] Preventing a first DVT and a recurrent event with thromboprophylaxis has been regarded by some experts as the best way to prevent PTS.[49] Elastic compression stockings (ECS) were widely accepted to be effective for the prevention and treatment of PTS. However, in a randomized placebo controlled (SOX) trial, Kahn *et al* did not find any benefit of ECS on the incidence of PTS two years after a proximal DVT.[51] Several approaches including ECS, intermittent compression devices, exercise training programs, and catheter directed thrombolysis for selected patients might be

considered for the treatment of PTS.[48] Overall, PTS is a substantial source of morbidity, disability and reduced quality of life after a DVT.[52,53]

Chronic thromboembolic pulmonary hypertension (CTPH) is a debilitating long-term complication of VTE, affecting 0.5-4% of PE patients.[54,55] CTPH is characterized by increased pulmonary vascular resistance secondary to fibrotic transformation of the non-resolved thrombus and pulmonary artery.[55] Symptoms such as hemoptysis and dyspnea may be present in CTPH, and eventually right-sided heart failure if the condition is left untreated. Previous splenectomy, infected ventriculo-atrial shunts, indwelling venous catheters and leads, thyroid replacement therapy, cancer and chronic inflammatory states have been proposed as risk factors for both VTE and CTPH, however, other more classic thromboembolic risk factors are lacking for CTPH.[56] Life-long anticoagulation is recommended for CTPH, and pulmonary endarterectomy is regarded as the only curative treatment in operable patients.[55] The main pathophysiological trigger for developing both CTPH and PTS after a VTE is impaired thrombus resolution.[56]

Within the European Union alone, it is estimated that 370 000 VTE-related deaths occur every year.[57] The 30-day risk of dying is about 2 to 3-fold higher in patients with PE (10-30%) compared to DVT (5-10%) in population-based and nationwide studies, and the overall one year all-cause mortality is about 22-30%.[23,36,58] The same studies show that the risk of death is strikingly high within the first month and year after VTE, and after that approaches the same mortality as the general population. Cancer is a major cause of death in VTE patients, and it is related to a particularly poor prognosis when discovered simultaneously or within one year of a VTE, being associated with a one-year mortality of up to 60-80%.[23,59] In a large population-based cohort study, the mortality of DVT has been shown to remain fairly constant over the last 30 years, while it has decreased for PE.[58] Considering VTE-related deaths of both incident and recurrent VTE and the associated morbidity in survivors, the overall burden of VTE is massive, despite advances in the VTE management.[57,60] Of note, the majority of deaths occur as a consequence of hospital-acquired VTE, leaving VTE an important source of preventable deaths in hospitals, given the availability of effective treatment and prevention.[57]

1.1.2 Pathophysiology

Human blood is kept fluid by dominance of anticoagulant activity within the intact vasculature. Ideally, clot formation is initiated only when procoagulant stimuli overcome the anticoagulant dominance at the site of and in response to a vascular injury. Primary

hemostasis includes the physiological processes of platelet activation, aggregation and adhesion at sites of vascular injury (platelet plug), while secondary hemostasis denotes the activation of coagulation factors, fibrin formation and plug stabilization. The hemostatic system has a vital role in orchestrating the cessation of a local bleeding, which requires a delicate balance of pro- and anticoagulant activity in time and space.[61] Deviation from the hemostatic balance may lead to thrombin generation and clot formation not intended for hemostatic function, i.e. thrombosis.[62,63]

Formation of thrombin takes place through a complex series of sequential activation of coagulation factors (F) known as the coagulation cascade. In a simplified overview, the cascade consists of an intrinsic, extrinsic and a common pathway that culminates in the formation of cross-linked fibrin (Figure 1). The extrinsic-, also called the tissue-factor-pathway, is obligatory for physiological hemostasis, while the intrinsic pathway is not crucial for initiation of blood coagulation *in vivo*.[63] Pathological activation of the extrinsic pathway may occur via tissue factor (TF) expression in activated monocytes, monocyte-derived microvesicles and possibly activated endothelial cells.[64] TF and activated FVII form a complex that facilitates the activation of FX. Cellular RNA and polyphosphate released from activated platelets or bacteria are identified as activators of the intrinsic pathway.[64] The intrinsic pathway was initially described by Davie and Ratnoff and MacFarlane as a waterfall-based model involving the sequential activation of coagulation factors (XII, XI, IX, VIII) leading to the activation of FX, and ultimately thrombin formation and fibrin deposition.[65,66] Hence, the two pathways converge at coagulation factor X, which in its activated form facilitates cleavage of prothrombin to thrombin when co-factored by FVa. Dependent on the thrombin concentration, fibrin may influence the formation, structure and stability of a blood clot, as it generally forms a mesh that provides structural scaffold to a thrombus.[67] Venous thrombi are typically composed of a larger proportion of red blood cells and fibrin (“red clot”) compared to arterial thrombi, which hold an abundance of platelet aggregates (“white clot”).

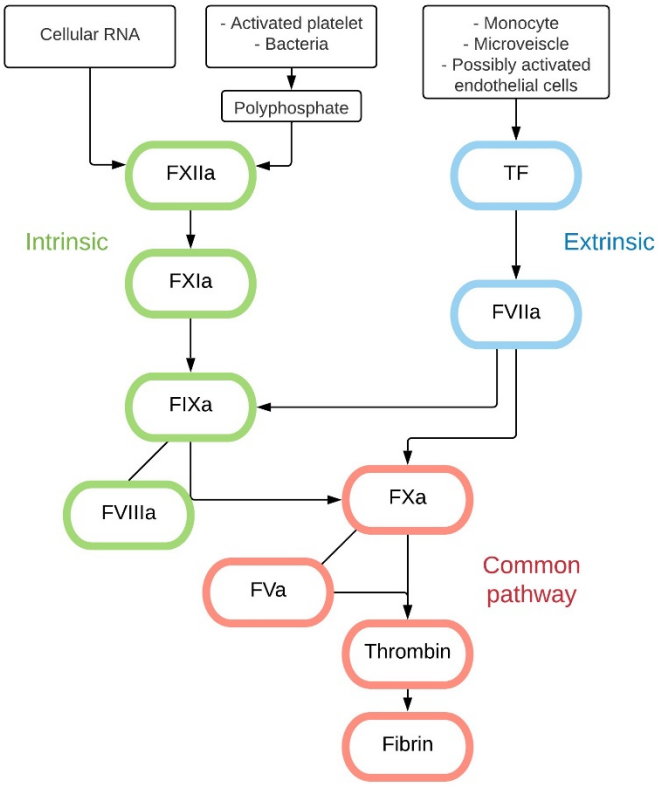

Figure 1 Simplified overview of the intrinsic (green), extrinsic (blue) and common pathway (red) of the coagulation cascade. Adapted from Mackman, J Clin Invest 2012[64]

Thrombin generation in the coagulation cascade is thoroughly regulated by feedback mechanisms, co-factors and perhaps most importantly, by the natural anticoagulants. Tissue factor pathway inhibitor (TFPI), antithrombin (AT) and protein C are the three major anticoagulants. TFPI blocks FXa and the TF/FVIIa complex.[68] AT may inhibit all procoagulant proteins dependent on the presence of heparin and heparin-like glycosaminoglycans. In their presence, the anticoagulant activity of AT is enhanced 100 to 1000-fold.[69,70] Activated protein C *(*APC) exerts an anticoagulant effect by inhibition of FVa and FVIIIa.[71] Animal studies have indicated the importance of these systems as mice lacking either of these anticoagulants do not survive.[72] Defects in the regulation of clot formation can lead to either bleeding or thrombosis.[72,73]

Rudolf Virchow postulated that physiological alterations in blood flow, blood coagulability and the vessel wall were involved in venous thrombus formation.[74] The procoagulant changes in these systems, i.e. reduction in blood flow/stasis, hypercoagulability and vessel wall injury/endothelial dysfunction are today collectively known as *Virchow's triad* (Figure 2).[75] Alterations in these systems remain important and relevant for our current understanding of thromboembolic disease, as most identified risk factors and thrombotic processes connect with one or more of the three pillars of the triad.[64] Hypercoagulability, i.e.

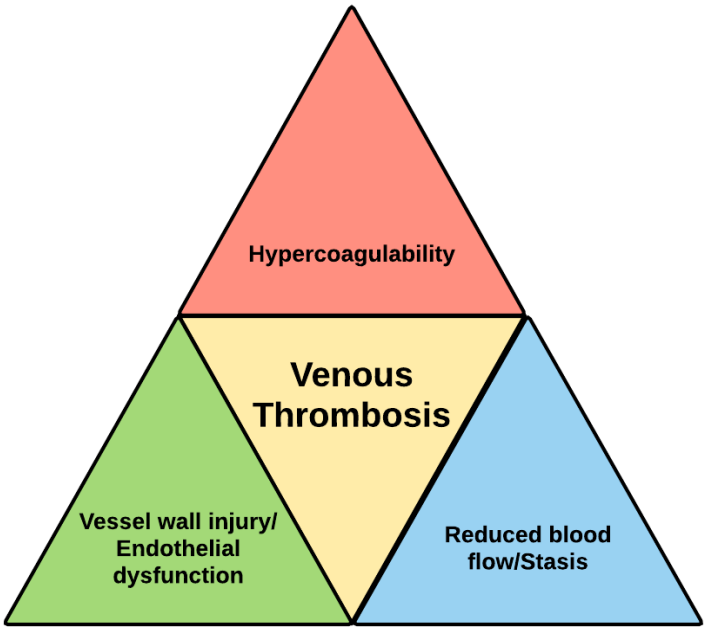

Figure 2 Virchow's triad

an abnormally increased tendency of blood clotting, may occur directly from acquired or inherited factors with an effect on thrombin generation, but also indirectly via the other elements of the triad. In the recess of venous valvular sinuses, where venous blood clots are most often formed, the endothelium is prone to dysfunction and subsequent downregulation of anticoagulant properties and promotion of prothrombotic processes.[64,76] Hypoxia and inflammatory mediators are identified as causes of endothelial dysfunction at this site.[64] As the innermost layer of the endothelium receives its oxygen supply from circulating erythrocytes, a gradient of increasing hypoxia towards the deepest recess of the valve pocket might occur due to vortical flow.[76] Moreover, the local hypoxia promotes activation of leukocytes and platelets, which in turn might release TF-containing microvesicles.[64] In sum, the local activation of the coagulation cascade overwhelm the protective anticoagulant pathways, initiating thrombus formation.

1.1.3 Heritable prothrombotic risk factors

There is a high heritability for VTE. Family and twin studies indicate that genetic factors account for 50-60% of the VTE risk.[77,78] Knockout mice technology and lessons from clinically abnormal levels of clotting factors, regulators and natural anticoagulants have been instrumental in determining the function and the relative importance of these factors, ranging from necessary for life *in utero* (e.g. TF) to severe, moderate and mild forms of thrombosis and bleeding diathesis.[68,79] For instance, increasing levels of FVIII are associated with increased risk of incident and recurrent VTE,[80,81] while severe FVIII deficiency may result in a bleeding-prone state known as hemophilia A. Genome-wide-association studies (GWAS) have contributed substantially in discovering genetic risk factors for VTE, uncovering around half of the loci that have been robustly associated with VTE so far.[82,83] In 2013, there were 16 single nucleotide polymorphisms (SNPs) occurring at these loci that had been firmly associated with VTE susceptibility, most of them affecting the coagulation cascade.[84,85] Larger, more recent GWAS and GWAS meta-analysis replicated previous findings, and found novel variants and loci associated with VTE risk. However, the strength of the associations and effect sizes suggest that the most important common variants associated with VTE risk have been discovered.[86-89]

Thrombophilia can be defined as a hypercoagulable state leading to a thrombotic tendency, and may occur either by gain of procoagulant or loss of anticoagulant function. In 1965, Egeberg described the first family with an inherited AT-deficiency causing thrombophilia.[90] The classical inherited loss-of-function thrombophilia occurs due to mutations leading to the quantitative or qualitative dysfunctional AT, protein C and protein S.[91] Around 200 mutations have been identified in each of the genes coding for AT (*SERPIN1*), protein C (*PROC*) and protein S (*PROS1*). Hereditary loss-of-function thrombophilia is relatively rare,[91] with a prevalence of AT, protein C and protein S deficiency in a healthy population of around 0.02-0.2% with variations across subtypes.[91] AT deficiency is the most severe among inherited loss-of-function thrombophilias, with a 3 to 7-fold higher risk of VTE compared to protein C or S deficiency, and more than 50-fold increased risk of VTE when compared to individuals carrying no loss-of-function defect.[69,91] In contrast, inherited gain-of-function thrombophilia is more common and typically less severe. In the 1990s, the genetic details leading to APC resistance due to FVL, and the prothrombin G20210A mutation, associated with higher levels of prothrombin, were elucidated.[92,93]

Bertina and colleagues found that a single point mutation in *F5* (G → A substitution) predicted the synthesis of the FVL, a variant of FV that was less susceptible to inhibition by the protein C system. Later, the prevalence of the FVL variant has been found to vary considerably in different ethnicities, from < 1% in populations of Asian origin to up to 7% in Caucasians.[33,94] Heterozygous carriers of the FVL variant have a 2 to 5-fold increased risk of venous thrombosis.[82] The prevalence of the prothrombin G20210A mutation is 1.7-3% in the European population.[95] In 1969, Jick *et al* reported that VTE was less frequent in patients with blood type OO compared to other ABO blood types,[96] an observation that later has been associated with lower levels of von Willebrand factor (vWF) and FVIII in those with blood type OO.[97,98] A recent large nationwide register based study on healthy blood donors in Sweden and Denmark found that the population attributable risk of non-O blood groups was more than 30% for VTE events.[99] Although the relative risk of VTE in subjects with non-O blood versus OO-blood type is fairly low, from 1.5 to 2.0-fold increased, the impact of ABO-blood type in the general population is extensive due to the high prevalence of non-O blood groups (approximately 60%).[84,99-101]

In a large case-control study, de Haan *et al* derived a parsimonious 5-SNP genetic risk score that could predict incident VTE with similar accuracy as a model of 31 SNPs associated with VTE.[102] Regardless of SNP composition, an increasing number of risk alleles showed a dose-response association with VTE risk. Compared to subjects with two risk alleles (reference), the odds ratios (OR) varied from 0.4 in subjects with zero risk alleles to an OR of 7.5 in those with ≥ 6 risk alleles. The same prothrombotic genotypes included in the 5-SNP risk score have later proved useful in predicting recurrent VTE in the MEGA study,[103] and were also associated with an increased risk of VTE in patients with stroke.[104] The five SNPs provided added predictive value when combined with acquired environmental VTE risk factors for both first and recurrent VTE.[102,103] For incident VTE, the area under the curve (AUC) improved from 0.71 to 0.77 with the addition of the 5-SNP model to non-genetic risk factors in the validation cohort.[102]

The 5-SNP model consisted of rs6025 (FVL) in *F5*, rs1799963 (prothrombin G20210A) in *F2*, rs8176719 (non-O blood type) in *ABO*, and two other genetic variants, one (rs2066865) in the fibrinogen gamma chain gene *(FGG),* and one (rs2036914) in the FXI gene *(F11).* The rs2066865 variant in *FGG* is present in 25-35% of Europeans and is associated with a 1.5 to 2-fold increased risk of DVT. The increased venous thrombosis risk is considered to be mediated by reduced plasma levels of fibrinogen gamma prime (γ').[84,102,105]

Fibrinogen γ' holds a unique binding site that appears critical for the expression of AT activity that develops during fibrin formation.[105] The rs2036914 SNP is one of the genetic variants in the *F11* locus that is independently associated with DVT risk.[106] The SNP is thought to modulate plasma levels of FXI and is associated with a 1.4-fold increased risk of VTE. However, the variant(s) responsible for the association is not entirely clear, as *F11* haplotypes are more strongly associated with VTE risk than individual SNPs.[107] The rs2036914 variant is very common, with a reported prevalence of 50-60% in European cohorts.[106]

1.1.4 Acquired risk factors

There are several acquired risk factors for VTE. Advanced age, obesity, cancer, hospitalization and surgery are among the high-impact risk factors that can readily be associated with blood hypercoagulability, reduced blood flow or changes in the vessel wall and placed in the conceptual framework of being a transient and/or persistent provoking factor.[41]

High and advancing age is associated with a persistent and increasing risk of VTE, being regarded as one of the strongest risk factors for VTE.[29,39,108,109] Although the basis for this association is uncertain, wear-and-tear on the vessels and their valves, higher levels of procoagulant factors in blood and accumulation of immobilizing conditions are proposed mechanisms that can explain the exponentially increased risk of VTE from the age of 45-55.[108,109] Age may be considered a risk factor in itself based on the observation that the same prothrombotic risk factors may lead to a VTE in an adult but not in a child.[109]

Obesity, defined as a body mass index (BMI) above 30 kg/m^2, is a modifiable risk factor for VTE associated with a 2 to 3-fold increased risk of VTE compared to persons with a normal BMI.[110] An increasing BMI is associated with an increased risk of VTE in a dose-response manner.[111] Other anthropometric measures of obesity, such as waist circumference, have been shown to detect patients at high risk of VTE more precisely than BMI.[112] Moreover, weight gain in itself is associated with increased risk of VTE, particularly in already obese individuals, having around 4-fold higher risk of VTE compared to obese subjects with no weight gain.[113] The underlying mechanisms for the association between obesity and VTE are not fully understood. However, venous stasis, chronic inflammation, increased coagulation activity and decreased fibrinolysis are proposed mechanisms.[111] Results from three recent Mendelian randomization studies imply that obesity is causally related to VTE.[114-116] Obesity has been found to account for around 30% of the population attributable

risk of unprovoked VTEs[28] and is arguably the most important preventable risk factor for VTE, considering the growing epidemic of obesity worldwide.[32]

Cancer is a major risk factor for VTE, associated with a 4 to 7-fold increased risk of VTE across all cancers, however with considerable variability according to type of cancer and treatment.[117,118] About 20% of VTE cases could be attributed to malignancy.[119] A differential risk of death in various cancers is however likely to impact the estimated rates of VTE in cancer patients unless corrected for.[120] The risk of VTE appears to be particularly high in the first few months after cancer diagnosis and in the presence of distant metastases. Proposed explanations for this association include use of therapeutic interventions such as surgery, chemotherapy, erythropoietin-stimulating agents and hospitalization.[121] Nevertheless, cancer seems to be a risk factor for VTE independent of these explanations as shown in the Scandinavian thrombosis and cancer study.[122] Here, the risk was shown to be similar six months prior to and after a cancer diagnosis when taking competing risk by death into account, thus presumably excluding the influence of risk factors related to cancer diagnosis and treatment.[122] Tumor cells can activate blood coagulation through multiple mechanisms, including expression of cancer procoagulant factors, proinflammatory cytokines, dysregulation of the fibrinolytic system, and by direct interaction with platelets and endothelial cells.[117] Moreover, common and cancer-type specific pathways appear to contribute to CAT.[123] For instance, lung cancer is associated with neutrophilia, which may enhance thrombosis by generating neutrophil extracellular traps (NETs), while TF-positive microvesicles appear to promote thrombosis predominantly in pancreatic cancer.[123] Among the RAMs developed for VTE risk in cancer patients, the Khorana score is arguably the most widely used.[124] The score has been expanded with the biomarkers D-dimer and soluble p-selectin in the Vienna score, which appeared to improve the prediction of VTE in cancer patients.[125]

Hospitalization represents an entity of VTE risk factors that can be associated with more than 50% of all VTE cases.[57] Infections, immobility, surgery and fractures are common among hospitalized patients who develop a VTE.[126] The population attributable risk is around 20% for surgery alone.[28] Compared with the occurrence of a community-acquired VTE, the rate of first or second hospital-related VTE was 35 times higher, i.e. 282 per 10000 person-years versus 8.1 per 10000 person-years, in a large retrospective study.[127] The length and number of hospitalizations and associated degree of immobilization affect the VTE risk considerably.[128] Given the high incidence of VTE in hospitalized patients, the identification of

patients in need of VTE prophylaxis should be emphasized. In order to improve identification of hospitalized patients at high risk of VTE, the Padua score has been proposed.[129] In this RAM, 11 VTE predictors are included, and those with a cumulative score of ≥ 4 (40% in the derivation study) are defined as being at high risk of VTE and in need of prophylaxis. Prophylaxis with low molecular weight heparin (LMWH) for two weeks has been found to significantly reduce the risk of VTE by 50% to 70% compared to placebo in RCTs.[130-132] Therefore, VTE prophylaxis with mainly LMWH or low dose unfractionated heparin (UFH) is recommended for hospitalized patients at high risk of VTE without risk of bleeding.[133] Severe and clinically relevant bleeding occurs in up to 3% of medically ill patients within 14 days after admission, and the IMPROVE RAM has been proposed to stratify bleeding risk in hospitalized medical patients.[134] The model identified about 10% of the population with a cumulative score ≥ 7, in which MB was more than 10 times more frequent compared to patients below this cut-off in the validation cohort. A gastroduodenal ulcer, bleeding 3 months before admission, platelet count < 50 x 10^9 cells/L and age ≥ 85 were the most strongly associated factors with bleeding among the 11 predictors in the IMPROVE model. The authors conclude that the model will allow physicians to identify patients in which pharmacologic thromboprophylaxis is safe. However, the results of a recent large validation study show that the model has low predictive power (AUC 0.63) and an IMPROVE score of ≥ 7 was only associated with approximately 2-fold increased risk of MB.[135]

The incidence of VTE is 2 to 5 times higher in pregnant women compared to non-pregnant women of similar age, and up to 20 times higher in the postpartum period.[136] The increased risk is explained by mechanical changes caused by the expanding uterus (i.e. increased venous stasis due to increased intra-abdominal pressure and compression of the vena cava) and hemostatic changes leading to blood hypercoagulability.[137] During **pregnancy** there is a marked increase in procoagulant activity characterized by elevation of coagulation factors (e.g. factors VII, X, VIII) and a decrease in physiological anticoagulants manifested by a significant reduction in protein S, leading to APC resistance.[138] Moreover, fibrinogen, tissue plasminogen activator, plasminogen activator inhibitor (PAI) 1 and PAI-2 levels are altered during pregnancy and at birth, with a net reduction in plasma fibrinolytic activity.[139,140] Shifting the hemostatic balance in favor of a hypercoagulable state during pregnancy has been thought to be important to minimize intrapartum blood loss.[140]

Use of **exogenous hormone supplements**, such as combined oral contraceptives (COCs) and hormonal replacement therapy, is very common among women worldwide and is

associated with a 2 to 4-fold increased risk of VTE compared to non-users.[141] The risk of VTE in COC users varies according to type of progestogen and dose of estrogen derivate, and the highest risk has been identified in the so-called third generation contraceptives.[141] In women using COCs, smoking, a high BMI and genetic risk factors, such as non-O blood group and FVL, have been identified as additional risk factors for VTE.[142] Women with FVL who use COCs have a 35-fold increased risk of VTE compared to non-users of COCs without FVL.[143] As the risk in women with only FVL is 2 to 7-fold increased, the combination of FVL and COC use is an example of gene-environmental interaction on the risk of VTE.[141]

Usually, more than one or a combination of inherited and acquired environmental risk factors are required in order to overcome the threshold leading to a VTE.[109] Rosendaal described a model that allows inherited and acquired thrombotic risk factors to interact in a dynamic manner over the course of a life-time, known as the thrombosis potential model.[109] The model emphasizes the strong age-dependency of VTE, as the same set of risk factors may lead to thrombosis in the elderly but not in children. Moreover, the model highlights how transient exposures with immediate and short-term effects on the risk of acute VTE can be triggers for a VTE event. Figure 3 shows the example of the thrombosis potential in a person with a fixed heritable risk factor (e.g. FVL) who is exposed to a transient provoking factor (e.g. hospitalization) in two occasions. The immediate effect of the transient risk factor is increased equivalently during the two occasions, however thrombosis only occurs when the thrombosis threshold is breached (Figure 3).

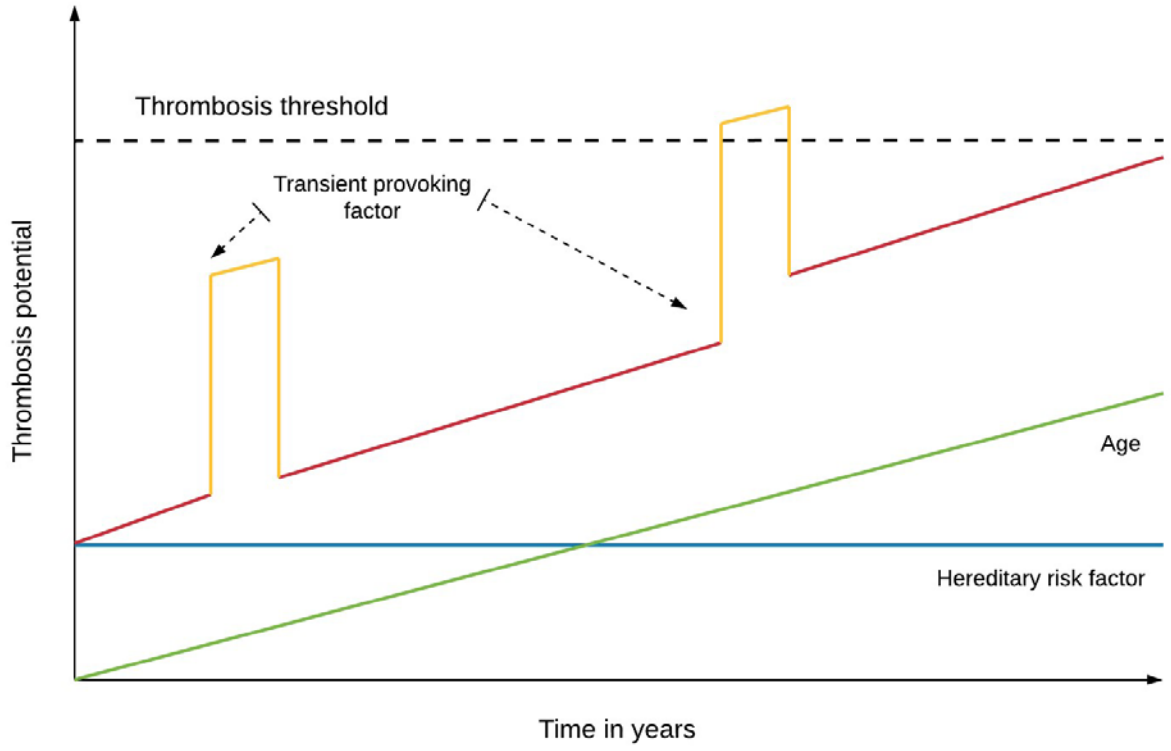

Figure 3 A thrombosis potential model showing the interactions between genetic (e.g. factor V Leiden) and environmental risk factors (e.g. hospitalization) for venous thrombosis over a life-span. The blue and green lines represent the thrombosis potential of a hereditary risk factor and age, respectively, and the red line indicates the combined thrombosis potential. The yellow lines represent the accumulated thrombosis potential taking provoking environmental risk factors into account. Note how the same provoking event leads to venous thrombosis only when the accumulated thrombosis potential surpasses the thrombosis threshold. Adapted from Rosendaal, Lancet 1999[109]

1.2 Anticoagulant treatment for venous thromboembolism and mechanisms of action

Advances in the management and pharmacologic treatment of VTE are reflected in evolving guidelines and recommendations published in the last decades.[5,144-147] Anticoagulation with vitamin K antagonists (VKA) and LMWH or UFH has until recent years been the mainstay of VTE treatment since the RCT of Barritt and Jordan in 1960.[4] Beneficial effects of heparin in VTE patients were documented years earlier in a few cases of PE in the pioneering work of Murray and Best.[148] However, Barritt and Jordan provided the first robust evidence of a dramatic and efficient risk-reduction of recurrent VTE and recurrence-related death that outweighed the risk of bleeding in the acute treatment of VTE. Initially, 35 cases with incident PE were randomized to either 14 days of treatment with intravenous heparin and VKA anticoagulation (n=19), versus no anticoagulation (n=16). At this stage, allocation of

patients to the no treatment arm was prematurely stopped as five cases had a fatal recurrent PE and another five had non-fatal recurrences, versus zero recurrent events in the treatment arm.[4] Although the primary study only included 35 cases, anticoagulant treatment was associated with a 90% risk reduction of recurrent VTE and all-cause mortality after two weeks of treatment, compared to no anticoagulation.

The direct thrombin inhibitor Dabigatran etexilate was the first among the direct oral anticoagulants (DOACs) to be approved by the U.S food and drug administration (FDA) in 2014 for the treatment of VTE in the United States (earlier for thromboprophylaxis in orthopedic surgery). Soon after, the direct factor Xa inhibtors, i.e. Rivaroxaban, Apixaban and Edoxaban, were approved for use in VTE patients and endorsed in the 2016 American college of chest physicians' (ACCP) guideline.[5] At least six RCTs have compared DOACs with LMWH and Warfarin in VTE patients, all showing non-inferiority with regards to efficacy in preventing recurrent VTE and VTE-related death, while some,[149,150] but not all,[151-154] show a statistically significant lower risk of MB in patients using DOACs compared to the standard treatment. In subsequent non-interventional studies on VTE patients, the DOAC-associated lower risk of bleeding has largely been reproduced, although some results indicate DOAC-specific variation with respect to effect sizes of risk reduction, both for overall MB and according to type of MB.[1,2,155] Still, the annualized rates of MB in these studies are 3-5% and underscore that MB remains a considerable clinical problem in VTE patients treated with DOACs.[1,2]

There is sound evidence supporting that an acute VTE should be treated with anticoagulants for a minimum of three months.[156,157] The 2016 ACCP-guidelines recommended three months of anticoagulation for provoked DVTs and PEs, and all isolated distal DVTs. However, for unprovoked VTE, either proximal DVT or PE, the ideal treatment duration is uncertain and dependent on the estimated bleeding risk.[5] Patients with moderate to low risk of bleeding are recommended extended treatment (no scheduled stop date), while patients estimated to have high risk of bleeding should stop at three months. RCTs show that extended treatment durations in unprovoked VTE are associated with substantial risk reduction of recurrence but also an increase in bleeding risk.[158-160] In a blinded RCT allocating unprovoked VTE patients that had completed three months of anticoagulation to either placebo or 2 years with VKA, authors reported a 95% risk-reduction of recurrent VTE (efficacy) in patients continuing anticoagulation.[158] However, 3.8% had an MB in the treatment arm versus zero in the placebo arm. In a similar RCT comparing 3 versus 6 months

of treatment duration, extended treatment (i.e. 6 months) was also associated with more MB events (3.5% versus zero).[160] There is also compelling evidence showing that the benefit of extended treatment in unprovoked VTE is lost when the anticoagulation is discontinued,[159] suggesting that indefinite treatment should be considered, given that the MB risk is low.

Low-intensity therapy with a target INR of 1.5-2.0 has been compared with conventional intensity (i.e. 2.0-3.0) as another strategy to optimize the net benefit of extended anticoagulant treatment.[161,162] However, lowering the intensity of warfarin treatment has largely resulted in decreased efficacy without less bleeding.[161] In contrast, low-dose Apixaban (2.5 mg twice daily) has been shown to be associated with similar safety and efficacy in preventing recurrence and recurrence-related deaths as the conventional dose (5 mg twice daily).[163] However, only seven MB events occurred during follow up in this RCT. As estimates of the risk of MB during extended oral anticoagulation are limited, balancing the risks of bleeding and recurrence with extended anticoagulation remains difficult.

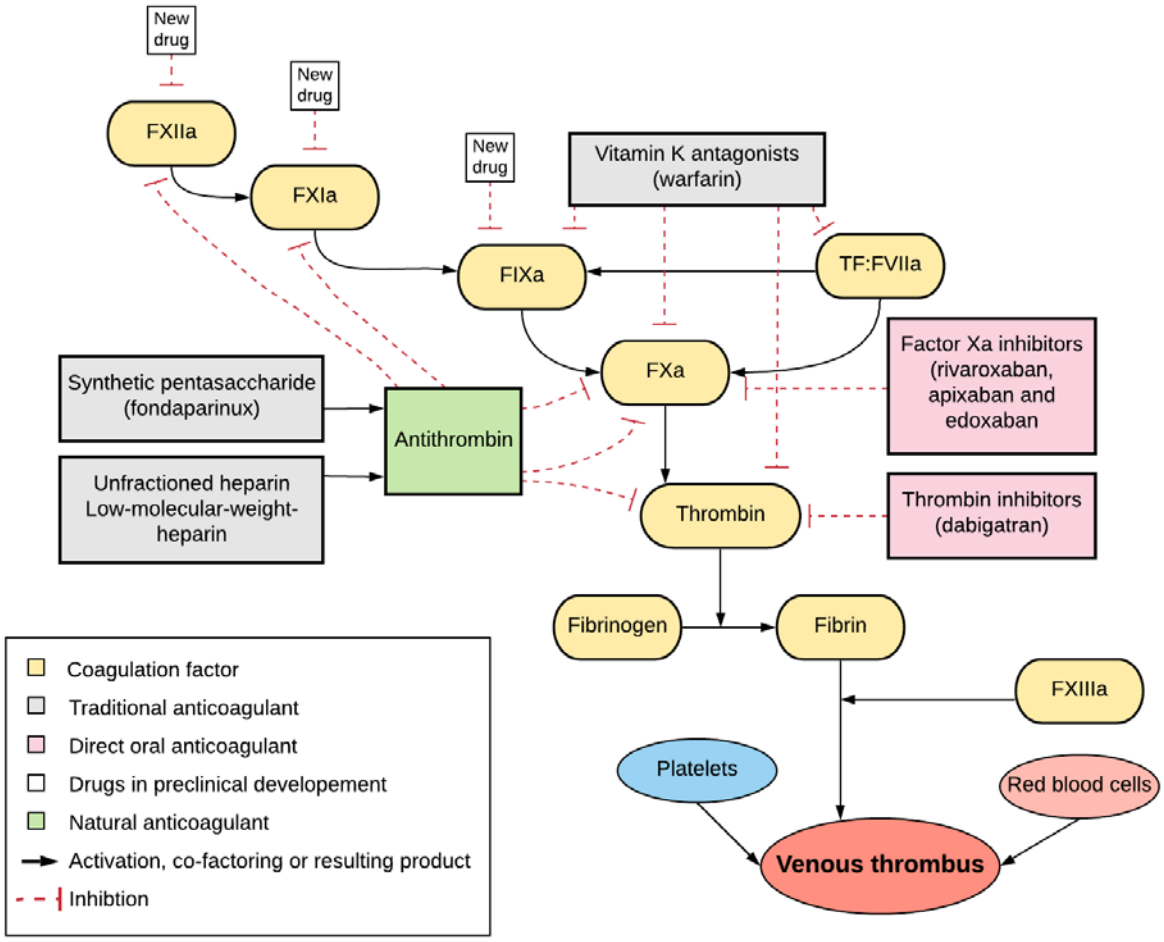

Figure 4 Overview of existing and emerging anticoagulant drugs and their targets in the coagulation cascade. Adapted from Wolberg et al. Nature 2015, Maclean and Tait, Drugs 2007[70,164]

Anticoagulant drugs exert their mechanisms of action mainly through indirect or direct inhibition of the coagulation factors in the coagulation cascade (Figure 4). This way, anticoagulants do not aid in dissolving a thrombus, but rather impair the ability of forming fibrin, thus limiting thrombus growth. There are four vitamin K dependent coagulation factors - FX, FIX, FVII and prothrombin. When synthesized in the liver, these factors require the enzyme Vitamin K epoxide reductase (VKOR) to catalyze the formation of reduced vitamin K in order to achieve a biologically active state, providing subsequent gamma-carboxylation of the specific coagulation factors.[165,166] VKAs, such as Warfarin, are inhibitors of VKOR. Thus, a VKA is technically not an antagonist to vitamin K, but an inhibitor of the enzyme that catalyzes its biologically active form. The heparins (LMWH and UFH) and synthetic heparin-like drugs, such as Fondaparinux inhibit FXa and thrombin by accelerating the anticoagulant effect of AT.[166] Heparins, being glycosaminoglycans, have varying molecular size and

saccharide chain length, which affect their anticoagulant capability.[167] Heparin molecules with fewer than 18 saccharides (e.g. Fondaparinux) possess mainly anti-FXa inhibition while larger molecules (e.g. UFH) effectively inhibit thrombin.[167] The DOACs, on the other hand, provide specific anticoagulant effect by inhibiting thrombin (Dabigatran) and FXa (Rivaroxaban, Apixaban and Edoxaban) in activated and bound forms. The pharmaco-dynamics and -kinetics of anticoagulants may therefore vary widely, and onset of anticoagulation, renal clearance, interactions and half-lives are among the important clinical issues that warrant consideration in the anticoagulant treatment of VTE patients.

1.2 Bleeding complications

VKAs and heparins were first discovered due to their anticoagulant effects leading to excessive bleedings.[168] The first direct thrombin inhibitor, Hirudin, was discovered in the medicinal leech *Hirudo medicinalis* due to its "lack of blood coagulation".[168] To date, bleeding complications have been inseparable from all known anticoagulants in the treatment of VTE. In the landmark study of Barritt and Jordan, the only death recorded in the anticoagulant-arm was due to a combination of suppurative pneumonia and gastrointestinal bleeding, and three patients had to be administered vitamin K in the treatment-arm.[4] Factors regarded to indicate risk of bleeding complications have changed over time, along with anticoagulant type, intensity and duration of anticoagulation recommended for VTE patients. For instance, while age > 60-75 years is among the most consistent predictors of MB risk in current RAMs,[12,13] age was unrelated to bleeding risk in a 7-year follow-up study of outpatients treated with VKAs conducted in 1979.[169] Moreover, early studies have included heterogeneous cases of bleeding with regards to bleeding severity and location, as definitions of "major, significant or serious" bleedings have varied widely due to lack of classification systems.[169-172] Previous studies have also typically included patients with a range of indications for anticoagulation (e.g. ischemic and valvular heart disease), with distinct clinical characteristics and co-morbidities, likely to affect the risk of bleeding.[169,171]

In the treatment of acute VTE, MB typically occurs during the first months after anticoagulant initiation.[6,7] In a clinical trial of VTE patients nearly half of all MBs occurred within 7 days, and 75% within 21 days after initiating anticoagulation.[173] Possible explanations for the clustering phenomenon of MB events at the start of anticoagulation include concomitant administration of LMWH and VKA until the INR is in therapeutic range. There is also a wide intra- and interindividual variability in the dose-requirements for a therapeutic effect in patients treated with VKAs that may increase the risk of over-

anticoagulation when anticoagulants are initiated for the first time.[174,175] Furthermore, patients with a bleeding predisposition are more likely to experience an MB early after initiation of anticoagulation. It is plausible that patients prone to bleeding will discontinue anticoagulation during the early phase of treatment, and those continuing might be perceived to tolerate the treatment better.[5,176]

1.2.1 Major bleeding

Bleedings may range from insignificant to fatal, and their clinical importance depends partly on the location and volume of the bleed. The International Society on Thrombosis and Haemostasis (ISTH) recommends specific criteria to define bleeding severities in non-surgical patients in order to enable valid comparisons between studies.[177,178] Bleedings that are fatal and/or symptomatic in a critical area/organ (e.g. intracranial, retroperitoneal and intramuscular with compartment syndrome) and/or caused fall in hemoglobin level of 20 g/L or more, or leading to transfusion of two or more units of whole blood or red blood cells are defined as major. Less severe relevant bleedings have been termed clinically relevant non-major bleeding (CRNMB), which in short are all symptomatic bleedings (e.g. more than expected for a clinical circumstance) that do not fit within the criteria for MB.[178]

Overall, MB occurs in 0.5-13% of VTE patients per year during anticoagulant treatment.[6,7,179,180] There are several potential reasons for the wide range of reported MB incidence, but striking differences are seen according to study design. In the Worcester VTE study, a community based study following 1567 VTE cases from 1999 to 2003, the cumulative incidence of MB was 12-13% each year, and 8% one month after VTE diagnosis.[7] In more recent observational studies of VTE patients treated with DOACs or VKAs, the annual rate of MB was 3-6%.[1-3] In contrast, interventional studies reported lower bleeding rates. In the AMPLIFY study, an RCT comparing the efficacy and safety of Apixaban versus conventional VKA and LMWH for the treatment of VTE, the 6 month cumulative incidence of MB was 0.6% and 1.8% in the Apixaban- and VKA-arm, respectively.[150] In a large meta-analysis from 2003 which included mostly RCTs, the annual incidence of MB was close to 7 per 100 patient-years.[6] A potential reason for the discrepancy might be that RCTs of VTE patients investigating the safety of anticoagulants tend to exclude patients perceived to be at risk of bleeding. Other potential explanations for incidence differences include MB definitions and anticoagulation type and duration. The case-fatality of MB is estimated to be 11-20% in a large register-based study and meta-analysis.[181,182] Although the rate and severity

of MB complications vary across studies, the overall associated cost and clinical impact in VTE patients are substantial.[6,183]

Patient-perceived risk of bleeding and physician's clinical uncertainty regarding the benefit versus risk of bleeding with anticoagulants are identified as leading causes for the underuse of anticoagulants in patients with AF.[184,185] It is therefore likely that uncertainties regarding MB risk contribute to underuse of anticoagulants in VTE patients due to fear of bleeding complications, ultimately limiting the benefit of anticoagulants. A recent Dutch study found that among VTE patients prescribed with a DOAC after a VTE, 20% had completely stopped taking DOACs after two months.[186] A Cochrane systematic review found that less than 20% of hospitalized patients, medical and surgical patients, received the appropriate VTE prophylaxis despite well-documented benefit of VTE risk reduction and lowered costs with VTE prophylaxis.[130-132,187] Accurate risk assessment of MB may therefore not only lead to a reduction in MB events, but also improve the adherence to the appropriate anticoagulant duration after an incident VTE. For unprovoked VTE specifically, MB risk assessment is a prerequisite to strike the ideal treatment duration and limit bleeding and recurrent VTE.[188,189]

1.2.2 Major bleeding risk assessment

The major determinants of MB risk currently include the intensity and length of anticoagulant effect, clinical patient characteristics and co-morbidities and concomitant use of drugs that interfere with hemostasis (Table 1).[5,176,190]

Table 1 Factors associated with major bleeding risk in venous thromboembolism patients.

Clinical patient characteristics	**Co-morbidities**	**Anticoagulant-specific factors**	**Concomitant medication**
Age[8,191,192]	Cancer[8,43,192]	Intensity/control[193,194]	Antiplatelet[195]
History of bleeding[191,196,197]	Metastatic cancer[196]	*Duration[158,198]	NSAIDS[195]
Previous stroke[191,199]	Reduced renal function/failure[196]		
Anemia[200-202]	Liver failure[134,203]		
Thrombocytopenia[204,205]	Diabetes[191,206]		
Alcohol abuse[196,207]	Hypertension[208,209]		

*Extended duration of anticoagulant treatment has been shown to be effective in preventing recurrent VTE but associated with an increased risk of MB.

Antiplatelet drugs, such as acetylsalicylic acid and clopidogrel, impair the primary hemostatic function of platelets by inhibiting the process of platelet activation.[210] Acetylsalicylic acid/Aspirin binds dose-dependently to cyclooxygenase enzymes (COX), leading to antiplatelet effects, especially via inhibition of thromboxane A_2, a prostaglandin with an important role in platelet activation and aggregation.[210] Clopidogrel binds irreversibly to the $P2Y_{12}$ receptor on platelets, thereby impairing the effect of ADP on platelet aggregation, which normally binds to the $P2Y_{12}$ receptor.[210] Other non-steroidal anti-inflammatory drugs (NSAIDS), such as Ibuprofen, are reversible and less selective inhibitors of COX-1. In a large RCT comparing Rivaroxaban with traditional anticoagulant treatment, 15% used acetylsalicylic acid and 23% used NSAIDS during follow-up.[195] The use of aspirin was associated with a 1.7-fold increased risk of MB compared to non-users, while NSAIDS use was associated with a 2.4-fold increased risk of MB.[195] Other medications (e.g. antibiotics) and foods may interact with anticoagulants, especially VKAs, either potentiating or inhibiting the anticoagulant effect.[211]

Although the liver synthesizes both pro- and anticoagulant factors, **chronic liver disease** is mostly characterized by clinical bleeding.[212] The bleeding tendency in patients with liver failure correlates poorly with routine laboratory tests of coagulation (INR and activated partial thromboplastin time [APTT]).[212] Hospitalized patients with chronic liver disease are not protected against VTE,[213] suggesting that the balance in hemostasis is not simply on a one-dimensional scale in this context. In patients with chronic liver failure (INR $\geq$ 1.5), the

use of anticoagulation for VTE prophylaxis was associated with 3.6-fold higher risk of major and minor hemorrhage in a retrospective register-based study.[203] Although liver function is a part of bleeding risk assessment,[214] to what extent liver failure/function affects the risk of MB in patients with incident VTE has not been thoroughly documented.

Alcohol consumption has been associated with altered levels of coagulation factors (e.g. FVII and fibrinogen), fibrinolytic factors (e.g. PAI-1) and platelet count in a crossover trial.[215] Some epidemiological studies have pointed towards an association between alcohol consumption and risk of bleeding in VTE patients. For instance, a diagnosis of alcohol abuse was associated with more than 3-fold higher risk of MB in a register-based study of VTE patients.[208] In a large cohort study in the United States, subjects with an alcohol-related hospitalization in the 18 months preceding a DVT diagnosis had a 2.6-fold higher risk of being re-admitted for bleeding compared to those without an alcohol-related hospital admission.[196]

A history of bleeding is one of the most consistent independent risk factors for MB risk in VTE patients and is associated with 2.5 to 3.0-fold increased risk of MB compared to those without a previous bleed.[191,196,200] Moreover, the association between a minor bleed and a subsequent MB was independent of the quality of anticoagulation in nearly 6000 VTE patients originating from a Dutch anticoagulation clinic.[197] In a large study of patients on VKAs with more than 900 MBs, Van Rein *et al* used a case-crossover design to disentangle the nature of the association between a previous minor bleed and MB.[216] Findings from the case-crossover study suggested that minor bleeds could be markers of fixed risk factors for MB, and the authors speculated that genetic coagulation-related factors could be candidate risk factors to explain the association.

The net (anti- or prothrombotic) effect of certain risk factors on hemostasis is not always easily predicted. This is the case in **cancer**, which is associated with higher risk of not only first and recurrent VTE but also MB.[40,43] The risk of both recurrence and MB was shown to correlate with cancer severity and could not be explained by sub-therapeutic or over-anticoagulation.[43] Observational studies have further revealed that genitourinary cancers are among the cancer sites that are most strongly associated with MB risk.[43,217] The annualized incidence of MB was 15.7 per 100 patient-years in 181 patients with concomitant cancer and VTE diagnosis in an Italian cohort.[43] During anticoagulant treatment for VTE, the relative risk of MB in patients with active cancer is about 2 to 3-fold higher compared to those without cancer.[8,192,196] LMWH has been the anticoagulant of choice for the anticoagulant treatment of

CAT.[5,218] However, results from at least four recent RCTs comparing DOACs with LMWH indicated that DOACs are non-inferior compared to LMWH with regards to efficacy, but possibly inferior with regards safety, i.e. MB risk.[219-222] Collectively, the increased bleeding risk of DOACs appeared in part to be driven by genitourinary and gastrointestinal bleeding, especially in patients with cancer in these organs. A meta-analysis has also shown that anticoagulant treatment of CAT with DOACs was associated with a higher risk of MB and a trend for a higher risk of CRNMB compared to LMWH.[223] DOACs may therefore be preferable for the treatment of CAT in patients with low bleeding risk and cancers not originating from gastrointestinal or genitourinary sites.

Age above 60-75 years has consistently been associated with a 1.5 to 3.0-fold increased risk of MB in VTE patients.[8,191,192] There are many potential factors associated with advanced age that are also related to MB risk, which may contribute to the association between high age and risk of bleeding in VTE patients. These include, but are not limited to, **polypharmacy**, **risk of falls** and a **low level of physical activity**.[224-226] Moreover, the elderly may accumulate co-morbidities and acquire reduced organ functions (e.g. renal function) that might influence the risk of over-anticoagulation. The risk of dying from MB during VTE treatment is increased in the elderly above 60 years.[227]

Chronic kidney disease and reduced renal function have been associated with around 2-fold increased risk of MB in VTE patients,[196,200] while other studies fail to find an independent association in multivariable analyses.[192] Reduced clearance of anticoagulant drugs could lead to increased risk of over-anticoagulation and subsequent MB risk. Moreover, the effect of impaired renal function or chronic kidney disease on the hemostatic system is complex and associated with pro- and antithrombotic pathophysiologic mechanisms.[228] Uremic toxins are thought to reduce platelet activation, recruitment, adhesion and aggregation, which may ultimately lead to the platelet dysfunction seen in these patients. On the other hand, plasma procoagulation activities of FXII, FXI, FVIII, FVII and thrombin are significantly enhanced, while the natural anticoagulant system is decreased in parallel.[228]

A **history of stroke** has been associated with bleeding risk in previous studies of patients using VKAs for different reasons,[191,199] including AF in the HAS-BLED model.[214] However, a history of stroke is not included in most RAMs derived exclusively from VTE patients, and a recent cohort study with over 13,000 VTE cases could not find any association between a previous diagnosis of stroke and MB during anticoagulation.[208] In contrast, a diagnosis of **hypertension** was independently associated with a 1.3-fold increased risk of MB

in the same study. Long-standing hypertension and periods of very high blood pressure can be linked to the occurrence of intra-cerebral hemorrhage via small and large vessel pathology.[229,230] High blood pressure has been included as a predictor (either uncontrolled hypertension in men or systolic blood pressure > 160 mmHg) of MB in recent RAMs derived from VTE patients.[209,231]

Data from RCTs and observational studies indicate that MB occurs more frequently in **women** compared to men during anticoagulation for VTE, regardless of anticoagulation type.[150,152,154,232,233] Notably, while men are at higher risk of recurrence, it seems that women are at higher risk of MB in crude analysis.[232] This association appears not to be related to sex *per se*, but other possibly sex-related factors, such as time in therapeutic range (TTR), body weight and co-morbidities.[232,233]

In a recent study of PE patients who completed at least 3 months of anticoagulation, **diabetes mellitus** was associated with MB, with a 2-fold increased risk of MB in those with diabetes compared to those without diabetes.[206] Previous studies have mainly shown diabetes to be associated with MB in univariate analysis but not in the presence of other risk factors.[191,194] In the derivation cohort of the outpatient bleeding risk index - OBRI, diabetes was initially a risk factor, associated with a 2.2-fold increased risk of MB. However, in multivariable analyses, the association did not meet the criteria for significance, and diabetes was included as a composite co-morbidity item in the final model.[191]

Clinical risk factors and markers have been combined in various constellations in several RAMs to predict MB risk in VTE.[191,199,200,234,235] The predictive capabilities have been promising in derivation stages, but have generally dropped drastically in external validation studies.[10-12] Although the AUC alone is not sufficient for the full evaluation of a RAM, the AUCs ranged from 0.49 to 0.63 in these validations studies, making them unsuitable for clinical use.

In summary, while MB risk assessment is pivotal to minimize risk of bleeding events and guide decisions on anticoagulant treatment duration, currently known predictors of MB risk have insufficient predictive capabilities when combined in RAMs for VTE. Identification of novel predictors of MB could improve the accuracy of MB risk assessment in VTE patients and ultimately the net benefit of anticoagulants in VTE patients.

1.2.3 Biomarkers of major bleeding risk

According to the Food and Drug Administration (FDA), a biomarker is "*a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions.*"[17]

Genetic factors have the potential of being useful biomarkers, as they are fixed within an individual, and not subjected to change after a VTE or VTE treatment. As an example, polymorphisms in the *CYP2C9* gene influence the achieved anticoagulant effect of warfarin and is associated with a 3 to 4-fold higher rate of MB compared to random controls.[236] Notably, when including genetic information related to polymorphisms in *VKORC1* and *CYP2C9* genes with age and height, the explained variability of the anticoagulant effect was considerably improved.[174] As discussed previously in section 1.3.3, the association between non-O blood groups and VTE risk has been explained by higher levels of vWF and FVIII in non-O blood groups carriers, compared to subjects with blood group OO.[98] Notably, several studies have reported that subjects with blood group OO bleed more frequently than other ABO blood groups in various study populations and settings.[237-240] These include patients with type 1 von Willebrand disease, patients with gastroduodenal ulcers, post-operative tonsillectomy bleeding and in patients hospitalized for epistaxis.[237-240] A systematic review and meta-analysis of 22 studies of various patient-groups treated with anticoagulants found that the prevalence of OO blood group was significantly higher in bleeding patients than in controls, with a pooled OR of 1.33.[241] A few studies have included VTE patients and investigated the risk of bleeding during anticoagulation according to ABO blood type.[242,243] The results have been somewhat conflicting in these studies, and there is a lack of data on the association between ABO blood groups and risk of MB in VTE patients.[244]

Carriers of FVL have recently been associated with a lower risk of MB compared to non-carriers in a registered-based study of thrombophilia-tested patients (RIETE).[245] Although the association was investigated in a VTE subgroup population and remains to be validated, the results substantiates a notion that genetic factors associated with increased VTE risk might be inversely associated with MB risk. FVL and non-O blood type were both included in the 5-SNP genetic risk score that have been associated with hypercoagulability and VTE risk, as previously mentioned.[102] When combined with the other three SNPs in the 5-SNP score, there was a dose response effect between increasing number of prothrombotic risk alleles and thrombosis risk. Although not fully elucidated for all 5 SNPs, the proposed

mechanisms for the association between each SNP and thrombosis risk likely reflect disturbances in different pathways of coagulation. To the best of our knowledge, no previous study has assessed the impact of multiple prothrombotic genotypes on the risk of MB in VTE patients. We hypothesized that an increasing number of prothrombotic risk alleles were protective of MB risk during the first year after an incident VTE.

A few **plasma biomarkers** related to the hemostatic system have previously been associated with risk of bleeding during anticoagulant treatment.[246,247] Case-control and cohort studies have reported that high levels of plasma soluble thrombomodulin are associated with a 2 to 4-fold increased risk of MB compared to low levels of thrombomodulin.[246,248] In a Dutch case-control study, patients treated with VKAs with thrombomodulin above ≥ 3.80 ng/mL (upper quartile) had a 3.3-fold increased risk of MB compared to thrombomodulin in the lower quartile.[246] Authors speculated that higher levels of thrombomodulin would indicate endothelial damage, or alternatively, would reflect the natural anticoagulant properties of the protein C system.[246] Elevated levels of plasma vWF have been associated with a 2.5 to 4.5-fold increased risk of MB in patients with mixed indications for anticoagulation in cohort studies.[249,250] vWF is considered an acute phase response protein involved in several inflammatory processes (e.g. recruitment of leukocytes) in addition to its essential role in hemostasis.[251] In a cohort of patients with AF, vWF was a prognostic biomarker of MB that improved the predictive value of RAMs for both stroke in the CHA_2DS_2-VASc score and MB in the HAS-BLED model during anticoagulation.[247] Based on these studies, plasma biomarkers of hemostatic system seem promising for the prediction of MB in VTE patients. However, blood samples were drawn at least 2-6 months after starting anticoagulation in the abovementioned studies, not allowing the assessment of the initial high risk period of MB in VTE.

Deficiencies of plasma PAI-1 have been shown to be associated with life-long increased risk of bleeding due to hyperfibrinolysis in case-reports and family pedigrees.[252,253] Inversely, subjects with a reduced plasma fibrinolytic potential, assessed by plasma-based assays of clot lysis time, have been associated with increased risk of incident DVT.[254] D-dimer is a degradation product of cross linked fibrin and reflects activation of coagulation and fibrinolysis.[255] Wells showed that a negative D-dimer test safely ruled out DVT in patients with a low clinical pre-test probability of DVT, which allowed to avoid unnecessary time- and cost consuming ultrasonography and hospital admissions.[14] Moreover, elevated levels one month after discontinuing anticoagulation were associated with a higher risk of VTE

recurrence.[256] D-dimer has been integrated in risk prediction models for VTE recurrence when measured at several time points after anticoagulation cessation.[257-259] Recently, D-dimer levels measured during VKA treatment were associated with MB risk in patients predominantly with prosthetic heart valves and AF in a Swedish cohort study.[260] Furthermore, the predictive accuracy of the HAS-BLED model improved with the addition of D-dimer in a large RCT-derived study of patients with AF.[261] Studies on VTE patients are lacking, and in the studies comprised of mainly other patient-groups, blood samples have been collected during anticoagulation. Whether D-dimer measured at VTE diagnosis can be used to assess risk of MB is largely unknown. As D-dimer is widely available for most VTE patients after the initial diagnostic work up, D-dimer represents a low-cost candidate biomarker of MB risk.

Among **hematological** parameters, hemoglobin and platelet count have previously been associated with MB risk in VTE patients. The presence of anemia (mild and moderate/severe) was associated with a 1.5 to 2.0-fold increased risk of MB compared to normal hemoglobin levels in a register-based study.[202] The risk of MB appeared to increase with the severity of anemia and remained statistically significant despite the fact that the cumulative incidence of discontinuing anticoagulation was higher in patients with moderate to severe anemia. Another register-based study found that patients with anemia, with or without cancer, had a 2.5 to 3-fold increased risk of MB compared to patients without anemia during anticoagulation.[201]

Platelets play a fundamental role in hemostasis and the use of platelet inhibitors is associated with increased risk of bleeding as previously discussed. However, the relationship between platelet count and risk of MB in VTE patients remains unclear. Previous data have shown that both low and high platelet counts were associated with increased risk of MB in VTE patients.[204,205,262] Elucidating the role of platelet count in MB risk assessment may be challenging, given the many diseases and conditions associated with abnormal platelet counts. Several conditions associated with VTE, such as cancer, liver disease, major surgery and trauma, can affect platelet count, but may also increase the bleeding risk during anticoagulation.[5,263-265] Despite the influence of environmental factors on platelet count, genetics largely contribute to the variation in platelet-related phenotypes.[266] Family and twin studies indicate a high heritability of platelet count and platelet size related indices, including mean platelet volume (MPV).[266-268] Moreover, the intra-individual variation in platelet count has been shown to be substantially less than the inter-individual variation in healthy subjects.[269] Given that platelet counts are relatively stable within an individual and largely

genetically determined, a potential influence on the MB risk in VTE patients might be clarified by assessing platelet counts measured at different time points prior to VTE.

2. Aims of the thesis

The overall aim of the thesis was to identify biomarkers of MB risk during the first year after an incident VTE.

The specific aims of the thesis were

A - To investigate the association between multiple prothrombotic genotypes and risk of MB during the first year after an incident VTE

B - To investigate the role of D-dimer, measured at venous thromboembolism diagnosis, as a predictive biomarker of MB events during the first year after an incident VTE

C - To investigate the association of platelet count measured prior to and at venous thromboembolism diagnosis with MB risk during the first year after an incident VTE

3. Methods

3.1 Study population

3.1.1 The Tromsø study – source population

The Tromsø study is a single-center, population-based cohort study with repeated health surveys of the inhabitants of the Tromsø municipality.[270] Since the first survey in 1974, the scope and research possibilities of the Tromsø study have expanded from a main focus on cardiovascular disease to a variety of health- and disease-related research questions, involving more than 50 research projects in Tromsø 7 (2015-16). The aim of the Tromsø study is to include a large, representative sample of the Tromsø population, with invitation of whole birth cohorts based on the official population registry. Overall, more than 45 000 unique individuals within the age range 25-97 years have participated in one or more of the Tromsø study surveys. The participation rates have been high, ranging from 66% to 85% of those invited. The papers in this thesis included participants from Tromsø 1-6 (paper II) and Tromsø 4-6 (papers I and III) who developed an incident VTE between January 1, 1994 and December 31, 2016 (2012 for paper I). The population is limited with regard to ethnic diversity as there are relatively few immigrants and the vast majority is of Caucasian descent.[270]

3.1.2 Identification and validation of VTE cases

All first lifetime VTE events were identified by searching the hospital discharge diagnosis registry, the autopsy registry and the radiology procedure registry at the University Hospital of North Norway (UNN). International Classification of Diseases (ICD) codes were searched for by means of manual and electronic text searches in the discharge registry. From 1994-98, the relevant ICD-9 codes were 325, 415.1, 451, 452, 453, 671.3, 671.4 and 671.9. From 1999-2016, the relevant ICD-10 codes were I26, I80, I81, I82, I67.6, O22.3, O22.5, O87.1, and O87.3. All relevant diagnostic procedures performed at the Department of Radiology at the UNN were systematically reviewed by trained personnel, and cases with objectively confirmed VTE were identified by the radiology procedure registry. VTE events causing or contributing to death were also identified in the autopsy registry, however not included in any analyses in this thesis.

Trained personnel reviewed the medical records of each potential VTE case and recorded a valid VTE event only if it was symptomatic (signs and symptoms of DVT or PE),

objectively confirmed by diagnostic procedures (i.e. ultrasound or computed tomography), resulted in a diagnosis of a DVT or PE, and treatment was initiated (unless contraindications for treatment were specified). VTEs were classified as either DVT or PE. DVTs were recorded in upper and lower extremities, inferior vena cava, mesenteric veins, portal vein and cerebral vein sinuses. In case of concurrent DVT and PE, the event was recorded as a PE.

3.2 Measurements

In the Tromsø study, measurements of height and weight were standardized with subjects wearing short-sleeved garments without shoes. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (km/m^2). Non-fasting blood samples were drawn from an antecubital vein when participants were enrolled in the Tromsø study. Analyses and preparations (for storage) were done at the Department of Clinical Chemistry at the UNN. If participants attended more than one Tromsø survey and had repeated measurements of the same variable, the measurement closest (and prior) to the VTE event of each individual was used.

The medical records of each VTE patient were reviewed for the 12 weeks preceding the diagnosis, and clinical information and VTE risk factors (provoking factors) were recorded. A VTE was classified as provoked in the presence of VTE risk factors within the 12 weeks preceding the VTE event, and as unprovoked if no such risk factor could be found. The provoking factors were major surgery, active cancer, trauma, or an acute medical condition (acute myocardial infarction, ischemic stroke, or major infectious disease), immobilization (bed confinement >3 days, wheel-chair confinement, or long-distance travel exceeding 4 hours within the last two weeks prior to VTE event), or any other factor specifically described in the medical records to have provoked the VTE (e.g. intravascular catheter or plaster cast).

3.3 Exposure assessment

3.3.1 Prothrombotic genotypes

DNA isolated from whole blood drawn at the Tromsø study was stored at -70° Celsius at the national CONOR biobank, located at the HUNT Biobank in Levanger, Norway. Genotyping was conducted at a collaborating institute at the University of California San Diego (UCSD), La Jolla, USA. Prothrombotic genotypes including rs6025 (FVL) in *F5*, rs1799963 (prothrombin G20210A) in *F2*, rs8176719 (non-O blood type) in *ABO* and rs2036914 in *F11* were genotyped using the Sequenom platform, and rs2066865 in *FGG* with

the TaqMan platform, as described by Horvei *et al*.[271] For Sequenom, which uses single-base extension followed by mass spectrometry to measure the molecular mass of extended primer, samples were genotyped with the sequenom iPlex Gold Assay according to the recommended protocol, with an initial input of 10-20 ng of DNA, and were analyzed with the MassARRAY Analyzer 4. For TaqMan, an initial input of 100 ng of DNA was used. Samples were genotyped with the Applied Biosystems 7900HT (Foster City, CA, USA) according to recommended protocol, and processed with SDS 2.4 (Thermo Fischer, Foster City, CA, USA).

3.3.2 D-dimer

D-dimer was measured in blood samples drawn for the initial diagnostic work-up in subjects suspected of VTE using two commercially available D-dimer assays. All blood samples were analyzed at the Department of Clinical Chemistry at the UNN. In the period from 1994-98, D-dimer was assessed with the NycoCard D-dimer (Nycomed Pharma, Oslo, Norway) assay, a bedside test based on immunofiltration principle. For the remaining period (1998-2016), D-dimer levels were determined using the STA-Liatest D-Di (Diagnostica Stago, Asnières, France) assay. The STA-Liatest is a quantitative, immuno-turbidimetric method using two monoclonal antibodies specific for D-dimer and covalently fixed onto latex microparticles.[272] The first measurement of D-dimer available in the medical records at the time of VTE diagnosis was used if more than one measurement were available.

3.3.3 Platelet count

As previously described in the Tromsø study,[273] blood was drawn into a vacutainer tube, containing EDTA as anticoagulant (K_3-EDTA 40 µL, 0.37 mol/L per tube), and analyzed within 12 hours by an automated blood cell counter (Coulter Counter®, Coulter Electronics, Luton, UK). Analyses and preparations (for storage) were done at the Department of Clinical Chemistry at the UNN. For platelet count measurements at VTE diagnosis, we considered the first blood sample drawn for the diagnostic work-up of VTE, as described in medical records of each VTE patient at the UNN. Blood samples were collected in vacutainers containing EDTA.

3.4 Assessment of major bleeding events

For each validated VTE, trained personnel searched the medical records at the UNN and recorded MB events during the 365 days following the thrombotic event. All advanced second-line care and emergency medicine, such as transfusion of blood products and surgical hematoma evacuation in the Tromsø region, is exclusively provided by the UNN. Situated in

the middle of Tromsø municipality, the UNN is the largest hospital in North Norway providing health services well beyond the borders of the municipality, with approximately 250 kilometers to the nearest hospital offering comparable health services. In order to be considered as an MB event in the present thesis, MB had to occur within 365 days of the VTE event and without close relation to a surgical or post-operative setting. The severity of bleeding was in accordance with the criteria of the International Society on Thrombosis and Haemostasis (ISTH).[177] In short, a bleeding event that was fatal, and/or symptomatic in a critical area or organ, and/or requiring blood transfusion of ≥ 2 units of whole blood or red blood cells or causing a fall in hemoglobin level of ≥ 2.0 g/dL, was considered major. In case of disagreement or uncertainty, the events were discussed in an endpoint committee to reach consensus.

4. Main results

4.1 Paper I – Prothrombotic genotypes and major bleeding risk

Genotypes associated with an increased risk of VTE could protect against MB during anticoagulant treatment for VTE due to a hypercoagulable state. Whether the risk of MB is reduced in parallel with an increasing number of prothrombotic genotypes after VTE diagnosis is unknown. Therefore, the aim of this study was to investigate the association between multiple prothrombotic genotypes and risk of MB in patients with VTE. There were 676 patients with incident VTE derived from the Tromsø study who were genotyped for rs6025 (*F5*), rs1799963 (*F2*), rs8176719 (*ABO*), rs2066865 (*FGG*) and rs2036914 (*F11*) SNPs. MB events were recorded from medical records during the first year after a VTE using ISTH criteria. Cox regression models were used to calculate hazard ratios for MB events according to individual SNPs and categories of risk alleles (5-SNP score; 0-1, 2, 3 and ≥4).

There were 50 MBs (IR 9.5 /100 person-years 95% CI 7.2-12.5) during follow-up. The median time to an MB was 33 days. The individual SNPs and number of risk alleles were not associated with MB risk. The HRs for MB per category increase of genetic risk score were 1.0 (95% CI 0.8-1.3) for the total study population and 1.1 (95% CI 0.8-1.5) when patients with active cancer were excluded. Analyses restricted to the first 3 months after VTE yielded similar results. Results were similar in subgroups of DVT, PE, provoked and unprovoked VTE, with HRs per category increase of the genetic risk score ranging from 0.9 to 1.2. The one-year cumulative incidences of MB were 6.7%, 9.1%, 9.4% and 7.4% in patients with 0-1, 2, 3, and ≥4 risk alleles, respectively. Our results indicate that prothrombotic genotypes, evaluated either as individual SNPs or as number of risk alleles included in a 5-SNP score, are not associated with a reduced risk of MB in VTE patients during anticoagulant treatment.

4.2 Paper II – D-dimer and major bleeding risk

Prediction of MB risk during anticoagulant treatment for VTE remains a challenge. Some studies suggest that D-dimer may predict MB during anticoagulation. However, data on VTE patients are scarce. The purpose of this study was to investigate the role of D-dimer, measured at VTE diagnosis, as a predictive biomarker of MB events during the first year after an incident VTE. D-dimer and clinical characteristics were registered in 555 participants of the Tromsø studies 1-6, who developed a community acquired VTE without concomitant active cancer from 1994 to 2016. Bleeding events were identified in medical records during the first year after a VTE diagnosis, and MBs were defined according to ISTH criteria. There were 29 MB events (IR 5.7/100 person-years, 95% CI 4.0-8.2) during follow-up, most of them (62%) occurring within the first three months after the VTE, at a median of 35 days. D-dimer levels were categorized at the 40th and 80th percentiles, setting the lowest category as the reference. The absolute risk of MB was highest during the first 3 months, especially in patients with D-dimer levels ≥80th percentile (≥8.3 µg/mL), with 28.8 MB events/100 person-years (95% CI: 13.7-60.4). In a model adjusted for age, sex and planned duration of anticoagulant treatment, the HR of MB was 2.6 (95% CI 1.1-6.6) in subjects with D-dimer value ≥8.3 µg/mL at VTE diagnosis as compared to the reference (D-dimer ≤ 2.3 µg/mL). In a generalized additive regression model, the risk of MB increased linearly as a function of D-dimer at levels > 7.0 µg/mL. The risk of MB was especially high in patients with a D-dimer above the 80th percentile presenting with DVT or provoked VTE. Results from subgroup analyses warrant careful interpretation due to few MB events. In this study, based on cancer-free subjects who developed a VTE in the community setting, we found that D-dimer might serve as a predictive biomarker of MB risk, especially during the first three months after a VTE. Future studies are warranted to confirm our findings and to investigate whether D-dimer could improve existing RAMs of MB during anticoagulant treatment for VTE.

4.3 Paper III – Platelet count and major bleeding risk

Platelets are fundamental in hemostasis, and platelet count represents an attractive candidate biomarker given its high availability at low cost and the fact that it has the potential to be a stable phenotype within an individual over time. Whether platelet count is associated with risk of MB in patients with VTE remains uncertain. We hypothesized that platelet counts measured at VTE diagnosis and several years before VTE were both associated with MB risk. The study population comprised 744 subjects with platelet count measurements available from Tromsø study enrollment (Tromsø 4-6) and at VTE diagnosis (1994-2016). MB events were recorded from medical records during the first year after a VTE using ISTH criteria. Cox-regression was used to calculate hazard ratios for MB across quartiles of platelet count based on the distribution measured at VTE diagnosis. There were 55 MBs (IR 9.1/100 person-years, 95% CI 7.0-11.8) within the first year, occurring at a median of 35 days. A dose-response trend for increased risk of MB by increasing quartiles of platelet count was observed. Compared to the lowest quartile of platelet count (≤192 $x10^9$L), a platelet count measurement in the highest quartile (≥300 $x10^9$L) was associated with a 4-fold (HR 4.3, 95% CI 1.7-10.9) increased risk of MB in an age- and sex-adjusted model. The corresponding HR in patients without active cancer was 4.0 (95% CI 1.4-11.4). When measured (on average) 7 years before a VTE, a platelet count in the highest quartile versus the lowest was associated with a 2.5-fold (95% 0.9-6.7) increased risk of MB. In this study, we found that higher platelet counts were consistently associated with increased risk of MB after a VTE, when measured either at diagnosis or years before the thrombotic event. Our results indicate that platelet count is a stable risk marker of MB in VTE patients that may improve stratification of MB risk.

5. General discussion

5.1 Methodological considerations

5.1.1 Study design

In the present thesis, a cohort of VTE patients was recruited from a population-based cohort study (the Tromsø study) and followed during the first year after VTE diagnosis, with the purpose to study biomarkers of MB.

In addition to being useful for determining the natural history and absolute risk of a disease,[274] cohort studies are suitable to study and obtain risk estimates for predictive purposes.[275] In a cohort study, a predefined group of people (cohort) is followed from the time of inclusion (baseline) until the outcome of interest or occurrence of other censoring events (e.g. death or study end). Study participants are classified according to exposure status, allowing differences in outcome to be investigated in exposed and non-exposed individuals during follow up. There are specific advantages inherent to the cohort study design. Because exposures are identified prior to the outcome, cohort studies enable a framework to study exposure and outcome with certainty regarding their temporal sequence. Although the question of causality (i.e. cause and effect) cannot be answered in a cohort study due to potential confounders (discussed in section 5.1.4), consistency and strength of the observed association, temporality between exposure and outcome, and a biological gradient (i.e. dose-response) strengthen the claim of a potential causal association in a cohort study.[276]

A clinical trial with random allocation of included subjects offers an “experiment” of causality which may support the hypothesis of causation most strongly, among Hills nine criteria of causality.[276] Together with the temporal sequence of exposure and outcome, the random allocation of study participants is the key feature that makes RCTs suitable to make inferences on causality or comparisons of drug efficacy and safety. RCTs require vast human and material resources and must ensure that patients are subjected to a minimum risk of harm to maintain strict ethical soundness. For instance, in an RCT investigating the risk of bleeding (as a secondary safety outcome) of a specific anticoagulant treatment in VTE patients, it would be unethical to include patients at high risk of bleeding. Systematic exclusion of certain study participants (lack of eligibility) may result in differences between the included study population and the background population the study is intended to reflect.[277] Implications of strict selection criteria include a potential lower representativeness of the study population compared to the target population. A high external validity (i.e. generalizability) is regarded as one of the main advantages of population-based cohorts.[278] The features of a study that are

important for its generalizability, in addition to selection of study participants, are further discussed in section 5.1.2.

Our design merits some considerations beyond those that apply to most cohort studies. First, we were able to assess platelet count on two occasions, first at time of Tromsø study enrollment, and second at VTE diagnosis. Comparing the same biomarker at two time points, with several years between them, offered important insights on the nature of the association between platelet count on MB risk in paper III. Moreover, as the association was consistent for both time-points prior to MB, it seems highly unlikely that the association could be a consequence of other MB risk factors that affect platelet count. Second, as VTE is a result of multiple risk factors, sampling a cohort based exclusively on subjects with incident VTE may induce dependence between risk factors with potential implications for measures of effect sizes on the risk of MB. This phenomenon, known as index event bias, and other important sources of bias in cohort studies including selection bias, information bias and confounding[279] will be discussed in the following sections.

5.1.2 Generalizability

Bias reduces the validity of a study. The internal validity denotes to what extent the observed findings lead to correct (free of bias) inferences in the reference population, while the external validity, i.e. generalizability refers to the degree to which results may apply, be generalized, or be transported to subjects beyond the study population.[280]

In the present thesis, VTE cases were identified with detailed procedures from a population-based cohort study with high participation rates to ensure the validity of the diagnosis. This likely yielded the identification of VTE patients that are representative of the VTE cases of the general population in Tromsø, which represents the source population in this thesis. As such, the characteristics of VTE patients included in trials investigating factors associated with MB risk might differ from the characteristics of our study population. Notably, the mean age across exposure categories was around 68 years in the papers of this thesis, and the proportion of patients with active cancer extended from 15% to 31%. It is therefore not surprising that the overall incidence rates of MB ranged from 5.7-9.5 per 100 person-year in the present thesis, which were considerably higher than the rates observed in RCTs. Yet, our incidence rate is in agreement with other observational studies on MB risk in VTE patients.[7,43]

5.1.3 Bias

A systematic error in the design or execution of a study resulting in incorrect estimates of a true association between exposure and outcome is termed bias. Numerous types of systematic errors have been identified and termed according to their mechanisms and sources, however most biases are related to selection of study participants (i.e. **selection bias**), measurements and classification of exposures and or outcomes (i.e. **information bias**), or the presence of **confounding factors**.[281]

Bias in the estimated association on an outcome that arises from the procedures used to select individuals into the study or analysis is termed selection bias.[280] Selection bias may overestimate or underestimate the true association depending on the net effect of selection on the exposures and outcomes. This type of bias is generally less likely to occur in cohort studies compared to case-control studies as non-exposed and exposed individuals are selected before the outcome has developed in cohort studies. Yet, there are subtypes of selection bias including differential loss to follow-up and non-response bias that warrant careful consideration in cohort studies. When there is a different probability of completing the study according to exposure status, there is a chance of bias due to differential loss to follow-up. For instance, if death occurs more frequently in an exposed group, the number of subjects at risk of developing the outcome of interest in the exposed group would be relatively lower as compared to those in the unexposed group. Consequently, crude cumulative incidences and relative risks may be overestimated.[120] The so-called competing risk of death is a problem mostly in studies with high mortality due to high age or cancer.[120,282] In the study populations included in our studies, the mean age and proportions of cancer across categorized groups ranged from 65-71 years and 15%-31%, respectively. Moreover, high levels of D-dimer and platelet count (paper II and III) have both been associated with increased mortality[283,284] and cancer[265,285] in previous studies. A statistical model conceived by Fine and Gray has been developed to take competing risk by death into account.[286] Using statistical methods, the model includes death as a competing event rather than merely a censoring event, and estimates the competing risk-corrected sub-distribution hazard ratio and cumulative hazard.[286] We applied the Fine-Gray model in papers II and III to address our concern for competing risk by death on the associations of D-dimer and platelet count with MB risk.

In population-based cohort studies like the Tromsø study, non-response bias may occur if the rate of non-responders is unequal across groups of people with different sets of

exposures and risks of outcomes. The distribution and relationship between exposure and outcome would potentially differ from the target population and therefore yield different associations in the study as compared to the target population. Participation rates in epidemiological studies have generally declined over the last decades, and investigations have recognized that marriage, female gender and high socioeconomic status consistently predict higher participation rates.[287] In accordance with the aforementioned data, participation rates in the Tromsø study have declined from 81-85% in the first three surveys (1974-87) to 66%-77% in the later three surveys (1994-2008),[270] and non-attendees tended to be younger, unmarried and men. However, since our study population comprised those who developed VTE during follow up, and the incidence of VTE is low in the young population, the effect of such non-responder bias would presumably be low in our study. The attendance rates of the Tromsø study are high compared to other similar population-based cohort studies conducted at similar time points.[287] Taken together, we considered the possibility of selection bias due to non-response bias in the Tromsø study to be low for the findings of the present thesis.

Index event bias is a special type of selection bias, also termed collider-stratification bias, which can occur when study participants are selected based on the occurrence of a multi-causal index event.[288,289] This phenomenon can typically be seen in recurrence studies, and could explain several of the so-called paradoxes or recurrence, including the thrombophilia paradox.[290] VTE is a result of multiple risk factors and a selection of only incident VTE events (as in a cohort of VTE patients) may induce dependence between these risk factors. For instance, VTE cases with a certain composition of risk factors, e.g. multiple prothrombotic risk alleles, might be more prone to lacking (or possessing) other specific risk factors (dependence), compared to those with another composition of risk factors. In our case, such potential dependence would theoretically only influence our results if the unknown risk factors were associated with MB risk. In that case, the effect on our results could be towards both under- and overestimation dependent on the direction of the potential association between the other specific unknown risk factors and MB.

Information bias, a result of a systematic tendency to erroneously place participants in different exposure/outcome categories, can arise at different stages in cohort studies.[281] Non-perfect sensitivity/specificity of the procedure(s) to detect or measure exposure or outcome may result in erroneous classification of exposures and diseases. Accordingly, information bias may lead to either differential or non-differential misclassification.[291] Non-differential misclassification occurs when the probability of exposure misclassification is not

related to the disease status, or vice versa, if exposed and non-exposed people are equally likely to be misclassified according to disease status. Non-differential misclassification usually, although not always, biases ratio measures of association like the relative risk towards the null.[291] Conversely, differential misclassification occurs when the probability of misclassification of exposure is different in diseased and non-diseased people, or the probability of misclassification of disease is different in exposed and non-exposed people. Differential misclassification can bias the observed effect estimate either towards or away from the null.[291] Information on exposure status was derived from blood samples drawn either at Tromsø study enrollment or at VTE diagnosis, for all papers in the present study, and their analyses were performed prior to assessment of MB.

In paper I, the quality of genotyping was assessed and classified according to several parameters, including Hardy-Weinberg equilibrium and call rate statistics. For both the Sequenom and TaqMan platforms, only genotypes of moderate or higher quality were eligible for use in paper I. When multiple attempts were made to genotype an individual, one of the highest quality genotypes across all attempts was chosen for each SNP.

In paper II, D-dimer was assessed in consecutive patients under suspicion of VTE using two different D-dimer assays. The risk of misclassification is increased when applying different methods to ascertain exposure status, especially if the methods are based on different technical principles. The NycoMed assay is based on immunofiltration, while the STA-Liatest is a quantitative, latex enhanced immuno-turbidimetric method. The STA-Liatest has consistently been reported to have excellent analytical properties for the diagnosis of VTE,[272,292] while there are conflicting results for the NycoMed assay.[293,294] In our study population, 93.5% had D-dimer determined by the STA-Liatest. To assess possible misclassification introduced by the two assays, we conducted a sensitivity analysis restricted to subjects with D-dimer determined by the STA-Liatest. The results remained essentially similar in this analysis, and therefore it is unlikely that any substantial misclassification was introduced because of using two D-dimer assays. Moreover, D-dimer was modelled in wide-ranging categories by initially dividing the study population into quintiles and then combining the quintiles into three categories. For most cases, small measurement errors in the D-dimer assessment would not impact on the category they resided in during the analysis.

In the third paper, we assessed platelet counts derived from the Tromsø study at enrollment and at VTE diagnosis. According to the protocol of the Department of Clinical Chemistry at the UNN, blood samples were collected in EDTA-containing vacutainers for

measuring platelet count in patients suspected of VTE, similarly to the procedure of the Tromsø study. Here, non-fasting blood samples were collected in vacutainers containing EDTA as an anticoagulation and analyzed within 12 hours in an automated cell counter (Coulter Counter©).[273] The time from blood sampling to analysis was probably substantially shorter for the measurement of platelet count at VTE diagnosis compared to the measurement at the Tromsø study.

MB events were identified retrospectively in medical records, and we therefore rely on the accurate and scrupulous documentation of health personnel at the UNN for the correct and accurate identification and classification of MB events. We sought to limit the risk of falsely classifying a minor bleed as a major bleed by adhering to the criteria of the ISTH in the assessment of bleeding events. However, as authors of the ISTH criteria point out, there is a subjective component to the assessment and the presence of bleeding and its severity, both at clinical center and adjudication levels.[177] There was uncertainty regarding the classification of a few bleeding events, in which case available data and information related to the bleeding was discussed in an endpoint committee. The source of doubt whether or not a bleeding event qualified as major was most often due to unclear or missing information in medical records of VTE patients. In some cases, medical records indicated that it was likely that the MB originated from or in relation to cancer-tissue, however it was unclear regarding the time-course of signs and symptoms of bleeding, making it hard to judge the acuteness of the bleeding. In this case, the event would be included as long as it fulfilled the ISTH criteria. In another case discussed in the endpoint committee, a patient was admitted with extensive bleeding in the abdominal cavity, apparently fulfilling ISTH MB criteria. However, the patient had undergone a gastrointestinal surgery in the recent past, and a concomitant anastomosis leakage was suspected. In this case, the bleeding event would not be included because the patient would be considered surgical. These issues will presumably be present in other studies investigating MB in VTE patients. Unfortunately, we were unable to assess causes of death in VTE patients outside the UNN, which might have resulted in misclassification of the outcome, with an underestimation of the MB events. However, fatal bleeding is rare, and the rate of fatal MBs in our data (0.5 per 100 person-years in paper III) was similar to the reported frequency in the literature.[295] Therefore, any MB due to sudden death that was not taken into account in our analysis would be very few, if any.

5.1.4 Confounding

Confounding is present when a non-causal association between an exposure and an outcome is observed as a result of the influence of a third variable (the confounder). As a general rule, a confounding variable is causally associated with the outcome, noncausally or causally associated with the exposure but is not an intermediate variable in the causal pathway between exposure and outcome.[296] Under these criteria, an association is distorted if the confounder is differentially distributed across exposure status. A confounder may strengthen, weaken or reverse the association under investigation. The difference between confounding and bias is that risks that are due to confounding are a result of indirect association, and are therefore real but not causal, while biased results are spurious associations.[297] Although randomization elegantly reduces the risk of differential distribution of confounders in exposed and non-exposed subjects, associations may also be confounded in RCTs due to randomly occurring differences with regards to important prognostic factors between compared groups.[296] Observational studies rely on statistical modeling, including multivariable adjustment and stratification to assess and reduce confounding.

The simplest method to assess the presence of confounding is by stratifying study participants according to the presumed confounder. It is achieved by dividing the data into subgroups on the presumed confounder of the association of interest. Stratification allows a straightforward and simultaneous examination of the possible presence of both confounding and effect modification (i.e. interaction).[296] An obvious disadvantage of stratification is the reduction of the statistical power. Multivariate analysis refers to a series of analytical techniques, based on mathematical modelling, which are frequently used to carry out statistical adjustment – that is, the estimation of a certain measure of association between an exposure and an outcome while controlling for one or more confounding variables.

In the present work, there were several factors that could be potential confounders of the investigated associations. In paper II and III, we regarded age and cancer as confounders and tried to limit the impact of these factors by adjusting and stratifying/excluding the respective confounders. In paper II, we regarded the associations between cancer, D-dimer, VTE and MB risk to be so complex and heterogeneous that we opted for excluding cancer patients from all analyses. There were other variables that could be considered potential confounders, including co-morbidities and provoking factors (e.g. major infectious diseases and acute medical conditions), and a differential treatment duration. In all papers, we conducted stratified analyses in patients with provoked and unprovoked VTE to assess the

impact of VTE provoking factors such as trauma, surgery and infectious diseases, rather than multivariable adjustment. We adjusted for the planned treatment duration to limit the potential of differential length of treatment duration across categories. Including covariates into a statistical regression model can have drawbacks and should be considered only when appropriate. Increasing the number of covariates in a statistical model will affect the statistical power. Furthermore, adjustment of a presumed confounder will also adjust for all other variables related to it, and the results may be "overadjusted".[296] Overadjustment is considered to occur when adjustment is inadvertently carried out for a variable that is either in the causal pathway between the exposure and the outcome (a mediator) or so strongly related to the exposure or the outcome that their true relationship is distorted. As many factors implicated for MB risk, including VTE provoking factors, are based on heterogeneous and not well-characterized associations, we regarded it most appropriate to conduct stratified analyses, as they offer the most straightforward interpretation. Given the observational nature of our studies, residual confounding can never be ruled out. Our results do not allow for any causal inferences but point towards the predictive potential of biomarkers of MB risk.

5.1.5 Missing data

Missing data may be present in almost all epidemiological studies, including ours.[298] Missing data can have many explanations, including malfunction of technical equipment and measuring devices, loss of laboratory data or lack of documentation/registration. The distribution of missing data is important for the risk of bias. Bias is less likely to be introduced if data are missing at random, compared to data not missing at random. There are mainly three ways to handle missing data in epidemiological studies, either omitting variables that contain missing data, omitting subjects with missing data or imputing missing data.[298]

In all papers of the present thesis, missing data regarding exposures were handled by excluding participants with lacking information. In paper I, 8% of subjects had missing data on genotypes; in paper II, 16% had missing data on D-dimer; in paper III, 17% had missing data on platelet count. For papers II and III, we compared the baseline characteristics of patients with non-missing with those with missing data on the exposure of interest. In both papers, no major differences were detected, indicating that data were most likely missing at random.

5.2 Discussion of main results

5.2.1 Prothrombotic genotypes and risk of major bleeding

In paper I, we found no association between an increasing number of prothrombotic risk alleles for rs6025 (FVL), rs1799963 (prothrombin G20210A), rs8176719 (non-O blood type), rs2066865 (*FGG*) and rs2036914 (*F11)* and the risk of MB during the first year after a VTE. The results were essentially similar in analyses restricted to patients without active cancer, and in subgroups of DVT, PE, provoked and unprovoked VTE. To the best of our knowledge, we were the first to address whether multiple prothrombotic genotypes were associated with MB risk in VTE patients derived from the general population.

Prior to the present thesis, a few case-control studies have investigated the association between ABO blood groups and risk of bleeding in anticoagulated patients.[242,243] Garcia *et al.* reported that that the risk of MB was 30% lower (OR 0.7, 95% CI 0.4-1.1) in carriers of non-OO genotypes compared to carriers of the OO genotype during anticoagulant treatment with VKA for various indications (e.g. mechanical heart valve, VTE and AF).[243] Franchini *et al.* found that the risk of bleeding was similar across phenotypes of ABO blood groups but noted differences in the distribution of ABO blood groups across indications for anticoagulation.[242] As the clinical characteristics and recommended duration/intensity of anticoagulation may vary considerably between subjects with AF, mechanical heart valves or VTE, the association between ABO blood types and MB risk may not necessarily be similar across subjects with various indications for anticoagulation. This may explain the apparently inconsistent results in the abovementioned studies. Moreover, cases and controls may differ from the general VTE population on baseline characteristics of potential importance for bleeding risk, as they were identified and selected from specialized anticoagulation/thrombosis clinics. Taken together, the generalizability to the VTE population may be limited since the proportion of VTE patients was 20-28% in the aforementioned studies. Tzoran *et al.* assessed the risk of MB according to the presence of FVL and prothrombin mutation in patients referred to thrombophilia-testing in the RIETE registry.[245] The authors found no clear association between prothrombin G20210A and MB but found that carriers of FVL had half the risk (HR, 0.50; 95% CI 0.25-0.99) of MB compared to non-carriers. The reasons for thrombophilia-testing may reflect patients with systematic differences in characteristics of potential importance for bleeding risk when compared to the general VTE population, such as age, presence of cancer and family history of thrombotic diseases.[245] For instance, the proportion

of cancer among carriers and non-carriers of the prothrombotic genotypes was relatively low (6%-11%) in their study compared to our study population (15%-28%).

Our results indicate that the five SNPs assessed in our study do not protect against MB in VTE patients when evaluated individually or in a cumulative manner (i.e. by counting risk alleles). There was no indication of a dose-response relationship with an increasing number of risk alleles and MB risk across patients with DVT, PE, unprovoked, provoked or non-cancer-associated VTE. The point estimates of the relative risk of MB in carriers of 2, 3 and ≥4, compared to 0-1 risk alleles ranged from 1.0 to 2.2 across main and subgroup analyses, and from 0.9 to 1.2 per category increase in the genetic risk score. Although we adjusted for planned treatment duration, we also estimated the risk of MB by number of alleles restricted to the first three months, when all participants were presumably on anticoagulation. The results of this sensitivity analysis were also close to unity per increase in risk allele category (HR 1.1, 95% CI 0.8-1.5), and we therefore regard it unlikely that potential differences across duration of anticoagulation could explain our results. Regarding the analyses of the individual SNPs, although carriership of ≥1 allele of the rs2036914 in the *F11* seemed to be associated with increased risk of MB, especially in cancer-free patients, we interpret this finding cautiously. The width of the 95% CIs were notably wider for the *F11* variant compared to the other SNPs, indicating particular uncertainty in this estimate.

We can speculate that the cumulative hypercoagulable state driven by increasing risk alleles would not be sufficient to balance the mechanisms that induce an MB associated with anticoagulant treatment. Compared to the potentially 1000-fold increased inhibition of FXa and thrombin by heparins[299] and the widespread reduction in functional coagulation factors (X, IX, VII and II) caused by VKAs, the hypercoagulable effects of prothrombotic genotypes on the coagulation system may be negligible. Taken together, it is unlikely that the prothrombotic variants included in the 5-SNP score would be of clinical relevance to improve discrimination between those with high and low risk of MB during anticoagulant treatment in VTE patients.

Even though our results suggest that the mild hypercoagulability driven by the prothrombotic genotypes is not able to restrain a severe bleeding associated with anticoagulant treatment, such hypercoagulability could still be associated with less severe bleeding. This way, the outcome of CRNMB might have offered the opportunity to perform a sensitivity analysis to address this question. Thus, a limitation in our study is that we did not have information on less severe bleedings, e.g. CRNMB. Nevertheless, our primary aim was

to investigate the combined impact of the five SNPs on the outcome of MB, as the severe and potentially fatal bleeding complications are most relevant for the harm and net benefit assessments of anticoagulant treatment.[5]

Other than chance, index event bias may be relevant for the observed null association. Our hypothesis was that multiple risk alleles would protect in a dose-response manner against MB risk in VTE patients. Such an effect would be similar but in an opposite direction to the effect on recurrent VTE.[103] VTE is a result of multiple risk factors, and as discussed in section 5.1.2, a selection of only incident VTE events may induce dependence between these risk factors. For example, an inverse association between number of prothrombotic risk alleles and other prothrombotic factors could arise in the incident VTE population. Given that these factors are also protective against bleeding, the effect measure of the risk of MB in carriers of prothrombotic genotypes compared to noncarriers may be shifted towards the null.

Finally, the null association in our study could be a result of chance. The probability of missing true associations (type II-error) is related to the statistical power of the study. Although the so-called post hoc power analysis has been used to estimate the probability of type II-error after a study has been conducted, confidence intervals are more appropriate to estimate the magnitude of effects after results are obtained.[300] The 95% CIs for the point estimate of 1.0 per category increase of the genetic risk score were 0.8 to 1.3 for the overall population. Across all subgroups in paper I, the results were essentially similar and consistently close to unity for the risk estimates by increasing category of risk alleles.

5.2.2 D-dimer and risk of major bleeding

In paper II, we found that high levels of D-dimer (≥ 8.3 µg/mL) were associated with increased risk of MB after a community-acquired VTE in patients without known active cancer. The association was especially pronounced during the initial three months after VTE diagnosis and in patients with provoked VTE. Among VTE cases with high D-dimer, the 3- and 12-month cumulative incidence of MB was 6.8% and 10.8% respectively, compared to 2.2% and 3.6% in patients with D-dimer ≤ 2.3 µg/mL. Our findings support that there is a potential for D-dimer as biomarker of MB risk in VTE patients, particularly in the initial phase of anticoagulant treatment.

Prior to the present thesis, some studies reported an association between D-dimer measured during anticoagulation and MB,[260] with data also showing that D-dimer could

improve prediction of MB in AF.[18,261] Studies on VTE patients have been scarce, and in these studies, D-dimer was measured after treatment initiation with anticoagulants. It is well established that the absolute risk of MB is highest during the initial phase of anticoagulant treatment for VTE.[6,173,194] Biomarkers of MB risk should therefore be assessed when anticoagulation is initiated in order to be relevant for the time-period in which there is the highest potential to avoid MB complications in VTE patients. Interestingly, a recent study compared bleeding RAMs in patients with acute PE and the ability of D-dimer measured at the day of admission to augment the discriminatory capacity of these RAMs for both MB and CRNMB.[301] Elevated levels of D-dimer were associated with in-hospital bleeding, both MB and CRNMB events, and addition of D-dimer to RAMs augmented their predictive capabilities for MB risk in this study.[301] Based on sensitivity and specificity assessment for MB, they reported an ideal cut-off of D-dimer at 5.8 µg/mL, which was associated with a 2.3-fold increased risk of any bleeding (95% CI 1.05-5.00) in patients with a D-dimer level above this threshold. In contrast to our study, only in-hospital MBs were recorded during a median of 6.5 days after an acute PE in the abovementioned study.[301] We found that the association between D-dimer and MB was less pronounced in subgroup analysis of PE patients compared to overall analysis. However, as there were few MB events in D-dimer categories of PE patients, the results of subgroup analysis warrant careful interpretation due to low statistical power.

In paper II we assessed D-dimer measured at VTE diagnosis and risk of MB. Because D-dimer has low specificity for the diagnosis of VTE as it is often elevated in patients hospitalized for other conditions or with cancer, we excluded subjects with active cancer and those already hospitalized for other conditions when the VTE occurred. After excluding these patients, there were still 38% provoked VTE events in our study. It is reasonable to assume that the MB risk might be overestimated due to competing risk of death in patients with higher D-dimer levels.[120,302] However, as the results were only slightly attenuated in the competing risk of death analyses, it is unlikely that the association could be explained by increased mortality in cases with D-dimer in the upper category. Other medical conditions associated with provoked VTE, but not necessarily with higher short-term mortality, could be relevant for the association between D-dimer and MB. Although including status as either provoked or unprovoked VTE and acute medical conditions in the multivariable adjusted models yielded similar results, we regarded it most appropriate and clinically relevant to report stratified analyses. Yet, we cannot fully exclude that other unmeasured factors could

explain the association between D-dimer and MB risk. In view of our analyses of non-hospitalized patients without known active cancer, we can conclude that the association between D-dimer and MB was unlikely driven by cancer, hospital-related factors or conditions associated with higher mortality in patients with elevated D-dimer levels.

The absolute risk of MB was high early after VTE diagnosis, and among patients with a D-dimer ≥ 8.3 µg/mL, the incidence rate was 28.8 per 100 person-years when analysis was restricted to the first 3 months. Although the relative risks between categories of D-dimer were similar in analyses of 12 months and 3 months of follow-up, the very high absolute risk in patients with high D-dimer levels indicate that D-dimer is most important as a biomarker for the short-term prediction of MB.

As D-dimer is already widely available for most VTE patients, our results may have future clinical implications for the risk prediction of MB risk at time of VTE diagnosis without added costs or interventions. With substantial improvement in the predictive power of MB risk assessment, high-risk patients could ultimately benefit from preventive measures during the initial treatment phase. This might include choice of anticoagulant, tighter follow-up, more aggressive approaches to target modifiable risk factors and stronger reluctance for co-medications such as antiplatelets and NSAIDS.

5.2.3 Platelet count and risk of major bleeding

In paper III, an increasing platelet count, measured at VTE diagnosis, was associated with a higher risk of MB in a dose-response fashion during the first year after the VTE. Subjects with a platelet count in the upper quartile (≥ 300 x 10^9/L) had a 3-fold higher risk of MB during the first year after VTE diagnosis, compared to subjects with a platelet count in the lowest quartile (≤192 x 10^9/L). We found similar results after the exclusion of subjects with active cancer at time of diagnosis and in unprovoked and provoked VTE. When platelet counts were assessed on average 7 years prior to the VTE event in the same subjects, a similar, albeit somewhat attenuated, association was observed between an increasing platelet count and MB risk. Taken together, our results suggest that platelet count is a stable phenotype within an individual over time that may influence the individual susceptibility to MB after treatment initiation for VTE.

The incidence rate of MB was 9.1 per 100 person-years in our study, which is in line with other observational studies conducted in the same time-period. In a population-based

observational study, Spencer *et al.* included 1567 incident VTE cases during 1999-2003 in the Worcester VTE study, and reported that MB occurred annually in 12-13% of patients.[7] In the Worcester VTE study, 30% of VTE cases were cancer-related and around 50% were classified as provoked VTE (i.e. hospitalization, surgery, pregnancy, trauma or fracture within 3 months of the event), which is similar to the proportions of active cancer and provoked events in our study.[7] In 2002, Prandoni *et al.* reported an incidence rate of 10.6 per 100 person-year in a prospective study of 842 VTE subjects, with a similar proportion of cancer-related VTE as in our study.

Prior to the present thesis, both platelet counts in the low (e.g. below 50-99 000/µL) and high ranges (e.g. above 450 000/µL) had been associated with increased risk of MB compared to platelet counts between these extremes, during anticoagulant treatment for VTE.[205,262] As authors found that a "high" or "low" platelet count was associated with other bleeding risk factors (e.g. renal impairment, cancer and anemia), they speculated that an abnormal platelet count could be a sign of greater frailty and subsequent increased MB risk during anticoagulant treatment for VTE.[262] Indeed, during derivation of the RIETE bleeding score, a platelet count below 100,000/mm^3 was associated with 2.3-fold (95% CI 1.4-3.9) increased risk of MB in univariate analysis, but not in multivariate analysis after inclusion of other risk factors, such as cancer, anemia and creatinine levels to the statistical model.[200] Another study from the RIETE-registry showed that high and low platelet counts were associated with increased MB risk in VTE cases with or without cancer, suggesting that the association was independent of cancer and cancer-related factors (e.g. chemotherapy).[204] Several other known or unknown factors at the time of VTE diagnosis might influence the association between platelet count and MB. Based on the previous available data, the role of platelet count at VTE diagnosis as a biomarker for MB during VTE treatment remained largely unclear.

Here, we found that increasing platelet counts were associated with higher MB risk in unselected VTE cases derived from the general population. The risk of MB increased around 50% per quartile increase in the overall population (HR 1.5, 95% CI 1.2-1.8), compared to the lowest quartile (p for trend .003). The risk was 4-fold higher in subjects with a platelet count in the upper quartile compared to the lowest quartile in an age- and sex- adjusted model (HR 4.3, 95% CI 1.7-10.9). In the fully adjusted model, in which competing risk by death and factors potentially associated with platelet count and MB were taken into account, a platelet count in the upper quartile was associated with a 3-fold increased risk of MB (SHR 3.2, 95%

CI 1.2-8.6) in comparison with the lowest quartile of platelet count. In VTE patients without cancer, the corresponding analysis yielded similar results (SHR 2.9, 95% CI 0.9-9.5). These findings suggest that the association could be in part, but not entirely, explained by other VTE-related factors with impact on the MB risk (e.g. cancer, acute medical conditions, renal function, hypertension, history of bleeding and anemia). A platelet count in the upper quartile was still associated with around 3-fold increased risk of MB in fully adjusted models in provoked and unprovoked VTE compared to a platelet count in the lowest quartile.

When platelet count was assessed on average 7 years prior to VTE within the same individuals and platelet count cut-offs, we found a similar but less pronounced association compared to a platelet count measured at VTE diagnosis. In an age- and sex- adjusted model, the risk of MB increased per category increase (p for trend 0.08) of platelet count, with a 2.5-fold higher risk of MB in the upper category versus the lowest category (HR 2.5, 95% CI 0.9-6.7). Because differences in platelet size could potentially explain the association between platelet count and MB, we further adjusted HRs for MPV, a marker of platelet size and function shown to be inversely correlated with platelet count.[303,304] After adjustment for MPV, risk estimates were somewhat attenuated (HR 2.0, 95% CI 0.7-5.6), suggesting the potential of platelet size and function, measured as MPV, to mediate the association between platelet count and MB. Platelet counts at VTE diagnosis correlated significantly with platelet counts measured at Tromsø study enrollment. Such findings reinforce the notion that platelet count is a stable phenotype within an individual over time, as previously demonstrated by others.[268,269]

In light of the strong heritability of platelet indices, including platelet count and MPV,[266,268,305] we can speculate that the association is driven by platelet phenotypes with different susceptibility to bleeding complications under anticoagulant treatment. Large and small platelets have been shown to substantially differ in their functional roles in the hemostatic system. Compared to small platelets, large platelets are associated with increased reactivity, shortened bleeding time, faster adhesion to collagen and aggregation *ex vivo*, and increased expression of glycoproteins on their membranes.[306] Reticulated platelets, which are large and hyperactive platelets, display a prothrombotic profile, as recently revealed in transcriptome analysis.[307] Moreover, results from epidemiological studies, including the Tromsø study, show that an increased MPV is associated with a higher risk of arterial cardiovascular disease[308] and VTE,[273] thereby supporting the higher prothrombotic potential of large platelets. It is of interest that in the presence of substantial thrombocytopenia (< 20 x 10^9/L), a low MPV has been shown to be a stronger predictor of bleeding than platelet

count.[309] Furthermore, small volume platelets are considered pathognomonic of Wiskott-Aldrich syndrome, an X-linked disorder most often presenting early in life with thrombocytopenia and spontaneous and post-traumatic bleeding episodes.[310] Although the pathophysiology and platelet function of Wiskott-Aldrich syndrome is not fully understood, reduced adhesiveness to collagen fibers and diminished ability to modulate membrane glycoprotein in response to thrombin have been reported,[311,312] which could play a role in the bleeding phenotype of the syndrome. Taken together, several data suggest that platelet phenotype and volume are important for the risk of bleeding in patients with thrombocytopenia. In light of our findings, we can speculate that an increasing platelet count, even within a normal range, would be associated with a lower platelet reactivity, as reflected by a decrease in MPV, which could predispose to MB during anticoagulant treatment after a VTE.

Platelet count, as a phenotype that is stable over time within an individual, seems a promising biomarker to improve stratification of major bleeding risk during anticoagulant treatment. However, the use of platelet count in risk assessment models in patients with VTE and AF has yielded controversial results, with studies using different cut-off values of platelet count.[170,295,313] Whether an elevated platelet count at the appropriate cut-off value can improve discrimination of VTE patients with high and low risk of MB would require further investigation.

6. Conclusions

- We found that the five common genetic variants, rs6024 (FVL), rs1799963 (prothrombin G20210A), rs8176719 (non-O blood type), rs2066865 (*FGG*) and rs2036914 (*F11),* associated with increased VTE risk, were not associated with MB risk when modelled individually or in a 5-SNP genetic risk score. The 5-SNP genetic risk score was not associated with MB risk in subgroups of DVT, PE, provoked or unprovoked VTE. Our findings suggest that the genetic variants included in the 5-SNP score do not protect against MB, individually or additively, in VTE patients derived from the general population during the first year after a VTE.

- We found that a high D-dimer (≥ 8.3 µg/mL), measured during the diagnostic work up for VTE, was associated with increased risk of MB, particularly during the first three months after diagnosis. Our results suggest that D-dimer assessed at the time of VTE diagnosis can predict MB risk in community-acquired VTE without known cancer. Additional studies are needed to validate the association and assess the predictive power of D-dimer in the presence of other relevant MB predictors.

- We found that an increasing platelet count, measured several years before and at VTE diagnosis, is associated with a higher risk of MB during the first year after an incident VTE. Our findings suggest that a platelet count measured at VTE diagnosis is a stable phenotype within an individual over time that has the potential to improve risk stratification of MB after a VTE.

7. References

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Paper I

Thrombosis Research 191 (2020) 82–89

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Prothrombotic genotypes and risk of major bleeding in patients with incident venous thromboembolism

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ARTICLE INFO

Keywords:
Venous thromboembolism
Anticoagulation
Genotype
Hemorrhage
Bleeding

ABSTRACT

Background: Genotypes associated with venous thromboembolism (VTE) may protect against bleeding due to a hypercoagulable state. Whether the risk of major bleeding is reduced in parallel with an increasing number of prothrombotic genotypes during anticoagulant treatment in VTE remains unknown.
Objectives: To investigate the association between multiple prothrombotic genotypes and risk of major bleeding in patients with VTE.
Methods: Patients with incident VTE (n = 676) derived from the Tromsø Study were genotyped for rs6025 (*F5*), rs1799963 (*F2*), rs8176719 (*ABO*), rs2066865 (*FGG*) and rs2036914 (*F11*) single nucleotide polymorphisms (SNPs). Major bleeding events were recorded during the first year after VTE according to the International Society on Thrombosis and Haemostasis criteria. Cox-regression was used to calculate hazard ratios with 95% confidence intervals (CIs) for major bleeding adjusted for age, sex and duration of anticoagulation according to individual prothrombotic SNPs and categories of risk alleles (5-SNP score; 0–1, 2, 3 and ≥4).
Results: In total, 50 patients experienced major bleeding (incidence rate: 9.5/100 person-years, 95% CI 7.2–12.5). The individual SNPs and number of risk alleles were not associated with major bleeding risk. The hazard ratios for major bleeding per category increase of genetic risk score were 1.0 (95% CI 0.8–1.3) for the total study population and 1.1 (95% CI 0.8–1.5) when patients with active cancer were excluded. Analyses restricted to the first 3 months after VTE yielded similar results.
Conclusion: Our findings suggest that an increasing number of prothrombotic risk alleles is not protective against major bleeding in VTE patients during anticoagulation.

1. Introduction

Bleeding is a dominant and potentially serious complication in patients undergoing anticoagulant treatment for venous thromboembolism (VTE) [1]. The annual rates of major bleeding (MB) in VTE patients during anticoagulant treatment have been reported to vary from 1% to approximately 10% depending on the type of anticoagulant, patient characteristics and study setting (clinical trials versus daily care) [2–7]. An accurate risk prediction of MB is crucial to make informed decisions on VTE management and particularly to guide the treatment duration in unprovoked VTE towards the highest net benefit. However, the current prediction models for MB in VTE are mainly based on clinical factors, and do not discriminate well between VTE patients at high and low risk of MB in validation studies [8–11]. Extended knowledge on risk factors associated with MB in VTE patients is a key step to improve risk stratification of MB events during anticoagulant treatment.

Single nucleotide polymorphisms (SNPs) associated with increased risk of VTE, such as factor V Leiden (FVL), prothrombin G20210A, and non-O blood type [12], could be plausible candidate risk factors for MB. The prothrombotic genotypes have been shown to be associated with a hypercoagulable state [12] and could therefore have the potential to counteract the risk of bleeding related to anticoagulation in VTE patients. For instance, FVL is associated with resistance to the natural anticoagulant protein C [13], prothrombin G20210A with increased plasma levels of prothrombin [14], and non-O blood type with increased plasma levels of von Willebrand factor and coagulation factor VIII [15]. The few studies addressing the impact of prothrombotic genotypes on the risk of MB during anticoagulant therapy have

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https://doi.org/10.1016/j.thromres.2020.04.008
Received 20 January 2020; Received in revised form 1 April 2020; Accepted 7 April 2020
Available online 22 April 2020
0049-3848/

suggested a decreased risk of MB in carriers of FVL [16] and non-O blood types [17].

In the context of VTE, de Haan and colleagues developed a genetic risk score originally based on 31 VTE-associated SNPs that was shown to improve the risk prediction of first lifetime VTE [18]. The number of prothrombotic risk alleles included in the genetic score was dose-dependently associated with VTE risk (i.e. the more risk alleles present, the higher the risk of VTE). A similar predictive performance and dose-response relationship was found when the authors conceived a more parsimonious score consisting of the 5 most strongly VTE-associated SNPs, i.e. rs6025 (FVL) in *F5*, rs1799963 (prothrombin G20210A) in *F2*, rs8176719 (non-O blood type) in *ABO*, rs2066865 in the fibrinogen gamma gene (*FGG*) and rs2036914 in *F11* [18]. Given that the mechanisms by which multiple prothrombotic genotypes affect the thrombosis risk likely reflect different pathways in blood coagulation, we hypothesized that an increasing number of prothrombotic risk alleles would have a dose-dependent protective effect on the risk of MB. To the best of our knowledge, no previous study has assessed the impact of multiple prothrombotic genotypes on the risk of MB. Therefore, we aimed to investigate the association between multiple prothrombotic genotypes and risk of MB during the first year after an incident VTE.

2. Methods

2.1. Study population

The study population originated from the Tromsø Study, a single-center, population-based prospective cohort, with repeated health surveys of the inhabitants of Tromsø, Norway [19]. Study participants were recruited from the fourth (1994–1995), fifth (2001–2002) and sixth (2007–2008) surveys of the Tromsø Study. The overall attendance rates were high, achieving 77% in the fourth survey, 79% in the fifth survey, and 66% in the sixth survey. In total, there were 30,371 unique individuals aged 25–97 years who participated in at least one of the surveys. The study was approved by the Regional Committee of Research and Medical Health Ethics, and all study participants provided informed written consent.

From the date of inclusion in one of the three surveys until the end of follow-up at December 31, 2012, all potential first lifetime venous thromboembolism (VTE) events were identified by searching the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry at the University hospital of North Norway (UNN). Identified cases were adjudicated by trained personnel and deemed as validated when signs and symptoms of deep vein thrombosis (DVT) or pulmonary embolism (PE) were combined with objective confirmation by radiological procedures, and resulted in a VTE diagnosis requiring treatment, as described in detail previously [20]. A total of 737 objectively confirmed VTE cases were identified during follow-up. Of these, 45 did not have blood samples available or of sufficient quality for DNA analysis, and 16 were not successfully genotyped for one or more of the SNPs of interest. Therefore, 676 VTE cases were included in our study.

2.2. Clinical characteristics of VTE events

Information on clinical and provoking factors at the time of and 12 weeks preceding the VTE diagnosis was obtained for all eligible patients by review of medical records at the UNN. In the case of a concurrent DVT and PE diagnosis, the VTE event was classified as a PE. Each VTE event was further classified as provoked if one or more of the following risk factors were present: major surgery, trauma or acute medical conditions (acute myocardial infarction, ischemic stroke, or major infectious disease) within 12 weeks prior to VTE event, marked immobilization (bed confinement > 3 days, wheel-chair, or long distance travel exceeding 4 h within the last 14 days prior to VTE event), or any other factor specifically described in the medical records to have provoked the VTE (e.g. intravascular catheter). Presence of known active cancer at the time of VTE diagnosis was regarded as a provoked VTE.

To account for type and duration of VTE treatment, we considered the planned treatment (i.e. heparin or vitamin k antagonist [VKA]) and duration of anticoagulation that were stated by the attending physicians in the medical records at the time of VTE diagnosis. Duration of anticoagulant therapy was categorized into 3, 6, 12, and > 12 months, as previously described [21].

2.3. Assessment of major bleeding (MB) events

The medical records for all participants were searched for MB events occurring during the 365 days following the VTE. All second-line care and advanced emergency medicine, such as transfusion of blood products, is exclusively provided by the UNN. The UNN is situated in the middle of Tromsø municipality, with a vicinity of approximately 250 km to the nearest hospital providing comparable health-care functions. Two reviewers (trained medical personnel from the UNN) adjudicated the bleeding events independently in accordance with the criteria proposed by the International Society on Thrombosis and Haemostasis (ISTH) [22]. In short, a bleeding event that was fatal, and/or symptomatic in a critical area or organ, and/or causing a fall in hemoglobin level of ≥20 g/L, or requiring transfusion of ≥2 units of whole blood or red blood cells, was considered major. In case of disagreement, the event was discussed in an endpoint committee (HSJ and JBH) to reach consensus.

2.4. Genotyping and quality control

Blood samples were collected from an antecubital vein at enrollment in the Tromsø Study and the initial preparation of samples was done at the department of Clinical Chemistry at the UNN. DNA was isolated from whole blood and stored at −70°C at the national CONOR biobank, located at the HUNT Biobank in Levanger, Norway. As previously described [23], we genotyped rs6025 (FVL) in *F5*, rs1799963 (prothrombin G20210A) in *F2*, rs8176719 (non-O blood type) in *ABO* and rs2036914 in *F11* with the Sequenom platform, and rs2066865 in *FGG* with the TaqMan platform. For Sequenom, which uses single-base extension followed by mass spectrometry to measure the molecular mass of the extended primer, samples were genotyped with the Sequenom iPlex Gold Assay according to the recommended protocol, with an initial input of 10–20 ng of DNA, and were analyzed with the MassARRAY Analyzer 4. Only genotypes with a high quality score of “A - Conservative” or “B - Moderate” were used. When multiple attempts were made to genotype an individual, one of the highest-quality genotypes across all attempts was chosen for each SNP. For TaqMan, an initial input of 100 ng of DNA was used. Samples were genotyped with the Applied Biosystems 7900HT (Foster City, CA, USA) according to the recommended protocol, and processed with SDS 2.4 (Thermo Fisher, Foster City, CA, USA).

Subjects were considered carriers of the prothrombotic risk gene when one or two risk alleles were present. Individual SNP assessment was done without differentiation between heterozygous and homozygous carriers due to small number of homozygotes for most SNPs. According to literature, risk alleles were defined as the variant associated with increased VTE risk [12,18]. Among the SNPs studied, rs2036914 in *F11* was the only one with a minor allele associated with reduced VTE risk, and in this case, we considered the common allele as the risk allele. Similar to de Haan et al., a 5-SNP score was conceived by counting the number of risk alleles from the five sequenced SNPs, with a theoretical maximum number of ten risk alleles for an individual (i.e. two risk alleles were counted for homozygous carriers) [18].

2.5. Statistical analysis

Subjects were followed from the date of their first lifetime VTE to the date of an incident MB, death, migration, or end of follow-up (i.e. 365 days after the first VTE), whichever came first. Subjects who died or migrated out of the municipality of Tromsø were censored at the date of the respective event. Statistical analyses were performed using STATA version 15.0 (Stata Corporation LP, College Station, TX, USA).

Based on the 5-SNP score, we created categories of 0–1, 2, 3 and ≥4 risk alleles instead of performing analyses per-risk-allele, due to small numbers. Crude incidence rates (IRs) with 95% confidence intervals (CIs) of MB were calculated for the individual SNPs and categories of the 5-SNP score, and expressed as number of events per 100 person-years at risk. Cox proportional hazards regression models were used to calculate hazard ratios (HRs) with 95% CIs for MB according to the individual SNPs (reference: 0 risk allele) and categories of the 5-SNP score (reference: 0–1 risk allele). HRs were adjusted for age and sex in a first model with the addition of planned duration of anticoagulant therapy in a second model. Risk estimates were adjusted for treatment duration because knowledge of thrombophilia or family history of early VTE onset might have resulted in the admitting physician planning longer treatment with anticoagulation, which could affect the risk of MB. In a separate sensitivity analysis, we also calculated HRs for MB by risk alleles restricted to the first three months after VTE diagnosis, when all subjects would be on anticoagulant treatment. For further sensitivity purposes, we assessed the risk of MB after excluding subjects with known cancer at time of VTE diagnosis, as cancer patients might be at increased risk of bleeding during anticoagulation [24]. Finally, we performed subgroup analyses according to clinical presentation (i.e. DVT or PE) and presence of provoking risk factors (i.e. provoked or unprovoked VTE events). The proportional hazard assumption was assessed by evaluating the parallelism of the log-log survivor function by categories of number of risk alleles, and tested with Schoenfeld residuals.

The 1-year cumulative incidences of MB across categories of the 5-SNP score were calculated and visualized in one minus Kaplan-Meier (1-KM) plots for the overall population and after excluding those with cancer at the time of VTE diagnosis.

3. Results

Baseline characteristics according to categories of prothrombotic risk alleles derived from the 5-SNP score are shown in Table 1. In the study population, the mean age and body mass index, the proportion of DVT and PE, and the planned duration of anticoagulation did not substantially differ across categories of risk alleles. Among patients in the highest category of the genetic score (i.e. ≥4 risk alleles), the proportion of men, subjects with active cancer at the time of VTE diagnosis and those treated with heparin tended to be lower, and the VTE events were more likely to be unprovoked in comparison with patients in the other categories.

Among the 676 patients with incident VTE, 50 had an MB within 1 year after the VTE event, resulting in an overall IR of 9.5 per 100 person-years (95% CI 7.2–12.5). The median time from incident VTE to MB was 33 days (interquartile range 11–180 days). Characteristics of MB events are presented in Table 2, showing that nearly half of the MB episodes was classified as major due to symptomatic bleeding in a critical area or organ.

As depicted in Fig. 1, the distribution of VTE patients across number of prothrombotic risk alleles of the 5-SNP score ranged from 0 to 6, with a median number of 3. The IRs and HRs for MB according to individual SNPs and categories of risk alleles of the 5-SNP score in the overall

Table 1
Baseline characteristics of venous thromboembolism (VTE) cases across categories of the 5-single nucleotide polymorphism (SNP) score.

	Number of risk alleles			
	0–1 (n = 109)	2 (n = 179)	3 (n = 205)	≥4 (n = 183)
Age (years), mean ± SD	71 ± 12	68 ± 14	69 ± 14	67 ± 13
Sex (males)	46.0 (50)	51.0 (92)	46.0 (94)	40.0 (74)
BMI (kg/m^2)[a], mean ± SD	27.2 ± 4.7	27.1 ± 4.8	27.4 ± 4.6	27.3 ± 4.3
DVT	56 (61)	56 (101)	59 (121)	57 (104)
PE ± DVT	44 (48)	44 (78)	41 (84)	43 (79)
Provoked VTE	63 (69)	63 (112)	57 (117)	51 (94)
Unprovoked VTE	37 (40)	37 (67)	43 (88)	49 (89)
Active cancer[b]	27 (29)	28 (50)	25 (52)	15 (28)
Initial thrombolytic therapy	6 (7)	4 (8)	5 (10)	5 (9)
Planned treatment type				
Heparin[c]	16 (17)	18 (32)	15 (30)	10 (19)
Heparin and VKA[d]	68 (74)	69 (124)	75 (153)	80 (147)
Planned duration of anticoagulation				
≤3 months	36 (39)	36 (65)	36 (73)	37 (67)
> 3 including 6 months	32 (35)	33 (59)	37 (76)	33 (61)
> 6 including 12 months	22 (24)	26 (47)	20 (42)	19 (35)
> 12 months	10 (11)	5 (8)	7 (14)	11 (20)

Abbreviations: body mass index (BMI); DVT, deep vein thrombosis; PE, pulmonary embolism; SD, standard deviation; VKA, vitamin K antagonist.
Categorical variables are shown as percentages with numbers in brackets, % (n).

[a] BMI was calculated as weight in kilograms divided by the square of height in meters (kg/m^2).
[b] Active cancer at the time of VTE diagnosis.
[c] Low molecular weight or unfractionated heparin.
[d] Low molecular weight or unfractionated heparin with VKA treatment.

Table 2
Characteristics of major bleeding in patients with venous thromboembolism.

Major bleeding site	% (n)
Gastrointestinal	36 (18)
Intramuscular/compartment syndrome	24 (12)
Intracranial	16 (8)
Urogenital	12 (6)
Retroperitoneal	6 (3)
Other[a]	6 (3)
ISTH major bleeding criteria	
Fatal bleeding[b]	6 (3)
Critical area or organ[c]	46 (23)
Blood transfusion[d] ± hemoglobin fall[e]	48 (24)

[a] Pericardial and subcutaneous/hematoma; ISTH, International Society on Thrombosis and Haemostasis.
[b] Fatal outcome within one week after major bleeding.
[c] Intracranial, retroperitoneal, pericardial and intramuscular with compartment syndrome.
[d] Bleeding leading to transfusion of ≥2 units of whole blood or red cells.
[e] Bleeding causing a fall in hemoglobin level of ≥20 g/L.

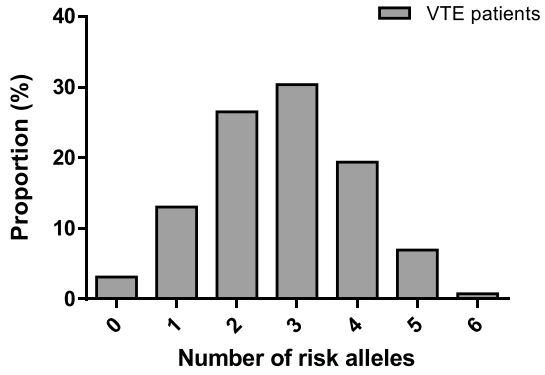

Fig. 1. Distribution (%) of patients with incident venous thromboembolism (VTE) across number of risk alleles of the 5-single nucleotide polymorphism (SNP) score.

population are shown in Table 3. None of the individual SNPs were associated with risk of MB. In the age- and sex-adjusted models, HRs for MB were within the range 1.0 to 1.1 for rs6025 (*F5*), rs8176719 (*ABO*), and rs2066865 (*FGG*). Further adjustment for planned duration of anticoagulation did not materially change risk estimates. For rs2036914 in *F11*, the HR for MB was 2.0 (95% CI 0.8–5.0) in the fully adjusted model, and for rs1799963 in *F2*, no individual carrying ≥1 risk allele experienced MB. Similarly to the individual SNPs, the number of prothrombotic risk alleles in the 5-SNP score had no impact on the risk of MB (Table 3). In the model adjusted for age, sex, and planned duration of anticoagulation, the HR for MB per category increase of genetic risk score was 1.0 (95% CI 0.8–1.3). Compared to subjects with 0–1 risk allele, the HRs for MB were 1.7 (95% CI 0.7–4.5), 1.6 (95% CI 0.6–4.0) and 1.3 (95% CI 0.5–3.5) in carriers of 2, 3 and ≥4 risk alleles, respectively. When analyses were restricted to the first 3 months of follow-up, again there was no consistent association of the individual SNPs or categories of the 5-SNP score with risk of MB (Supplementary Table 1).

In sensitivity analysis, in which we excluded patients with known active cancer at the time of VTE diagnosis, results were essentially similar to the main analysis, with no significant associations between prothrombotic genotypes and risk of MB (Table 4). Table 5 shows the risk of MB by categories of the 5-SNP score according to subgroups of VTE (provoked, unprovoked, DVT and PE). For unprovoked VTE, the HR for MB per category increase of genetic risk score was 1.0 (95% CI 0.6–1.6) in the fully adjusted model. Analyses of the other subgroups did not reveal any consistent association of MB risk across number of prothrombotic risk alleles.

The 1-year cumulative incidences of MB across categories of the 5-SNP score were estimated by 1-KM for the overall study population (Fig. 2A) and after excluding subjects with active cancer at the time of VTE diagnosis (Fig. 2B). As shown in Fig. 2A, the majority of the MB events occurred in the first 3 months after the VTE. The 3-month cumulative incidences of MB for subjects with 0–1, 2, 3 and ≥4 risk alleles were 2.0% (95% CI 0.5–7.9), 6.6% (95% CI 3.7–11.5), 6.7%, (95% CI 3.9–11.2), and 4.1% (95% CI 2.0–8.5), respectively. Similar results were found in patients without cancer (Fig. 2B).

Table 3
Risk of major bleeding (MB) by individual single nucleotide polymorphisms (SNPs) and number of prothrombotic risk alleles in patients with venous thromboembolism.

Risk allele	MB events (n = 50)	IR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]
SNP (gene)				
rs6025 (*F5*)				
0	43	9.6 (7.1–13.0)	Ref.	Ref.
≥1	7	8.5 (4.1–17.9)	1.0 (0.4–2.2)	1.0 (0.4–2.2)
rs1799963 (*F2*)				
0	50	9.7 (7.4–12.8)	Ref.	Ref.
≥1	0	–	–	–
rs8176719 (*ABO*)				
0	17	10.2 (6.3–16.4)	Ref.	Ref.
≥1	33	9.1 (6.5–12.8)	1.0 (0.5–1.7)	1.0 (0.5–1.7)
rs2066865 (*FGG*)				
0	26	9.1 (6.2–12.4)	Ref.	Ref.
≥1	24	9.8 (6.6–14.7)	1.1 (0.7–2.0)	1.2 (0.7–2.0)
rs2036914 (*F11*)				
0	5	5.3 (2.2–12.8)	Ref.	Ref.
≥1	45	10.4 (7.7–13.9)	2.0 (0.8–5.0)	2.0 (0.8–5.0)
5-SNP score				
0–1	6	7.0 (3.2–15.7)	Ref.	Ref.
2	15	11.3 (6.8–18.7)	1.7 (0.7–4.5)	1.7 (0.7–4.5)
3	17	10.8 (6.7–17.4)	1.6 (0.6–4.0)	1.6 (0.6–4.0)
≥4	12	7.9 (4.5–13.9)	1.3 (0.5–3.5)	1.3 (0.5–3.5)
Per category increase			1.0 (0.8–1.3)	1.0 (0.8–1.3)

[a] IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval.
[b] Adjusted for age and sex.
[c] Adjusted for age, sex and planned duration of anticoagulation.

Table 4
Risk of major bleeding (MB) by individual single nucleotide polymorphisms (SNPs) and number of prothrombotic risk alleles in patients with venous thromboembolism (VTE) without active cancer at the time of VTE diagnosis.

Risk allele	MB events (n = 35)	IR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]
SNP (gene)				
rs6025 (*F5*)				
0	28	7.5 (5.2–10.9)	Ref.	Ref.
≥1	7	9.5 (4.5–19.9)	1.5 (0.7–3.5)	1.6 (0.7–3.6)
rs1799963 (*F2*)				
0	35	8.1 (5.8–11.3)	Ref.	Ref.
≥1	0	–	–	–
rs8176719 (*ABO*)				
0	13	9.6 (5.5–16.5)	Ref.	Ref.
≥1	22	7.1 (4.7–10.6)	0.8 (0.4–1.5)	0.8 (0.4–1.5)
rs2066865 (*FGG*)				
0	17	7.1 (4.4–11.3)	Ref.	Ref.
≥1	18	8.8 (5.5–13.9)	1.3 (0.7–2.5)	1.4 (0.7–2.6)
rs2036914 (*F11*)				
0	2	2.6 (0.6–10.4)	Ref.	Ref.
≥1	33	8.9 (6.4–12.6)	3.6 (0.9–15.0)	3.5 (0.8–14.7)
5-SNP score				
0–1	4	5.8 (2.2–15.5)	Ref.	Ref.
2	8	7.2 (3.6–14.4)	1.4 (0.4–4.8)	1.4 (0.4–4.7)
3	14	10.9 (6.5–18.4)	2.0 (0.6–6.0)	2.0 (0.7–6.2)
≥4	9	6.5 (3.4–12.5)	1.3 (0.4–4.3)	1.3 (0.4–4.4)
Per category increase			1.1 (0.8–1.5)	1.1 (0.8–1.5)

[a] IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval.
[b] Adjusted for age and sex.
[c] Adjusted for age, sex and planned duration of anticoagulation.

Table 5
Risk of major bleeding (MB) by number of prothrombotic risk alleles in subgroups of venous thromboembolism.

DVT (n = 387)	MB events	IR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]
5-SNP score				
0–1	3	6.2 (2.0–19.2)	Ref.	Ref.
2	10	13.9 (7.5–25.8)	2.2 (0.6–8.0)	2.2 (0.6–8.1)
3	10	10.7 (5.7–19.8)	1.7 (0.5–6.2)	1.7 (0.5–6.2)
≥4	5	5.7 (2.4–13.1)	1.0 (0.2–4.0)	1.0 (0.2–4.0)
Per category increase			0.9 (0.6–1.3)	0.9 (0.6–1.3)
PE (n = 289)				
5-SNP score				
0–1	3	8.2 (2.6–25.4)	Ref.	Ref.
2	5	8.2 (3.4–19.6)	1.2 (0.3–5.3)	1.3 (0.3–5.5)
3	7	11.0 (5.3–23.1)	1.5 (0.4–5.7)	1.6 (0.4–6.1)
≥4	7	10.9 (5.2–22.9)	1.8 (0.5–7.0)	1.9 (0.5–7.6)
Per category increase			1.2 (0.8–1.8)	1.2 (0.8–1.9)
Provoked (n = 392)				
5-SNP score				
0–1	4	8.2 (3.1–21.7)	Ref.	Ref.
2	11	14.6 (8.1–26.4)	2.0 (0.6–6.3)	2.0 (0.6–6.5)
3	12	15.6 (8.9–27.5)	1.9 (0.6–6.0)	2.0 (0.6–6.2)
≥4	8	11.5 (5.7–23.0)	1.6 (0.5–5.4)	1.7 (0.5–5.6)
Per category increase			1.1 (0.8–1.5)	1.1 (0.8–1.5)
Unprovoked (n = 284)				
5-SNP score				
0–1	2	5.5 (1.4–22.1)	Ref.	Ref.
2	4	6.9 (2.6–18.3)	1.4 (0.3–7.9)	1.4 (0.3–7.9)
3	5	6.2 (2.6–14.9)	1.2 (0.2–6.3)	1.2 (0.2–6.3)
≥4	4	4.8 (1.8–12.9)	1.0 (0.2–5.6)	1.0 (0.2–5.6)
Per category increase			1.0 (0.6–1.6)	1.0 (0.6–1.6)

SNP, single nucleotide polymorphism; DVT, deep vein thrombosis; PE, pulmonary embolism.
[a] IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval.
[b] Adjusted for age and sex.
[c] Adjusted for age, sex and planned duration of anticoagulation.

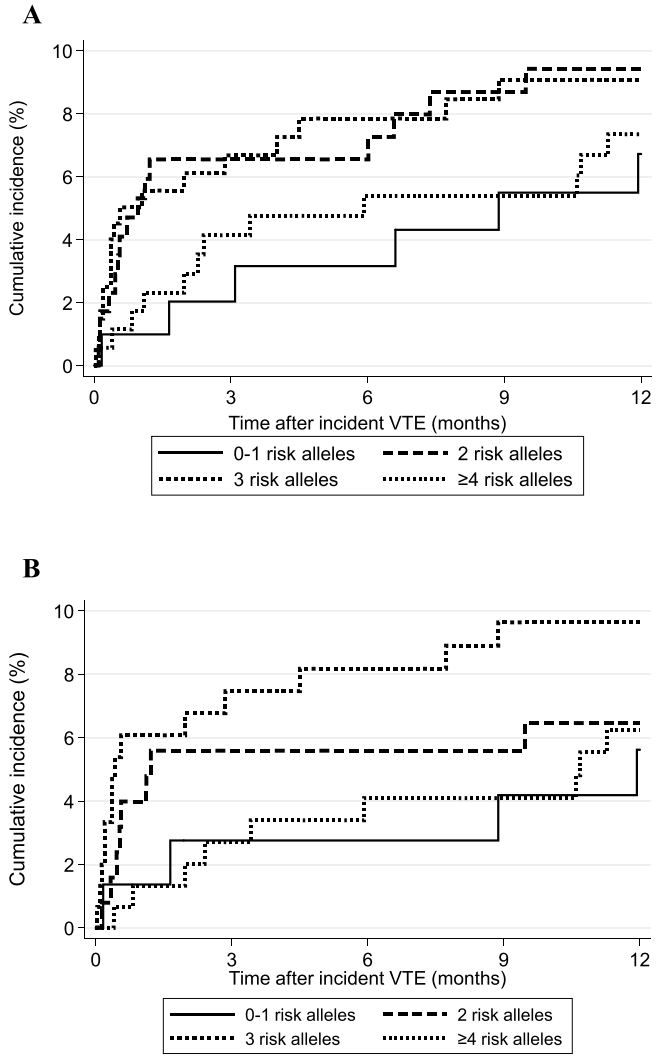

Fig. 2. Cumulative incidences (1-Kaplan-Meier) of major bleeding according to categories of risk alleles of the 5-single nucleotide polymorphism (SNP) score in overall venous thromboembolism (VTE) (A) and after excluding subjects with active cancer at the time of VTE diagnosis (B).

4. Discussion

In this population-based cohort of 676 patients followed for 1 year after their first lifetime VTE, we investigated whether the risk of MB was stepwise reduced in parallel with an increasing number of prothrombotic risk alleles. Using a genetic risk score based on five prothrombotic SNPs, we found that the number of risk alleles was not associated with risk of MB. Likewise, none of the individual prothrombotic SNPs were associated with risk of MB. Exclusion of patients with active cancer at the time of VTE diagnosis, restriction to the first 3 months after VTE diagnosis, or subgroup analyses stratified by unprovoked or provoked VTE and location (i.e. DVT or PE) yielded essentially similar results. Our findings suggest that a hypercoagulable state driven by prothrombotic genotypes is not able to restrain a severe bleeding associated with anticoagulant treatment.

In our study, originated from the general population, the overall rate of MB was 9.5 per 100 person-years, which was considerably higher than the rate of about 1.0 per 100 person-years reported in randomized controlled trials (RCTs) comprising VTE patients [2]. However, our results are not unexpected, as unselected patients derived from the general population have more often serious comorbidities and are managed under less intensive surveillance as compared to patients selected into RCTs. Moreover, RCTs on the efficacy and safety of anticoagulant treatment tend to exclude patients with a bleeding predisposition. Of note, 24% of our patients (159 out of 676) had active cancer at the time of VTE diagnosis, which is a well-known risk factor for bleeding during anticoagulation [24]. Our rate of MB is in line with a similar prospective cohort consisting of 842 VTE patients treated with anticoagulant therapy during daily care, of whom 21.5% had known cancer [7]. In the above mentioned study, the rate of MB was 10.6 per 100 person-years, and it was especially high in analysis restricted to VTE patients with cancer (15.7 per 100 person-year). Another noteworthy finding of our study was the fact that the majority of the MB events occurred within the first 3 months after VTE. These results are in accordance with previous data [25] and with the notion that patients with an underlying predisposition to bleeding are more likely to develop a MB event early after initiation of anticoagulation. Still, the prothrombotic genotypes studied were not associated with risk of MB even within the initial 3 months after VTE when the majority of MB events occurred.

The association between prothrombotic genotypes and risk of MB has been scarcely investigated in a VTE population under anticoagulant treatment [16,17]. In a Dutch case-control study that used data from the FACTors in ORal anticoagulation Safety (FACTORS) study [26], Garcia et al. [17] found that the risk of MB during anticoagulation with VKAs in non-OO blood group carriers was 30% lower than in carriers of OO blood group. Even though risk estimates pointed towards a protective effect (odds ratios = 0.7, 95% CI 0.4–1.1), the 95% CIs were somehow wide and included unity in the aforementioned report [17]. Furthermore, only 20% of 110 cases with MB and 220 controls without MB presented VTE as an indication for anticoagulation, and bleeding leading to hospitalization was also among the criteria to define MB [17,26]. Admission to hospital can be influenced by other important factors, such as support in the community and presence of comorbidities [22], thus not necessarily reflecting the severity of a bleeding event. In another study, Franchini et al. assessed the ABO blood group phenotypes instead of genotypes in 183 cases with bleeding complications and 366 controls without bleeding during treatment with VKA, of whom about 25% had VTE [27]. Even though bleeders met at most grade 2 (mild blood loss) of a 4-grade World Health Organization grading system, no association between ABO blood group and bleeding was found [27]. Our results interpreted in light of the existing studies indicate that there are no consistent data supporting a protective effect of non-O blood type on the risk of MB in VTE patients during anticoagulation.

In a study derived from the RIETE-registry comprising 10,139 VTE patients tested for thrombophilia, Tzoran et al. [16] investigated the impact of FVL on the risk of MB during anticoagulation. Compared to noncarriers, FVL carriers (n = 1384) had a 50% (HR 0.50, 95% 0.25–0.99) lower risk of MB. It is important to address that patients undergoing thrombophilia testing generally do not share the clinical characteristics of unselected VTE patients derived from a general population. For instance, in the above mentioned study [16], the proportion of VTE patients with cancer was relatively low (6%–11% among carriers and noncarriers of the prothrombotic genotypes studied) as compared to the present study (24%). Therefore, the study from Tzoran et al. is not necessarily comparable to our population-based cohort.

It is of interest that minor bleeding has been shown to be associated with subsequent increased risk of MB in patients treated with VKA, independent of the quality of anticoagulation and other known risk factors [28]. The association was confirmed and further explored by van Rein et al. [29], who used a case-crossover design to untangle the nature of such an association. Results from the case-crossover study suggested that minor bleeds could be markers for fixed risk factors for MB events, like genetic variants affecting blood coagulation. For instance, the mild hypercoagulable state associated with FVL [13] has been proposed to have offered evolutionary advantages in face of life-threatening bleeding, such as during childbirth, warfare, or other

activities carrying high risks of trauma [30]. However, in the context of bleeding related to anticoagulant treatment after a VTE, our study revealed that neither the individual SNPs nor the increasing number of prothrombotic risk alleles included in the 5-SNP score [18] were protective against an MB event in VTE patients. We can speculate that the mild hypercoagulability driven by FVL and the other prothrombotic genotypes would probably not be able to balance the mechanisms that induce an MB associated with anticoagulant treatment. Alternatively, our findings could be explained by a phenomenon called index event bias [31]. VTE is a result of multiple risk factors, and a selection of only incident VTE events (as in a cohort of VTE patients) may induce dependence between these risk factors. For example, an inverse association between carriership of prothrombotic genotypes and other prothrombotic factors could arise in the incident VTE population. If these other prothrombotic factors are also protective against bleeding, the effect measure of the risk of MB in carriers of prothrombotic genotypes compared to noncarriers may be shifted towards the null. Taken together, based on our findings, it is unlikely that the prothrombotic genotypes included in the 5-SNP score would be of clinical relevance to improve discrimination between those with high and low risk of MB during anticoagulant treatment in VTE patients.

The inclusion of subjects derived from the general population is among the main strengths of the present study. In comparison to subjects included in RCTs, the clinical characteristics, comorbidities and prevalence of cancer in our study participants are similar to those found in real-life patients, thereby increasing the external validity of our results. Other strengths include the complete and validated registry of VTE events, the exclusivity of UNN as the sole health care provider, likely to receive all relevant MB events, and the strict criteria used to define MB based on the ISTH recommendations [22]. Limitations also need to be addressed. Given our sample size and number of MB cases, the statistical power could have been limited to detect slight protective effects of prothrombotic genotypes, especially among those in the uppermost category of the genetic score (i.e. carriers of ≥ 4 risk alleles) and in subgroups of VTE. Of note, the risk estimates of MB were above 1.0 in some subgroups, but the 95% CIs of these estimates were considerably wide and included unity. It is important to address, that when the 5-SNP score was analyzed per category increase in genetic risk score, the risk estimates were 1.0 or around 1.0 both in overall and subgroup analyses, as well as in sensitivity analyses, thereby suggesting no protective effect of prothrombotic genotypes on MB risk in VTE patients. We were not able to perform analyses stratified by planned type of anticoagulant treatment (i.e. heparin or heparin and VKA) due to the low number of participants in the heparin group ($n = 98$) and the low number of MB events ($n = 10$). Moreover, we did not have information on concomitant use of drugs that might have affected the bleeding risk, such as antiplatelet agents. However, it is very unlikely that the type of anticoagulant, and the use of concomitant drugs, would differ across categories of prothrombotic risk alleles and thereby serve as potential confounders for the relationship between prothrombotic genotypes and risk of MB. In this study, we did not have access to information on the actual duration of anticoagulant treatment. Nonetheless, when we restricted our analyses to the first three months after VTE diagnosis (i.e. a period during which all patients would be on anticoagulant treatment), results were essentially similar to the overall analyses, showing no consistent association between prothrombotic risk alleles and risk of MB. Finally, an increasing number of prothrombotic risk alleles could still be protective against clinically relevant non-major bleedings (CRNMB). However, our study was not designed to evaluate CRNMB, and future research is required to address this question.

In conclusion, our study suggests that prothrombotic genotypes, evaluated either as individual SNPs or as number of risk alleles included in a 5-SNP score, do not protect against MB in VTE patients during anticoagulant treatment. Our findings further suggest that assessment of prothrombotic genotypes is not helpful to improve patient stratification of MB risk during anticoagulation for a VTE.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2020.04.008.

Addendum

H.S. Johnsen contributed to data collection, statistical analyses, data interpretation, and drafted the manuscript.

E. Bjøri and K. Hindberg contributed to statistical analyses, data interpretation, and revision of the manuscript.

S.K. Brækkan contributed to data collection, data interpretation and critical revision of the manuscript.

V.M. Morelli contributed to data interpretation and critical revision of the manuscript.

J.B. Hansen provided study concept and design, and contributed to data interpretation and critical revision of the manuscript.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgements

The K. G. Jebsen Thrombosis Research and Expertise Center is supported by an independent grant from Stiftelsen Kristian Gerhard Jebsen.

H. S. Johnsen receives a grant from the Northern Norway Regional Health Authority (Helse-Nord).

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Supplementary material

Supplementary Table 1 Risk of major bleeding (MB) by individual single nucleotide polymorphisms (SNPs) and number of prothrombotic risk alleles during the first three months after venous thromboembolism

Risk allele	MB events (n = 33)	IR (95% CI)	HR (95% CI) †
SNP (gene)			
rs6025 (*F5*)			
0	29	23.8 (16.6-34.3)	Ref.
≥1	4	18.0 (6.8-47.9)	0.8 (0.3-2.4)
rs1799963 (*F2*)			
0	33	23.5 (16.7-33.0)	Ref.
≥1	0	-	-
rs8176719 (*ABO*)			
0	9	19.7 (10.3-37.9)	Ref.
≥1	24	24.4 (16.4-36.4)	1.3 (0.6-2.8)
rs2066865 (*FGG*)			
0	15	19.3 (11.6-32.0)	Ref.
≥1	18	27.2 (17.1-43.2)	1.5 (0.7-3.0)
rs2036914 (*F11*)			
0	3	11.7 (3.8-36.4)	Ref.
≥1	30	25.3 (17.7-36.2)	2.2 (0.7-7.0)
5-SNP score			
0-1	2	8.2 (2.2-34.6)	Ref.
2	11	29.5 (16.5-53.7)	3.8 (0.8-17.2)
3	13	29.9 (17.4-51.6)	3.5 (0.8-15.7)
≥4	7	17.3 (8.2-36.3)	2.2 (0.5-10.7)
Per category increase			1.1 (0.8-1.5)

† Adjusted for age and sex

IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval

Paper II

D-Dimer Measured at Diagnosis of Venous Thromboembolism is Associated with Risk of Major Bleeding

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TH Open 2019;3:e77–e84.

Abstract

Identification of patients at risk of major bleeding is pivotal for optimal management of anticoagulant therapy in venous thromboembolism (VTE). Studies have suggested that D-dimer may predict major bleeding during anticoagulation; however, this is scarcely investigated in VTE patients. We aimed to investigate the role of D-dimer, measured at VTE diagnosis, as a predictive biomarker of major bleeding. The study population comprised 555 patients with a first community-acquired VTE (1994–2016), who were identified among participants from the Tromsø study. Major bleeding events were recorded during the first year after VTE and defined according to the criteria of the International Society on Thrombosis and Haemostasis. Cox-regression was used to calculate hazard ratios (HRs) with 95% confidence intervals (CIs) adjusted for age, sex, and duration of anticoagulant therapy. In total, 29 patients experienced major bleeding (incidence rate: 5.7/100 person-years, 95% CI: 4.0–8.2). The major bleeding risk was highest during the first 3 months, especially in patients with D-dimer $\geq$8.3 µg/mL (upper 20th percentile), with 28.8 major bleedings/100 person-years (95% CI: 13.7–60.4). Patients with D-dimer $\geq$8.3 µg/mL had a 2.6-fold (95% CI: 1.1–6.6) higher risk of major bleeding than patients with D-dimer $\leq$2.3 µg/mL (lower 40th percentile). Major bleeding risk according to D-dimer $\geq$8.3 versus $\leq$2.3 µg/mL was particularly pronounced among those with deep vein thrombosis (HR: 4.6, 95% CI: 1.3–16.2) and provoked events (HR: 4.2, 95% CI: 1.0–16.8). In conclusion, our results suggest that D-dimer measured at diagnosis may serve as a predictive biomarker of major bleeding after VTE, especially within the initial 3 months.

Keywords

- venous thromboembolism
- anticoagulants
- major bleeding
- D-dimer
- biomarker

Introduction

Anticoagulant therapy (AT) is the cornerstone in the treatment of venous thromboembolism (VTE). Extended AT effectively prevents recurrent events, but at the cost of bleeding complications.[1–3] The reported annual risk of major bleeding (MB) varies in the range of 1 to 4%[4–6] and is dependent on the choice of anticoagulant, intensity of anticoagulation, and duration of treatment.[6–8] The MB risk is particularly high within the first months of AT,[2] with a case-fatality rate of 11% during the initial 3 months of anticoagulation.[9]

received
September 24, 2018
accepted after revision
February 1, 2019

DOI https://doi.org/10.1055/s-0039-1683395.
ISSN 2512-9465.

Even though initially promising in derivation studies,[10–12] prediction models developed to stratify risk of MB in VTE patients have demonstrated inconsistent discriminative powers in validation studies.[13–16] The existing prediction models mainly apply the same traditional predictors for bleeding such as age, history of bleeding, previous stroke, and cancer.[10–12] Identification of novel predictors for bleeding in VTE patients is therefore an essential step for the development of a more accurate prediction score for MB, capable of guiding clinical decision-making in the future.

D-dimer, a global biomarker of activation of the coagulation and fibrinolytic systems, is useful to exclude a VTE-diagnosis in the diagnostic work-up of suspected acute VTE.[17] Moreover, elevated D-dimer is used to identify patients at high risk of VTE-recurrence after discontinuation of AT.[18,19] Interestingly, elevated D-dimer levels have also been shown in conditions associated with increased bleeding risk, such as disseminated intravascular coagulation and acute abdominal aortic dissection.[20,21] Furthermore, high D-dimer levels appeared to predict MB during AT.[22] However, studies on VTE patients are scarce,[23] and whether D-dimer measured at VTE diagnosis can be used to assess risk of MB is largely unknown. Information on D-dimer at the time of VTE diagnosis is easily available for most patients with community-acquired VTE, as D-dimer is frequently used for the diagnostic work-up of VTE.[24] We aimed to investigate the role of D-dimer, measured at VTE diagnosis, as a predictive biomarker of MB events during the first year after an incident VTE.

Methods

Study Population

The source population comprised subjects participating in $\geq$1 of the six currently completed surveys of the Tromsø study (Tromsø 1–6), who were still alive and inhabitants of Tromsø by January 1, 1994 (n = 33,885). The Tromsø study is a single-center, population-based prospective cohort, with repeated health surveys of the inhabitants of Tromsø, Norway.[25] Overall, participation rates were high, ranging from 85% in Tromsø 2 to 66% in Tromsø 6, with an average of 78.5% for the six surveys. The study was approved by the Regional Committee of Medical and Health Research Ethics, and all participants gave their informed written consent.

All potential first lifetime VTE cases were identified from January 1, 1994 to December 31, 2016 by searching the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry at the University Hospital of North Norway (UNN). The UNN is the only hospital serving the source population and all outpatient care for diagnostic assessment and treatment of VTE is exclusively provided at this hospital. The medical records of each potential VTE case were reviewed by trained personnel, and a VTE event was confirmed and registered as a validated VTE when clinical signs and symptoms of proximal or distal deep vein thrombosis (DVT) or pulmonary embolism (PE) were combined with objective confirmation by diagnostic procedures, and resulted in a VTE diagnosis requiring treatment, as described in detail previously.[26] Using the aforementioned strategy, a total of 986 objectively confirmed VTE cases were identified. D-dimer has low specificity for the diagnosis of VTE as it is often elevated in patients hospitalized for other conditions or with cancer.[27–29] We therefore excluded subjects with active cancer (n = 230) and those already hospitalized for other conditions (n = 108) when the VTE occurred. Moreover, subjects with high clinical suspicion leading to a VTE diagnosis without the aid of D-dimer measurement were also excluded (n = 93), leaving 555 VTE patients eligible for this study. These patients were followed for 365 days, and all bleeding events occurring in this period were recorded by thorough review of medical records.

Clinical Characteristics

Information on clinical and provoking factors at the time of and 12 weeks preceding the VTE diagnosis was obtained for all eligible patients. Patients with provoked VTE were those with major surgery, trauma, or an acute medical condition (acute myocardial infarction, ischemic stroke, or major infectious disease) within 12 weeks prior to VTE event, marked immobilization (confined to bed >3 days, wheelchair, or long-distance travel exceeding 4 hours within the last 14 days prior to VTE event), or any other factor specifically described in the medical records to have provoked the VTE (e.g., intravascular catheter).

Even though the study population was originated from a prospective cohort study (the Tromsø study), data collection for the present study was conducted retrospectively. To account for treatment duration in the present study, we considered the planned duration of anticoagulation that was objectively described by the attending physicians in the medical records at the time of VTE diagnosis. Duration of AT was categorized into 3, 6, and 12 months according to the preplanned length of AT. When the treatment duration was not specified by the treating physician (n = 42), subjects were categorized into 3 months if the incident event was a provoked DVT, 6 months if it was an unprovoked DVT, and 12 months if it was a PE.

Outcome Assessment of Major Bleeding

For each study participant, MB events occurring during the 365 days following the VTE were identified by thorough review of the medical records at the UNN. This hospital is the exclusive provider of advanced health care, including transfusion of blood products and emergency medicine in a vicinity of 250 km, and all subjects with a significant bleeding event in the Tromsø region are likely to be admitted at this hospital. Two reviewers (trained medical personnel from the UNN) adjudicated the bleeding events independently in accordance with the criteria proposed by the International Society on Thrombosis and Haemostasis.[30] In short, a bleeding event that was fatal, and/or symptomatic in a critical area or organ, and/or requiring blood transfusion of $\geq$2 units of red blood cells or causing a fall in hemoglobin level of $\geq$20 g/L was considered major. In case of disagreement, the event was discussed in an endpoint committee (H.S.J. and J.B.H.) to reach consensus.

D-Dimer Measurement

Blood samples were drawn for the diagnostic work-up of VTE, before initiation of AT. D-dimer was determined using two commercially available kits at the Department of Clinical Chemistry at the UNN,[31] and a D-dimer value <0.5 µg/mL was defined as a negative test in the diagnostic work-up of patients with suspected VTE. The NycoCard D-dimer assay (Nycomed Pharma, Oslo, Norway), based on immunometric flow-through principle, was used in the period 1994 to 1998. It was succeeded by the STA-Liatest D-Di assay (Diagnostica Stago, Asnières-sur-Seine, France) for the remaining period (1998–2016). The Stago assay quantified D-dimer by the immuno-turbidimetric method (liquid reagent) within a range of 0.27 to 20 µg/mL, which determined the levels of D-dimer available in this study.

Statistics

Subjects were followed from the date of their first VTE to the date of an incident MB, death, migration from Tromsø, or end of follow-up (i.e., 365 days after the first VTE), whichever came first. The patients were followed for 1 year, regardless of the length of anticoagulation. Thus, the follow-up time included both time-on and time-off anticoagulant treatment. Subjects who died or migrated were censored at the time of the respective event. Statistical analyses were performed with STATA version 15.0 MP (Stata Corp. College Station, Texas, United States).

D-dimer levels were initially divided into quintiles. The two lowest (Q1–2) and the two middle-upper (Q3–4) quintiles were combined to achieve better statistical power (i.e., a more robust reference category), and to enable the assessment of MB risk according to the highest D-dimer levels (Q5). The two lowest quintiles (Q1–2) were set as the reference.

Crude incidence rates (IRs) with 95% confidence intervals (CIs) of MB were calculated across D-dimer categories and expressed as number of events per 100 person-years at risk. Cox proportional hazards regression models were used to estimate hazard ratios (HRs) with 95% CIs for MB. The HRs were estimated using the following models: the first model was adjusted for age and sex, and the second was additionally adjusted for the planned duration of AT. Since a large severe thrombus could result in both high D-dimer levels and prolonged treatment, the treatment length was added as a potential confounder in the second model. The proportional hazards assumption was verified by evaluating the parallelism in the log–log survivor function by the categorical division of D-dimer. Further, the association between D-dimer levels and MB, adjusted for age, sex, and duration of AT, was visualized by a generalized additive regression plot using R version 3.4.4, to assess potential nonlinear effects of D-dimer levels on MB risk. D-dimer was modeled with a smoothing spline fit in a Cox proportional hazards model.

Due to potentially higher all-cause mortality rates in the upper D-dimer category, we additionally performed competing risk by death analyses and calculated subdistribution hazard ratios (SHRs) to limit overestimation of the relative risk differences of MB between D-dimer categories.[32,33] The 1-year cumulative incidences of MB across D-dimer categories were visualized in traditional one minus Kaplan–Meier (1-KM) plots and in cumulative incidence function plots corrected for competing risk by death.

We performed subgroup analyses stratified by clinical presentation (i.e., DVT and PE with or without DVT) and presence of provoking risk factors at the time of VTE diagnosis (i.e., unprovoked and provoked events). For overall VTE, we also assessed the risk of MB in analyses restricted to the first 3 months after VTE diagnosis (i.e., the period in which all patients would be on anticoagulant treatment). For sensitivity purposes, we performed analyses where we excluded patients who received thrombolytic therapy (systemic or catheter-directed) for VTE treatment, as these patients might be at increased bleeding risk.[3] We also did sensitivity analyses where patients were censored at the time they stopped anticoagulant treatment (estimated according to the planned duration of anticoagulation), to assess the risk of bleeding according to D-dimer restricted to the time on anticoagulant treatment.

Results

Baseline characteristics according to D-dimer categories are shown in ►**Table 1**. D-dimer levels were in the ranges of ≤2.3, 2.4 to 8.2, and ≥8.3 µg/mL in the lowest (Q1–2), middle (Q3–4),

Table 1 Baseline characteristics of venous thromboembolism (VTE) cases across categories of D-dimer

D-dimer, quintiles	Q1–2 (n = 225)	Q3–4 (n = 219)	Q5 (n = 111)
Range (µg/mL)	≤2.3	2.4–8.2	≥8.3
Age (y), mean ± SD	64 ± 15	67 ± 14	69 ± 14
Sex (males)	44.0 (99)	56.2 (123)	50.5 (56)
Previous stroke	3.6 (8)	5.5 (12)	10.8 (12)
Thrombolytic therapy	1.8 (4)	6.9 (15)	10.8 (12)
Planned duration of anticoagulation			
≤ 3 mo	24.9 (56)	16.9 (37)	9.9 (11)
> 3 including 6 mo	43.6 (98)	48.0 (105)	38.7 (43)
> 6 including 12 mo	24.4 (55)	26.0 (57)	37.8 (42)
> 12 mo	7.1 (16)	9.1 (20)	13.5 (15)
VTE characteristics			
DVT	57.8 (130)	60.7 (133)	46.0 (51)
PE ± DVT	42.2 (95)	39.3 (86)	54.0 (60)
Unprovoked	64.0 (144)	59.8 (131)	60.4 (67)
Provoked	36.0 (81)	40.2 (88)	39.6 (44)
Trauma	12.0 (27)	10.0 (22)	10.8 (12)
Surgery	12.4 (28)	13.7 (30)	12.6 (14)
Acute medical condition	4.4 (10)	7.8 (17)	13.5 (15)
Confined to bed >3 days	1.8 (4)	1.8 (4)	2.7 (3)

Abbreviations: DVT, deep vein thrombosis; mo, months; PE, pulmonary embolism; SD, standard deviation.
Note: Categorical variables are shown as percentages with numbers in brackets, % (n).

Table 2 Sites of major bleeding (MB) in patients with venous thromboembolism

Bleeding site	MB, % (*n*)
Intramuscular/compartment syndrome	27.6 (8)
Gastrointestinal	27.6 (8)
Intracranial	17.2 (5)
Urogenital	13.8 (4)
Other[a]	13.8 (4)

[a]Other sites of MB included pericardial, retroperitoneal, and subcutaneous (hematoma).

and upper (Q5) categories, respectively. The mean age and proportion of subjects with acute medical conditions preceding the VTE were higher in the upper than in the lower categories of D-dimer. Moreover, a higher proportion received thrombolytic therapy, and the planned duration of AT was longer in the highest category of D-dimer. The proportion of patients with PE was higher in the upper category, whereas DVTs were more frequent in the two lowest categories. Of note, missing information on duration of AT was similarly distributed across the lowest (7.6%), middle (7.3%), and upper (8.1%) D-dimer categories. The baseline characteristics of the overall study population can be found in ►**Supplementary Table S1** .

Of the 555 patients with incident VTE, 29 had a MB event within 1 year after the incident VTE, yielding an overall IR of 5.7 per 100 person-years (95% CI: 4.0–8.2). The median and mean times from VTE diagnosis to MB were 35 and 113 days, respectively. MBs that were intramuscular with symptoms of compartment syndrome and gastrointestinal bleedings were most frequent (27.6%), followed by intracranial MBs (17.2%) (►**Table 2**).

The 1-year cumulative incidences of MB across categories of D-dimer were estimated by 1-KM (►**Fig. 1A**), and in the presence of death as competing risk (►**Fig. 1B**), as displayed in ►**Fig. 1**. The cumulative incidence of MB was considerably higher for the upper D-dimer category than for the lower and middle D-dimer categories (►**Fig. 1A**). The results remained essentially similar after taking competing risk by death into account (►**Fig. 1B**). The majority of the MB events occurred in the first 3 months after the VTE, and the 3-month cumulative incidences of MB were 2.2%, 2.5%, and 6.8% for patients in the lower, middle, and upper categories of D-dimer, respectively (►**Fig. 1B**). At 12 months the cumulative incidences for the lower, middle, and upper categories were 3.6%, 4.1%, and 10.8%, respectively (►**Fig. 1B**).

The 3-month and 12-month IRs and HRs of MB in the overall population according to D-dimer categories are presented in ►**Table 3**. At 12 months, the crude IR of MB was 13.1 per 100 person-years (95% CI: 7.5–23.1) in the upper category of D-dimer, versus 4.6 (95% CI: 2.4–8.9) and 3.8 (95% CI: 1.9–7.5) per 100 person-years in the middle and lower categories, respectively. In the model adjusted for age, sex, and duration of AT, patients with a D-dimer in the upper category (≥8.3 µg/mL) had a 2.6-fold higher risk of MB (HR: 2.6, 95% CI: 1.1–6.6) compared with those with a D-dimer in the lowest category (≤2.3 µg/mL). When analyses were restricted to the first 3 months of follow-up, the crude overall IR of MB was 13.9 per 100 person-years (95% CI: 8.8–22.1), and likewise the risk increased across categories of D-dimer, with an IR in the upper category of 28.8 per 100 person-years (95% CI: 13.7–60.4). Exclusion of patients who received thrombolytic therapy ($n = 31$) yielded similar results as the main analyses (►**Supplementary Table S2**). The generalized additive regression plot revealed that the risk of MB started to increase at D-dimer levels >7.0 µg/mL (►**Fig. 2**).

Stratification according to specific subgroups (provoked, unprovoked, DVT, and PE) revealed that the association between D-dimer and MB was particularly pronounced among patients with DVTs and provoked events. In age-, sex-, and duration of AT-adjusted models, the HRs of MB according to D-dimer ≥8.3 versus ≤2.3 µg/mL were 4.6 (95% CI: 1.3–16.2) for patients with DVT and 4.2 (95% CI: 1.0–16.8) for those with provoked VTE (►**Table 4**). In contrast, a D-dimer ≥8.3 µg/mL was associated with a marginally increased risk of MB in patients with PE (HR: 1.7, 95% CI:

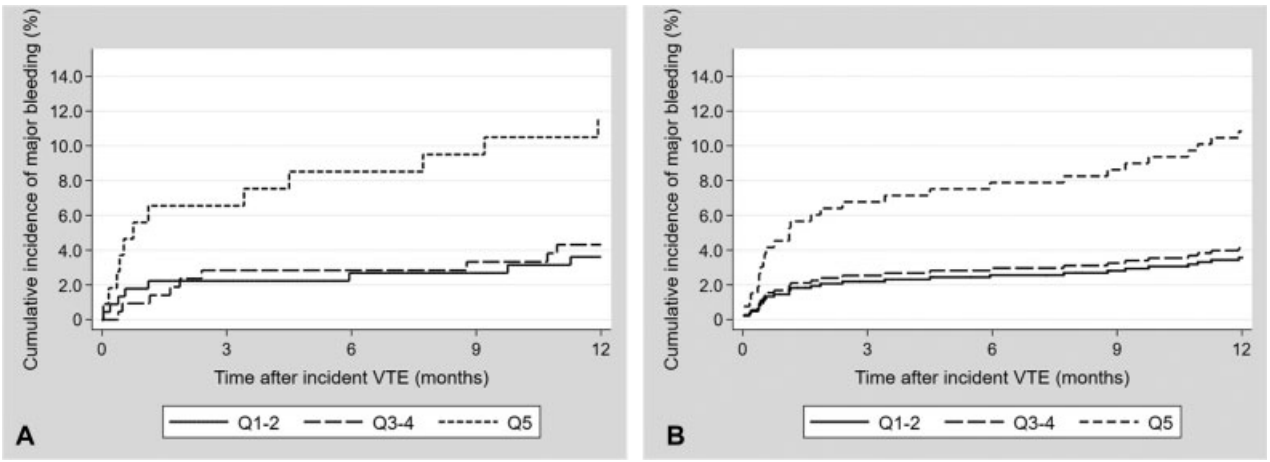

Fig. 1 One year cumulative incidence of major bleeding by categories of D-dimer estimated by 1-Kaplan–Meier (**A**) and in the presence of death as competing event (**B**).

Table 3 Incidence rates (IRs) and risk of major bleeding (MB) by categories of D-dimer at 12 and 3 months of follow-up after incident venous thromboembolism

12 mo	(D-dimer, μg/mL)	MB	IR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
Q1–2	≤2.3	8	3.8 (1.9–7.5)	Ref.	Ref.	Ref.
Q3–4	2.4–8.2	9	4.6 (2.4–8.9)	1.1 (0.4–2.9)	1.1 (0.4–2.8)	1.0 (0.4–2.6)
Q5	≥8.3	12	13.1 (7.5–23.1)	2.9 (1.1–7.1)	2.6 (1.1–6.6)	2.5 (1.0–6.3)
3 mo						
Q1–2	≤2.3	5	9.3 (3.9–22.4)	Ref.	Ref.	Ref.
Q3–4	2.4–8.2	6	11.7 (5.3–26.1)	1.2 (0.4–3.9)	1.2 (0.4–3.9)	1.2 (0.4–3.8)
Q5	≥8.3	7	28.8 (13.7–60.4)	2.6 (0.8–8.4)	2.6 (0.8–8.6)	2.5 (0.7–8.8)

Abbreviations: CI, confidence interval; HR, hazard ratio; mo, months; SHR, subdistribution hazard ratio.
[a]Per 100 person-years.
[b]Adjusted for age and sex.
[c]Adjusted for age, sex, and planned duration of anticoagulation. SHR denotes the HR after taking competing risk by death into account.

0.4–6.9) or unprovoked events (HR: 1.5, 95% CI: 0.4–5.9), but the results were not statistically significant. As in the overall analyses (►**Table 3**), the results were only slightly attenuated in the competing risk model (►**Table 4**), as demonstrated by the SHRs.

Among the 29 MB events, only one case occurred after the preplanned treatment length. Sensitivity analyses restricted to the time on anticoagulant treatment showed essentially similar results (►**Supplementary Table S3** and ►**Supplementary Fig. S1**).

Discussion

In this population-based cohort study of patients with a first lifetime community-acquired VTE, we found that patients with high D-dimer levels (≥8.3 μg/mL) had 2.6-fold higher risk of a MB during the 1 year of follow-up compared with those with D-dimer levels ≤2.3 μg/mL. The risk of MB was highest during the first few months after the VTE. In patients with high D-dimer, the 3-month cumulative incidence of MB was 6.8%, whereas the cumulative incidence for the entire 12-month follow-up was 10.8%. The risk of MB among patients with D-dimer ≥8.3 μg/mL was especially high for those with DVTs and provoked events. Our findings suggest that a high D-dimer value at VTE diagnosis identifies patients at increased risk of MB events, particularly in the initial phase of anticoagulant treatment.

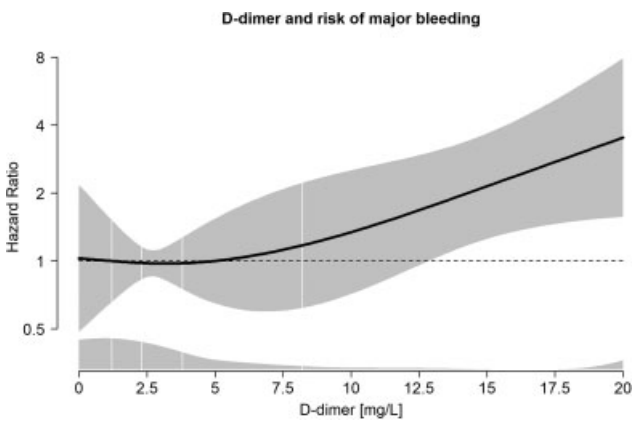

Fig. 2 The risk of major bleeding (MB) as a function of D-dimer adjusted for age, sex, and planned treatment duration in a generalized additive regression model. The solid line shows hazard ratios (HRs), enclosed by shaded area showing 95% confidence intervals. The distribution of D-dimer is shown as density plots at the bottom, and in quintiles at the vertical lines.

In the present study, the overall 1-year IR of MB was 5.7 per 100 person-years, which is comparable to previously reported rates of MB in patients treated with warfarin.[34] Consistent with previous data,[2] we found that the IR of MB was notably high during the first 3 months after VTE, especially in those with high D-dimer (28.8 MBs per 100 person-years). Possible explanations for the initially high bleeding risk may include overanticoagulation due to the wide intra- and interindividual variability in the dose-requirements in patients treated with vitamin K antagonists (VKAs),[35,36] and the initial administration of concomitant low molecular weight heparin. Furthermore, patients with a bleeding predisposition are more likely to experience a MB early after initiation of AT.[3]

Our results are consistent with previous studies on atrial fibrillation,[37,38] such as the ARISTOTLE-trial,[37] where patients with a D-dimer ≥1,123 μg/L had a twofold increased risk of MB compared with those with a D-dimer <423 μg/L. To date, only a few studies, with substantial differences in designs and sample sizes, have examined the association of D-dimer with risk of MB in VTE patients.[22,23] In line with our results, a study of 1,707 PE patients from the RIETE-registry found that a D-dimer ≥4.2 μg/mL was associated with increased risk of MB within 15 days of PE diagnosis.[23] In a study comprising 719 patients treated with VKA for at least 2 months before inclusion, of whom only 11% had VTE, Lind et al found that high D-dimer levels, measured during AT, were associated with increased risk of MB.[22] To the best of our knowledge, the present study is the first to provide data on the association between D-dimer and risk of MB in a cohort of community-acquired VTE, encompassing both DVT and PE, without active cancer at the time of VTE diagnosis.

Table 4 Incidence rates (IRs) and risk of major bleeding (MB) by categories of D-dimer in subgroups of venous thromboembolism

DVT ($n = 314$)	(D-dimer, μg/mL)	MB	IR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
Q1–2	≤2.3	4	3.2 (1.2–8.5)	Ref.	Ref.	Ref.
Q3–4	2.4–8.2	6	4.9 (2.2–10.9)	1.5 (0.4–5.3)	1.1 (0.3–4.1)	1.1 (0.3–3.9)
Q5	≥8.3	8	19.9 (10.0–39.8)	5.0 (1.4–17.7)	4.6 (1.3–16.2)	4.4 (1.1–18.1)
PE ($n = 241$)						
Q1–2	≤2.3	4	4.5 (1.7–11.9)	Ref.	Ref.	Ref.
Q3–4	2.4–8.2	3	3.8 (1.2–11.8)	0.8 (0.2–3.6)	0.8 (0.2–3.6)	0.8 (0.2–3.3)
Q5	≥8.3	4	7.5 (2.8–20.0)	1.7 (0.4–6.8)	1.7 (0.4–6.9)	1.5 (0.3–7.8)
Unprovoked ($n = 342$)						
Q1–2	≤2.3	5	3.6 (1.5–8.7)	Ref.	Ref.	Ref.
Q3–4	2.4–8.2	5	4.1 (1.7–9.9)	1.1 (0.3–3.9)	1.1 (0.3–3.9)	1.1 (0.3–3.7)
Q5	≥8.3	4	6.7 (2.5–17.8)	1.7 (0.5–6.5)	1.5 (0.4–5.9)	1.5 (0.3–6.1)
Provoked ($n = 213$)						
Q1–2	≤2.3	3	3.9 (1.3–12.2)	Ref.	Ref.	Ref.
Q3–4	2.4–8.7	4	5.0 (1.9–13.2)	1.0 (0.2–4.5)	0.8 (0.2–3.7)	0.8 (0.2–3.5)
Q5	≥8.8	8	23.9 (12.0–47.8)	4.2 (1.0–16.9)	4.2 (1.0–16.8)	3.6 (0.9–14.8)

Abbreviations: CI, confidence interval; DVT, deep vein thrombosis; HR, hazard ratio; PE, pulmonary embolism; SHR, subdistribution hazard ratio.
[a]Per 100 person-years.
[b]Adjusted for age and sex.
[c]Adjusted for age, sex, and planned duration of anticoagulation. SHR denotes the HR after taking competing risk by death into account.

In our study, the risk of MB in patients with high D-dimer levels was most pronounced among those with provoked events. It is reasonable to assume an overestimation of the MB risk for comorbidities with high mortality rates.[33,39,40] However, the risk estimates for MB remained essentially similar when the competing risk of death was taken into account. Hence, our findings suggest that the association between high D-dimer and MB risk in subjects with provoked VTE could not be explained by an overestimation due to high mortality rates. Still, other medical conditions associated with increased risk of provoked DVT, but not necessarily with higher mortality within the first year after an incident VTE, could be relevant for the association between D-dimer and MB.[29,41]

Several studies have shown that elevated D-dimer is associated with increased risk of recurrence in patients with unprovoked VTE.[18,19] Using data from the Tromsø study, we recently reported that a low D-dimer (≤1.5 μg/mL), measured at first VTE diagnosis, was associated with a low recurrence risk, particularly among patients with DVTs and unprovoked events.[31] In the present study, we found that a high D-dimer, also measured at VTE diagnosis, was associated with risk of MB. Intuitively, for the purpose of discriminating between VTE patients at high risk of either MB or recurrent VTE, such a predictive factor may appear noninformative, as D-dimer is associated in the same direction for both outcomes. However, the risk of MB and recurrence seemed to be most pronounced in different parts of the spectrum of D-dimer values. Only the very high levels of D-dimer (>7.0 μg/mL) were predictive of MB, whereas the recurrence risk did not further increase for D-dimer levels >1.5 μg/mL (threshold effect).[31] Moreover, the risk of MB and recurrence displayed different patterns in cumulative incidence curves. For recurrence, the risk gradually increased over years in patients with a D-dimer ≥1.5 μg/mL,[31] whereas for MB, the risk rapidly increased within the first 3 months after the incident VTE in patients with a D-dimer ≥8.3 μg/mL.

Our findings have potential clinical implications for the management of VTE during anticoagulation. D-dimer may be used as a predictive biomarker for MB to guide decisions on duration of AT, particularly in combination with clinical predictors of MB.[13] Importantly, this may be achieved without the need for additional blood sampling or cost as D-dimer is measured at VTE diagnosis in most patients. Given that the absolute risk and potential to prevent MB is highest during the first few months of anticoagulation, D-dimer may have clinical utility for the short-term management of VTE. Improved assessment of individual bleeding risk may impact clinical management, such as the choice of anticoagulant drug, careful supervision of anticoagulation, prompt investigation of minor gastrointestinal or urogenital bleeding to eliminate possible sources of future MB, or avoidance of concomitant therapies that may cause bleeding (e.g., antiplatelet agents and nonsteroidal anti-inflammatory drugs).[42] Moreover, after a first unprovoked event, identification of patients at high risk of bleeding is of utmost importance for decisions on extended duration of AT.[43] Even though we found relatively weak associations between D-dimer levels and risk of MB in patients with unprovoked events, the potential of D-dimer as a contributing building block in a prediction model in this specific patient group remains to be determined. Finally, during the study period, the majority of the patients in our study were treated with VKAs, as direct oral anticoagulants (DOACs)

became available for clinical practice in Norway around 2012. Therefore, future studies are needed to confirm if D-dimer also is a predictive biomarker of bleeding in patients treated with DOACs.

The inclusion of subjects derived from the general population is among the main strengths of the present study. In contrast, bleeding complications are often studied as a safety outcome in randomized clinical trials, which tend to include selected patients compared with those from population-based studies, whose clinical characteristics more likely reflect real-life patients. Other strengths include the prospective design, complete and validated registry of VTE events, and the exclusivity of UNN as the sole health care provider, likely to receive all relevant MB events. The study also has some limitations. Our results are not generalizable to patients already hospitalized for other conditions or with active cancer when the VTE occurred. For this patient group, however, D-dimer may already be of limited clinical utility given its reduced specificity in the diagnostic work-up of VTE.[27,28] Even though the UNN is the sole health care provider within a geographically well-defined region, we cannot rule out the unlikely possibility that a MB event was not captured due to the retrospective collection of data. In this study, we did not have access to information on the actual duration of anticoagulant treatment, and we based our adjustments on the preplanned treatment length. This could have led to misclassification of treatment length in some patients. Nevertheless, when we restricted our analyses to the first 3 months after VTE diagnosis (i.e., the period in which all patients would be on anticoagulant treatment), the results were essentially similar to the overall analyses, showing an increased risk of MB associated with a high D-dimer value ($\geq$8.3 µg/mL). Unfortunately, we did not have information on the concomitant use of drugs that might affect the bleeding risk, such as the use of antiplatelet medication. Approximately 14% of eligible patients were excluded because of missing D-dimer values. Of these, there were three MB events, yielding an IR of 3.5 per 100 person-years (95% CI: 1.1–10.9), which was lower compared with the rate of the included population. However, this rate was based on few MBs, and clinical characteristics were not widely different in those with measured and missing D-dimer (data not shown). Taken together, missing values of D-dimer was presumably at random, and would unlikely introduce selection bias. We used two different assays to measure D-dimer levels, which might have led to misclassification due to varying analytical properties across D-dimer assays.[44] However, the STA-Liatest, which has consistently reported excellent analytical properties,[45,46] was used for 93.5% of the study population, thus limiting misclassification. Moreover, in sensitivity analysis restricted to subjects with D-dimer determined by the STA-Liatest, the results remained essentially the same (data not shown). Finally, our results should be interpreted with caution due to low numbers of MB events and limited statistical power, mainly in subgroup analyses.

In conclusion, our findings suggest that high levels of D-dimer ($\geq$ 8.3 µg/mL), measured at the time of first VTE diagnosis, identify patients at increased risk of MB, particularly during the first 3 months of AT. Future studies are warranted to confirm our findings and to investigate whether D-dimer at VTE diagnosis could improve risk stratification of MB when added to existing prediction models.

Funding
K.G. Jebsen TREC is supported by an independent grant from Stiftelsen K.G. Jebsen. The publication charges for this article have been funded by a grant from the publication fund of UiT - The Arctic University of Norway.

Conflict of Interest
None declared.

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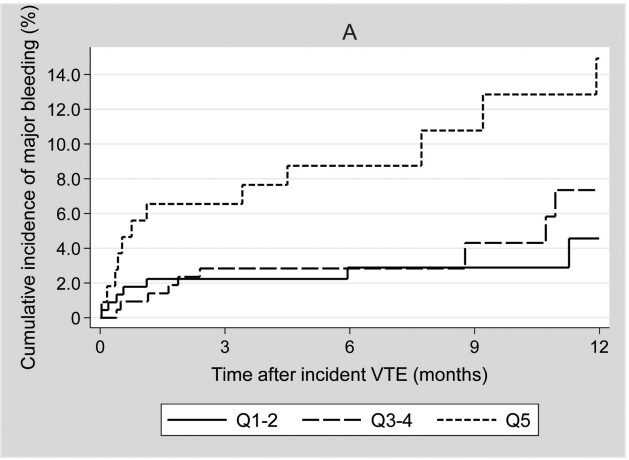
Supplementary Table S1 Baseline characteristics of the overall population

Number of patients	555
Age (y), mean ± SD	66 ± 15
Sex (males)	50.0 (278)
Previous stroke	5.8 (32)
Thrombolytic therapy	6.6 (31)
Duration of anticoagulation	
≤ 3 mo	18.7 (104)
> 3 including 6 mo	44.3 (246)
> 6 including 12 mo	27.8 (154)
> 12 mo	9.2 (51)
VTE characteristics	
DVT	56.6 (314)
PE ± DVT	43.4 (241)
Unprovoked	61.6 (342)
Provoked	38.4 (213)
Trauma	11.0 (61)
Surgery	13.0 (72)
Acute medical condition	7.6 (42)
Confined to bed >3 days	2.0 (11)

Abbreviations: DVT, deep vein thrombosis; mo, months; PE, pulmonary embolism; SD, standard deviation.
Note: Categorical variables are shown as percentages with numbers in brackets, % (*n*).



Supplementary Fig. S1 One year cumulative incidence of major bleeding by categories of D-dimer estimated by 1-Kaplan–Meier with follow-up restricted to time on anticoagulant therapy.

Supplementary Table S2 Incidence rates (IRs) and risk of major bleeding (MB) by categories of D-dimer after incident venous thromboembolism, with the exclusion of patients who received thrombolytic treatment ($n = 31$)

Overall ($n = 524$)	(D-dimer, µg/mL)	MB	IR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
Q1–2	≤2.3	8	3.8 (1.9–7.6)	Ref.	Ref.	Ref.
Q3–4	2.4–8.2	9	4.8 (2.5–9.2)	1.2 (0.4–3.0)	1.1 (0.4–3.0)	1.1 (0.4–2.8)
Q5	≥8.3	10	11.8 (6.4–22.0)	2.6 (1.0–6.7)	2.4 (0.9–6.3)	2.2 (0.8–6.0)

Abbreviations: CI, confidence interval; HR, hazard ratio; SHR, subdistribution hazard ratio.
[a]Per 100 person-years.
[b]Adjusted for age and sex.
[c]Adjusted for age, sex, and planned duration of anticoagulation.

Supplementary Table S3 Incidence rates (IRs) and risk of major bleeding (MB) by categories of D-dimer with follow-up restricted to time on anticoagulant therapy

	(D-dimer, µg/mL)	MB	IR (95% CI)[a]	HR (95% CI)[b]	SHR (95% CI)[b]
Q1–2	≤2.3	7	5.7 (2.7–12.1)	Ref.	Ref.
Q3–4	2.4–8.2	9	7.2 (3.7–13.8)	1.2 (0.4–3.3)	1.2 (0.5–3.2)
Q5	≥8.3	12	17.8 (10.1–31.4)	2.9 (1.1–7.4)	2.7 (1.0–7.1)

Abbreviations: CI, confidence intervals; HR, hazard ratio; SHR, subdistribution hazard ratio.
Note: Time on anticoagulant therapy was determined according to the planned duration of anticoagulation described in the medical records at venous thromboembolism diagnosis.
[a]Per 100 person-years.
[b]Adjusted for age and sex.

Paper III

platelets

http://www.tandfonline.com/iplt
ISSN: 0953-7104 (print), 1369-1635 (electronic)

Platelets, Early Online: 1–9

DOI: https://doi.org/10.1080/09537104.2020.1769052

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Platelet count and risk of major bleeding in venous thromboembolism

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Abstract

The relationship between platelet count and risk of major bleeding in patients with venous thromboembolism (VTE) during anticoagulation remains unclear. We therefore investigated the association between platelet count, measured at VTE diagnosis and before the thrombotic event, and risk of major bleeding. Participants comprised 744 patients with incident VTE derived from the Tromsø Study. Major bleedings were recorded during the first year after VTE. Cox-regression was used to calculate hazard ratios (HRs) for major bleeding across platelet count quartiles.

There were 55 major bleedings (incidence rate 9.1/100 person-years, 95% confidence interval [CI] 7.0–11.8). The major bleeding risk increased across quartiles of platelet count measured at VTE diagnosis (*P* for trend<0.02). In the age- and sex-adjusted model, subjects with platelet count in the highest quartile (≥300x10^9/L) had a 4.3-fold (95% CI 1.7–10.9) higher risk of major bleeding compared to those with platelet count in the lowest quartile (≤192x10^9/L), and exclusion of patients with cancer yielded similar results. When platelet count was measured on average 7 years before a VTE, the corresponding HR was 2.5 (95% CI 0.9–6.7). Our results suggest that increasing platelet count, assessed several years before and at VTE diagnosis, is associated with a higher risk of major bleeding, and could be a stable individual marker of major bleeding risk in VTE-patients.

Keywords

anticoagulation, hemorrhage, bleeding, platelet count, venous thromboembolism

History

Received 21 January 2020
Revised 8 May 2020
Accepted 8 May 2020
Published online 6 June 2020

Introduction

Major bleeding events are feared and severe complications of anticoagulant therapy associated with high costs, morbidity and mortality in the treatment of venous thromboembolism (VTE) [1–4]. Depending on type, intensity and duration of anticoagulation, major bleeding has been reported to occur annually in 3 to 9 per 100 person-years in non-interventional studies of VTE patients [5–7]. The assessment of major bleeding risk is essential to guide decisions regarding treatment duration in unprovoked VTE [3]. Furthermore, an accurate risk stratification of major bleeding may identify patients at high risk of bleeding, who would benefit from targeted preventive measures during the initial period of anticoagulant treatment. Known risk factors for major bleeding are predominantly of clinical nature, such as advanced age, active cancer and co-morbidities, which display only modest discriminatory ability when combined in risk assessment models for major bleeding risk [8–10]. The addition of biomarkers to clinical risk factors and age improved prediction of major bleeding in patients with atrial fibrillation in the ABC-model [11]. Therefore, biomarkers could be promising candidates to improve discrimination between those at high and low risk of major bleeding during anticoagulant treatment in VTE.

Platelets are potential attractive biomarkers for bleeding given their crucial role in hemostasis [12], and the fact that measurement of platelet count is inexpensive and easily obtainable. However, the relationship between platelet count and risk of major bleeding in VTE patients remains unclear. For instance, previous data have shown that both low and high platelet counts were associated with increased risk of major bleeding in VTE patients [13,14]. Elucidating the role of platelet count in the risk of major bleeding may be challenging, as several conditions associated with VTE can affect platelet count but also increase the bleeding risk during anticoagulation, such as cancer, liver disease, major surgery, and trauma [3,15–18]. It is noteworthy that even though environmental factors influence platelet count, genetics largely contribute to variation in platelet-related phenotypes [19]. Indeed, family and twin studies indicate a high heritability of platelet count and indices related to platelet size, including mean platelet volume (MPV) [19–21]. Moreover, the intra-individual variation in platelet count has been shown to be substantially less than the inter-individual variation in healthy subjects [22]. In light of available data, platelet count appears to be a stable phenotype within an individual over time.

As a potentially stable phenotype, an individual's platelet count could influence the predisposition to bleeding during exposure to anticoagulant therapy. In order to clarify the association of platelet count with risk of major bleeding in VTE patients under

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anticoagulant treatment, we hypothesized that platelet count measured at the time of VTE diagnosis and several years before the thrombotic event were both associated with major bleeding. To address our study hypothesis, we investigated the association between platelet count, measured at VTE diagnosis, and risk of major bleeding during the first year after an incident VTE. Then, using the same study population, we explored whether platelet count assessed several years prior to the incident VTE was associated with major bleeding.

Methods

Study Population

Study participants originated from the fourth (1994–95), fifth (2001–02) and sixth (2007–08) surveys of the Tromsø study, a single-center, population-based cohort in Tromsø, Norway [23]. Members of the population living in the municipality of Tromsø were invited to participate in the surveys, and altogether 30 371 unique individuals aged 25–97 years participated. Identification and subsequent adjudication of potential VTE cases from the source population have been previously described in detail [24]. In short, potential VTE cases were identified by searching the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry at the University hospital of North Norway (UNN). Identified cases were adjudicated by trained personnel and only included when signs and symptoms of deep vein thrombosis (DVT) or pulmonary embolism (PE) were combined with objective confirmation by radiological procedures that resulted in a VTE diagnosis requiring treatment. The study was approved by the Regional Committee of Research and Medical Health Ethics, and all study participants provided informed written consent.

From the date of inclusion in one of the three surveys until December 31 2016, a total of 918 participants developed an incident VTE (Figure 1). Seventeen participants who died on the same day of their VTE diagnosis were excluded, leaving 901 eligible study participants. Among these, 29 participants did

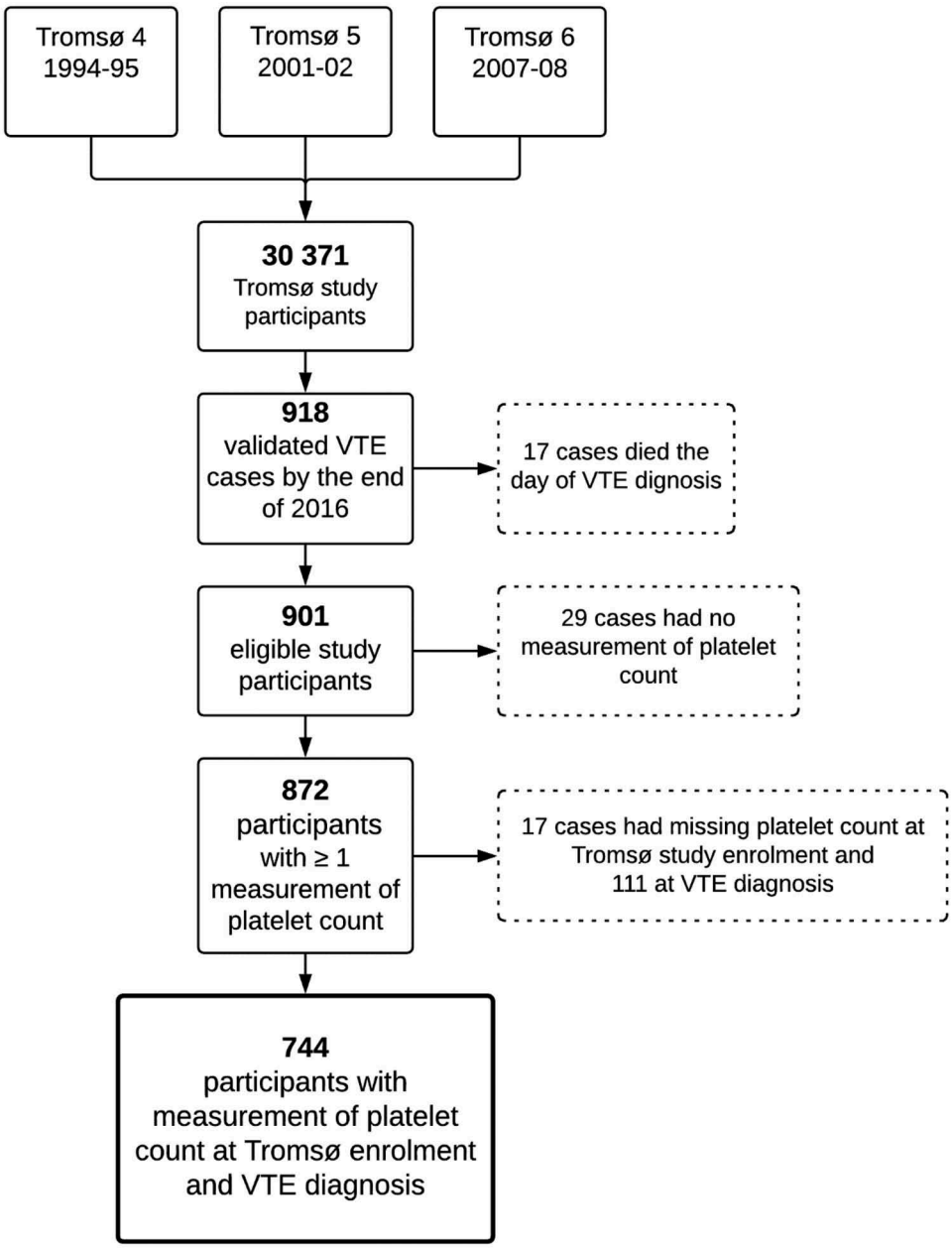

Figure 1. Flowchart illustrating the composition of the study population. VTE, venous thromboembolism.

not have any measurement of platelet count. Of the remaining 872 participants, 17 had missing platelet count values at Tromsø study enrollment and 111 at VTE diagnosis and were therefore excluded. The resulting study population consisted of 744 VTE cases with available platelet count measurement at Tromsø study enrollment and VTE diagnosis (Figure 1).

Clinical Characteristics of VTE Events

Medical records were searched at time of and 12 weeks preceding the VTE diagnosis for clinical information on VTE characteristics and provoking factors. Factors that classified the VTE event as provoked were major surgery, trauma or acute medical conditions (acute myocardial infarction, ischemic stroke, or major infectious disease) within 12 weeks prior to VTE event, marked immobilization (confined to bed >3 days, wheel-chair, or long-distance travel exceeding 4 hours within the last 14 days prior to VTE event), or any other factor(s) specifically described in the medical records to have provoked the VTE (e.g. intravascular catheter or plaster cast). Presence of known active cancer at the time of VTE diagnosis was regarded as a provoked VTE. In the case of a concurrent DVT and PE diagnosis, the VTE event was classified as a PE.

The presence of comorbidities, such as hypertension, renal dysfunction, and anemia, was assessed in medical records of VTE patients. A systolic blood pressure above 160 mmHg defined hypertension. The estimated glomerular filtration rate (eGFR) was calculated with the chronic kidney disease epidemiology collaboration equation based on creatinine levels, age, gender and race [25]. Anemia was defined as a hemoglobin level below 11.5 g/dL for women and below 13.0 g/dL for men at VTE diagnosis. A history of bleeding was recorded if a previous bleeding event was specifically noted in the medical records of VTE cases.

To account for type and duration of VTE treatment, we considered the planned treatment (i.e. heparin, vitamin k antagonist [VKA] or direct oral anticoagulant) and duration of anticoagulation that were stated by the attending physicians in the medical records at the time of VTE diagnosis. Duration of anticoagulant therapy was categorized into 3, 6, 12, and more than 12 months, as previously described [26].

Platelet Count Measurements

Measurement of platelet-related phenotypes at Tromsø study enrollment, i.e. platelet count and MPV, has previously been described elsewhere [24]. Briefly, non-fasting blood samples were collected from an antecubital vein into 5-mL vacutainers containing EDTA as an anticoagulant (K3- EDTA 40 μL, 0.37 mol/L per tube), and analyzed within 12 hours in an automated blood cell counter (Coulter Counter®, Coulter Electronics, Luton, UK). For the platelet count measurement at VTE diagnosis, we considered the first blood sample drawn for the diagnostic work-up of VTE, as described in the medical records of each VTE patient at the UNN. According to the protocol of the Department of Clinical Chemistry at the UNN, blood samples were collected in vacutainers containing EDTA.

Major Bleeding Events

The medical records for all study participants were searched for bleeding events during the 365 days following the VTE at the UNN. All second-line care and advanced emergency medicine, such as transfusion of blood products, is exclusively provided by the UNN. The UNN is situated in the middle of Tromsø municipality, with a vicinity of approximately 250 km to the nearest hospital providing comparable health-care functions. Two reviewers (trained medical personnel from the UNN) adjudicated the bleeding events independently in accordance with the criteria proposed by the International Society on Thrombosis and Hemostasis (ISTH) [27]. In short, a bleeding event that was fatal, and/or symptomatic in a critical area or organ, and/or requiring blood transfusion of ≥2 units of whole blood or red blood cells or causing a fall in hemoglobin level of ≥20 g/L, was considered major. In case of disagreement, the event was discussed in an endpoint committee (HSJ and JBH) to reach consensus.

Statistical Analyses

Subjects were followed from the date of their first VTE to the date of an incident major bleeding, death, migration, or end of follow-up (i.e. 365 days after the first VTE), whichever came first. Subjects who died or migrated out of the municipality of Tromsø were censored at the time of the respective event. Statistical analyses were performed with STATA version 15.0 MP (Stata Corp. College Station, Texas, United States).

Platelet count was divided into quartiles based on platelet count distribution measured at VTE diagnosis, and the first quartile was set as the reference. Crude incidence rates (IRs) with 95% confidence intervals (CIs) of major bleeding were calculated across quartiles of platelet count and expressed as number of events per 100 person-years at risk. Cox proportional hazards regression models were used to estimate hazard ratios (HRs) with 95% CIs for major bleeding. HRs were adjusted for age and sex in a first model, with the addition of body mass index (BMI) and planned duration of anticoagulation therapy to a second model. Risk estimates were adjusted for treatment duration because knowledge of platelet count at VTE diagnosis might have influenced the decision on preplanned treatment length. In a final fully adjusted model, we included surgery, acute medical conditions, eGFR, hypertension, history of bleeding and anemia. The proportional hazards assumption was assessed by evaluating the parallelism of the log-log survivor function across quartiles of platelet count, and tested using Schoenfeld residuals. In order to assess potential non-linearity between platelet count and major bleeding risk, the association was visualized by a generalized additive regression plot using R version 3.6.1. Platelet count was modeled with a smoothing spline fit in a Cox model adjusted for age, sex, BMI and planned duration of treatment.

The risk of death has been reported to be higher in elderly with high and low platelet counts compared to those with a normal platelet count [28]. We therefore additionally performed competing risk by death analyses and calculated the sub-distribution hazard ratios (SHRs) to limit overestimation of the relative risk differences of major bleeding between platelet count categories [29]. The 1-year cumulative incidences of major bleeding across platelet count quartiles were visualized in traditional one minus Kaplan-Meier (1-KM) plots and in cumulative incidence function plots corrected for competing risk by death.

In addition to possible chemotherapy-induced thrombocytopenia, cancer has the potential to induce thrombocytosis [17]. Cancer is also a well-known risk factor for bleeding during anticoagulation in VTE [3], and we therefore conducted a sensitivity analysis excluding all patients with active cancer at VTE diagnosis. Using a similar rationale, we stratified analyses according to provoking status, as cases with provoked VTE are more likely to be exposed to factors that may affect platelet count and risk of major bleeding, such as major surgery and trauma [3,18].

Platelet count measured at Tromsø study enrollment was categorized using the same cutoff values as for platelet count at VTE diagnosis. HRs for major bleeding were adjusted for age and sex in a first model, and BMI was added to a second model. It is well established, and previously shown in the Tromsø study [24], that platelet count is negatively correlated with MPV, an indice of platelet size. Platelet size is regarded as a marker of platelet function, with studies showing that large platelets are more

reactive and adhere and aggregate faster *ex vivo* than small platelets [30,31]. Therefore, differences in platelets size could potentially explain the association between platelet count and major bleeding. In order to investigate the potential of platelet size, measured as MPV, to mediate the association between platelet count and major bleeding, we further adjusted HRs for MPV measured at Tromsø study enrollment.

Results

Baseline characteristics across quartiles of platelet count measured at VTE diagnosis are presented in Table I. The mean age and proportion of male subjects decreased across increasing quartiles of platelet count. Subjects in the highest quartile were more likely to have anemia, active cancer, provoked VTE, recent surgery and acute medical conditions compared to those in the lower quartiles. The planned treatment type and duration of anticoagulant therapy did not appear to vary in any consistent manner across quartiles of platelet count.

Among the 744 patients with incident VTE, there were 55 major bleeding events within 1 year of VTE diagnosis during 605 person-years (IR 9.1 per 100 person-years, 95% CI 7.0–11.8), with a median time from VTE events to major bleeding of 35 days (interquartile range [IQR] 11–183 days). Major bleeding characteristics and classification according to ISTH criteria are presented in Supplemental Table I. Three bleeding events were fatal (within 1 week), and 40% of the major bleedings were symptomatic in critical areas or organs.

The crude IRs and relative risks for major bleeding according to quartiles of platelet count measured at VTE diagnosis are presented in Table II. IRs for major bleeding increased across quartiles of platelet count, from 3.8 per 100 person-years (95% CI 1.7–8.6) in the lowest category (≤192 x10^9/L) to 12.8 per 100 person-years (95% CI 8.1–20.3) in the highest category (≥300 x10^9/L). Likewise, in the age- and sex-adjusted model, HRs for major bleeding increased with increasing platelet count in a dose-response fashion. Compared to the first quartile, HRs for major bleeding were 2.7 (95% CI 1.0–6.9), 3.1 (95% CI 1.2–8.0) and 4.3 (95% CI 1.7–10.9) for quartiles 2 to 4, respectively. Further adjustment for BMI and planned duration of anticoagulant treatment yielded essentially similar results (Table II). With additional adjustments for surgery, acute medical conditions, eGFR, hypertension, history of bleeding and anemia, the risk estimates were somewhat attenuated, with an HR of 3.4 (95% CI 1.3–8.8) for the highest vs. the lowest quartile of platelet count, with virtually no changes in risk estimates after taking the presence of death as competing event (HR 3.2, 95% CI 1.2–8.6). In all models of the overall population, there was a trend (*P* for trend ≤0.02) for increased risk of major bleeding by increasing quartiles of

Table I. Baseline characteristics according to quartiles of platelet count measured at venous thromboembolism diagnosis.

	Quartiles of platelet count (10^9/L)			
	≤192 (n = 192)	193–239 (n = 192)	240–299 (n = 174)	≥300 (n = 186)
Age (years), mean±SD	71 ± 12	70 ± 13	70 ± 13	66 ± 14
Sex, males	61 (117)	51 (98)	42 (73)	40 (75)
BMI[a] (kg/m^2), mean±SD	27.2 ± 4.4	27.6 ± 4.3	27.7 ± 4.9	27.2 ± 5.0
Hypertension[b]	18 (34)	18 (35)	22 (38)	12 (23)
eGFR (ml/min/1.73^2), mean±SD	67.1 ± 24.4	70.7 ± 22.8	73.6 ± 23.1	78.6±24.5
Previous stroke	8 (15)	6 (11)	8 (14)	9 (16)
History of bleeding[c]	4 (8)	6 (11)	4 (7)	9 (17)
Anemia[d]	36 (69)	26 (49)	29 (51)	47 (87)
Active cancer[e]	26 (49)	19 (36)	21 (37)	31 (57)
VTE characteristics				
DVT	55 (106)	58 (111)	62 (108)	56 (105)
PE ± DVT	45 (86)	42 (81)	38 (66)	44 (81)
Unprovoked	47 (91)	44 (85)	47 (82)	33 (61)
Provoked	53 (101)	56 (107)	53 (92)	67 (125)
Trauma	9 (18)	8 (16)	9 (15)	9 (17)
Surgery	10 (20)	12 (23)	16 (28)	21 (39)
Acute medical conditions[f]	11 (21)	12 (23)	9 (16)	19 (35)
Immobilization[g]	24 (47)	24 (46)	18 (31)	20 (38)
Initial thrombolytic therapy	7 (13)	4 (8)	4 (7)	4 (7)
Planned treatment type				
Heparin[h]	21 (40)	18 (34)	16 (28)	23 (43)
Heparin[h] and VKA	63 (120)	71 (136)	68 (118)	61 (114)
DOAC	6 (11)	3 (6)	7 (13)	6 (12)
Planned duration of anticoagulation				
≤3 months	19 (36)	23 (45)	26 (46)	30 (55)
>3 including 6 months	39 (75)	42 (80)	37 (64)	28 (52)
>6 including 12 months	29 (56)	26 (49)	24 (42)	27 (51)
>12 months	13 (25)	9 (18)	13 (22)	15 (28)

Categorical variables are shown as percentages with numbers in brackets, % (n).
SD, standard deviation; BMI, body mass index; VKA, vitamin K antagonist; DOAC, direct oral anticoagulant; DVT, deep vein thrombosis; PE, pulmonary embolism; eGFR, estimated glomerular filtration rate.
[a]BMI was calculated as weight in kilograms divided by the square of height in meters (kg/m^2) in the Tromsø study.
[b]Systolic blood pressure >160 mmHg.
[c]A history of bleeding was present if a previous bleeding event was specifically noted in the medical records of VTE cases.
[d]hemoglobin <11.5 g/dL for women and <13.0 g/dL for men.
[e]Active cancer at the time of VTE diagnosis.
[f]Acute myocardial infarction, ischemic stroke or major infectious disease within 12 weeks prior to VTE.
[g]Confined to bed >3 days, wheel-chair, or long-distance travel exceeding 4 hours within the last 14 days prior to VTE event.
[h]Low molecular weight heparin or unfractionated heparin.

Table II. The 1-year risk of major bleeding according to quartiles of platelet count measured at venous thromboembolism diagnosis.

Quartiles of platelet count (10^9/L)	Major bleeding	IR (95% CI)	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
All VTE patients						
≤192	6	3.8 (1.7–8.6)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193–239	15	9.2 (5.6–15.3)	2.7 (1.0–6.9)	2.7 (1.0–7.0)	2.4 (0.9–6.3)	2.5 (1.0–6.5)
240–299	16	11.0 (6.7–17.9)	3.1 (1.2–8.0)	3.2 (1.2–8.2)	2.8 (1.1–7.4)	3.1 (1.2–8.0)
≥300	18	12.8 (8.1–20.3)	4.3 (1.7–10.9)	4.4 (1.7–11.2)	3.4 (1.3–8.8)	3.2 (1.2–8.6)
p for trend			.002	.002	.01	.02
VTE patients without cancer						
≤192	5	4.1 (1.7–9.8)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193–239	9	6.3 (3.3–12.2)	1.9 (0.6–5.6)	2.0 (0.7–5.9)	1.8 (0.6–5.4)	1.9 (0.6–5.7)
240–299	9	7.2 (3.7–13.8)	2.0 (0.7–6.1)	2.1 (0.7–6.4)	1.6 (0.5–5.2)	1.8 (0.5–6.1)
≥300	13	11.6 (6.8–20.0)	4.0 (1.4–11.4)	4.2 (1.5–12.1)	2.8 (0.9–8.5)	2.9 (0.9–9.5)
p for trend			.01	.01	.08	.09

IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval; SHR, sub-distribution hazard ratio.
[a]Adjusted for age and sex.
[b]Adjusted for age, sex, body mass index and planned duration of anticoagulant therapy.
[c]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery, acute medical conditions (acute myocardial infarction, ischemic stroke, or major infectious disease), estimated glomerular filtration rate, hypertension (systolic blood pressure >160 mmHg), history of bleeding (specifically noted in the medical records of VTE cases), and anemia (hemoglobin level below 11.5 g/dL for women and below 13.0 g/dL for men at VTE diagnosis).
SHR denotes the HR after taking competing risk by death into account.

platelet count. In the sensitivity analysis, the risk estimates were slightly attenuated after excluding patients with cancer, and the HRs were 1.9 (95% CI 0.6–5.7), 1.8 (95% CI 0.5–6.1) and 2.9 (95% CI 0.9–9.5) for quartiles 2 to 4, respectively, compared to the first quartile in the fully adjusted model (Table II). Supplemental Table II describes the stepwise adjustment for the aforementioned covariates for the overall population and those without cancer. In analyses stratified according to planned type of anticoagulant, the results did not appear to differ substantially between subjects treated with heparins+VKAs compared to subjects treated with heparins only, even though there were relatively few individuals in the heparin group (Supplemental Table III).

Figure 2a shows the risk of major bleeding as a continuous function of platelet count. As indicated in the density plot, the major bleeding risk increased linearly within the 25–75th percentile range of platelet count (192–299 x 10^9/L). The 1-year cumulative incidences of major bleeding across quartiles of platelet count were estimated by 1-KM (Figure 3a), and by the cumulative incidence function in the presence of competing risk by death (Figure 3b). The cumulative incidences of major bleeding increased with increasing quartiles of platelet count (Figure 3a), and the results remained essentially similar after taking competing risk by death into account (Figure 3b). The majority of the major bleeding events occurred in the first 3 months after the VTE, and the 3-month cumulative incidences of major bleeding were 1.6%, 4.3%, 5.2% and 6.2% for quartiles 1–4, respectively (Figure 3b). Of note, in the six major bleeding events occurring among patients in the lowest quartile, platelet counts ranged from 123 to 181 x 10^9/L.

In subgroups (Table III), the HRs for major bleeding in patients with provoked VTE were similar in quartiles 2–4 compared to the reference, ranging from 3.5 (95% CI 1.0–12.7) to 4.5 (95% CI 1.3–16.0) in the age- and sex-adjusted models. For unprovoked VTE, subjects with a platelet count in the two highest quartiles had a 2- to 3-fold higher risk of major bleeding compared to those with a platelet count in the first quartile, but CIs were wide and included unity.

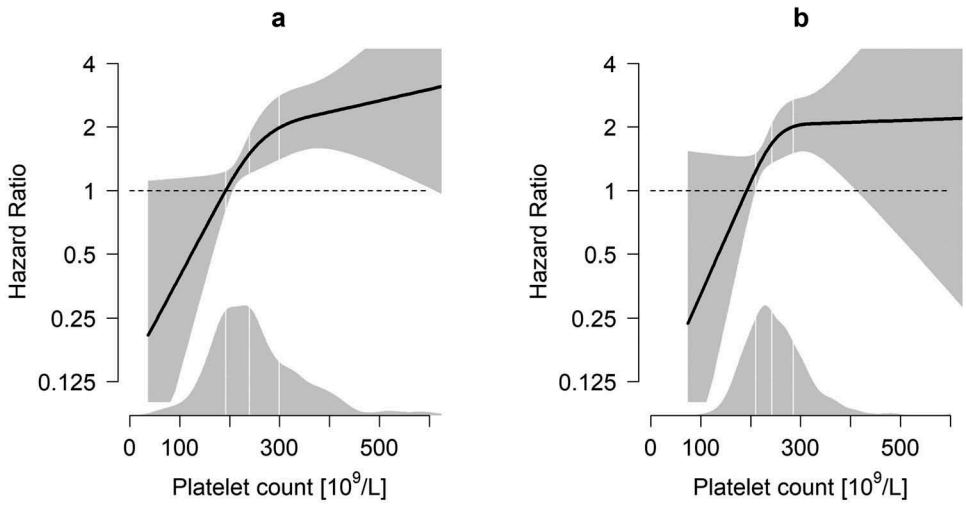

Figure 2. The risk of major bleeding as a continuous function of platelet count measured at venous thromboembolism diagnosis (a, adjusted for age, sex, body mass index and planned duration of anticoagulation) and at Tromsø study enrollment (b, adjusted for age, sex and body mass index) in a generalized additive regression model. The solid line shows hazards ratios, enclosed by shaded areas indicating 95% confidence intervals. The distribution of platelet count is shown in a density plot at the bottom, and vertical lines indicate quartile cutoffs.

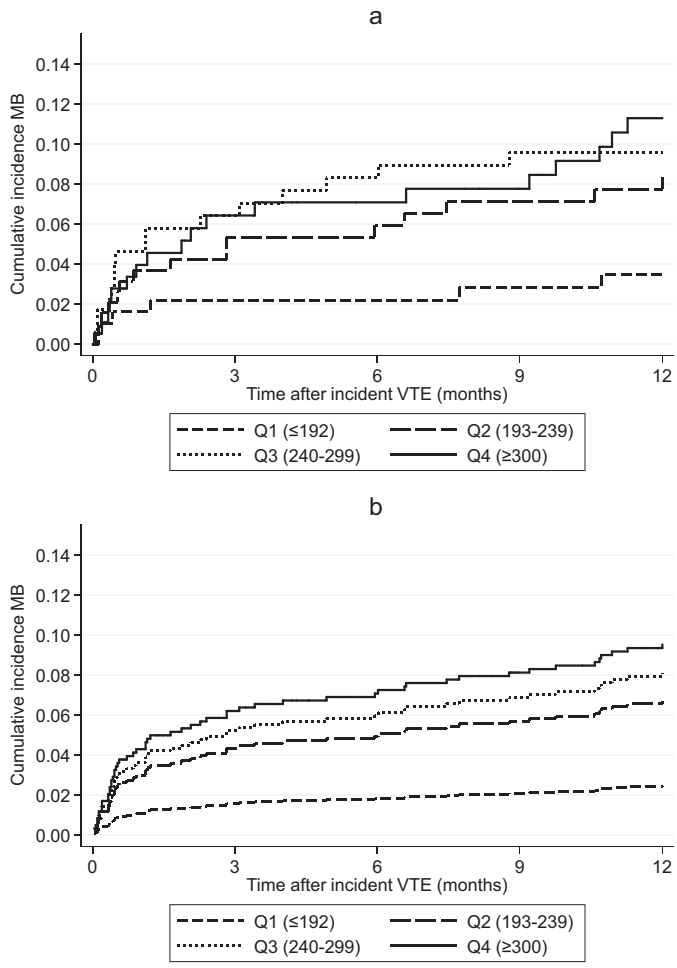

Figure 3. One-year cumulative incidence of major bleeding by quartiles of platelet count measured at venous thromboembolism diagnosis estimated by 1-Kaplan-Meier (a) and in the presence of death as competing event (b).

In our cohort, the median time from blood sampling in Tromsø 4–6 to VTE diagnosis was 6.8 years (IQR 3.1–9.2). There was a positive correlation between platelet count assessed at Tromsø study enrollment and VTE diagnosis (Spearman's rho = 0.52, $P < .001$). Moreover, as expected, platelet count at Tromsø study enrollment was negatively associated with MPV (Spearman's rho = −0.42, $P < .001$). As described in Table IV, the risk of major bleeding increased with increasing platelet count measured several years before the incident VTE. In the age- and sex adjusted model, subjects in the highest category of platelet count (i.e. ≥300 x 10^9/L) had a 2.5-fold (HR 2.5, 95% 0.9–6.7) higher risk of major bleeding after developing a VTE compared to subjects with a platelet count in the lowest category (i.e. ≤192 x 10^9/L). Risk estimates did not materially change after adjusting for BMI, but were somehow attenuated when MPV was added to the regression models, mainly for the highest category of platelet count (HR 2.0, 95% CI 0.7–5.6). When the risk of major bleeding was visualized as a function of platelet count measured at Tromsø study enrollment, the association displayed a pattern similar to the results for platelet count assessed at VTE diagnosis (Figure 2b).

Discussion

In this study, we found that an increasing platelet count measured at VTE diagnosis was associated with a higher risk of major bleeding in a dose-response fashion during the first year after the VTE. Exclusion of patients with active cancer and subgroup analyses stratified by provoked and unprovoked VTE yielded similar results. When platelet count was measured in the same study participants several years prior to the development of the incident VTE, an increasing platelet count was also associated with a higher risk of major bleeding. Our results suggest that platelet count is a stable phenotype within an individual over time that may influence the susceptibility to bleeding during anticoagulant treatment after a VTE.

In our study of patients derived from the general population, the overall major bleeding rate of 9.1 per 100 person-years was considerably higher than the rate of about 1.0 per 100 person-years found in randomized controlled trials (RCTs) involving VTE patients [32]. However, unselected patients derived from the general population have more often serious comorbidities and are managed under less intensive surveillance as compared to patients selected into RCTs. In addition, RCTs evaluating the efficacy and safety of anticoagulants are more likely to exclude patients with a bleeding predisposition. Notably, 24% of our patients had active cancer at the time of VTE diagnosis, which is an established risk factor for bleeding during anticoagulation [3]. Our rate of major bleeding is in agreement with a prospective cohort comprising 842 VTE patients treated with anticoagulant therapy in the community [33]. In this study, the rate of major bleeding was 10.6 per 100 person-years, and it was particularly high in analysis restricted to VTE patients with cancer (15.7 per 100 person-year). Another clinically relevant finding in our study was the fact that the median time from incident VTE to major bleeding was 35 days, with the majority of the major bleeding events occurring within the first 3 months after VTE. These results are consistent with previous data [1] and underscore the concept that patients with an underlying predisposition to bleeding are more likely to develop a major bleeding shortly after initiation of anticoagulant therapy.

To the best of our knowledge, we are the first to investigate the association of platelet count, measured within the same subjects prior to and at VTE diagnosis, with risk of major bleeding. A few previous studies have explored the relationship between platelet count and major bleeding in VTE [13,14,34]. In a study comprising 3012 VTE patients, Yamashita *et al*. assessed the influence of platelet count at VTE diagnosis on the risk of major bleeding [34], and reported a higher risk of major bleeding in patients with a moderate to severe thrombocytopenia (<100 x 10^9/L) compared to those with no thrombocytopenia (>150 x 10^9/L). Apparently, these findings are in contrast with our results. However, the study by Yamashita *et al*. is not necessarily comparable to the present study. For example, the platelet count cutoffs were determined according to clinical preferences in their study (i.e. 100 x 10^9/L and 150 x 10^9/L) and these cutoffs are included within the lowest quartile in our analyses. Furthermore, the authors did not treat death as a competing risk in their analyses, which could have led to an overestimation of the association between thrombocytopenia and major bleeding. The relationship between platelet count measured at VTE diagnosis and major bleeding was also investigated in the RIETE registry [13,14]. Di Micco *et al*. assessed the three-month risk of major bleeding in 43078 VTE patients according to categories of platelet counts ranging from <80 x 10^9/L to >450 x 10^9/L [13]. In patients with and without cancer, both the highest (>450 x 10^9/L) and lowest (<80 x 10^9/L) categories were associated with increased risk of major bleeding when compared to a platelet count ranging from 150 to 300 x 10^9/L [13]. Similar to our findings, Di Micco *et al*. found that a high platelet count was associated with increased risk of major bleeding.

Here we found a dose-response relationship between an increasing platelet count, assessed at VTE diagnosis, and risk of major bleeding. Although the statistical power in sensitivity and

Table III. The 1-year risk of major bleeding according to quartiles of platelet count measured at venous thromboembolism diagnosis in provoked and unprovoked cases.

Quartiles of platelet count (10^9/L)	Major bleeding	IR (95% CI)	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
Provoked						
≤192	3	4.0 (1.3–12.5)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193–239	13	15.8 (9.2–27.2)	4.1 (1.2–14.4)	4.3 (1.2–15.1)	3.5 (1.0–12.5)	3.8 (1.1–13.6)
240–299	10	14.0 (7.5–25.9)	3.5 (1.0–12.7)	3.7 (1.0–13.6)	3.3 (0.9–12.4)	3.6 (1.0–13.2)
≥300	13	15.4 (8.9–26.5)	4.5 (1.3–16.0)	4.7 (1.3–16.6)	3.7 (1.0–13.5)	3.4 (1.0–12.3)
p for trend			.04	.04	.08	.08
Unprovoked						
≤192	3	3.7 (1.2–11.4)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193–239	2	2.5 (0.6–10.0)	0.8 (0.1–4.7)	0.8 (0.1–4.6)	0.7 (0.1–4.5)	0.7 (0.1–4.1)
240–299	6	8.1 (3.6–18.0)	2.4 (0.6–10.1)	2.3 (0.6–9.9)	1.7 (0.4–7.6)	1.8 (0.3–9.9)
≥300	5	8.9 (3.7–21.4)	3.3 (0.8–14.4)	3.3 (0.8–14.6)	2.7 (0.6–12.4)	2.7 (0.5–13.7)
p for trend			.05	.05	0.1	0.2

IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval; SHR, sub-distribution hazard ratio.
[a]Adjusted for age and sex.
[b]Adjusted for age, sex, body mass index and planned duration of anticoagulant therapy.
[c]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, estimated glomerular filtration rate, hypertension (systolic blood pressure >160 mmHg), history of bleeding (specifically noted in the medical records of VTE cases), and anemia (hemoglobin level below 11.5 g/dL for women and below 13.0 g/dL for men at VTE diagnosis)
SHR denotes the HR after taking competing risk by death into account.

Table IV. The 1-year risk of major bleeding in patients with venous thromboembolism according to platelet count measured at Tromsø study enrollment.

Categories of platelet count (10^9/L)	Major bleeding	IR (95% CI)	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
≤192	6	6.0 (2.7–13.3)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193–239	18	9.3 (5.9–14.8)	1.7 (0.7–4.2)	1.7 (0.7–4.2)	1.6 (0.6–4.0)	1.5 (0.6–3.8)
240–299	19	9.7 (6.2–15.2)	1.9 (0.8–4.9)	1.9 (0.8–4.9)	1.7 (0.6–4.4)	1.6 (0.6–4.1)
≥300	12	10.5 (5.9–18.4)	2.5 (0.9–6.7)	2.5 (0.9–6.8)	2.0 (0.7–5.6)	1.9 (0.6–5.5)
p for trend			.08	.07	.2	.3

IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval; SHR, sub-distribution hazard ratio.
[a]Adjusted for age and sex.
[b]Adjusted for age, sex and body mass index.
[c]Adjusted for age, sex, body mass index and mean platelet volume.
Categories of platelet count measured at Tromsø study enrollment were established using the same cutoff points of platelet count assessed at VTE diagnosis.
SHR denotes the HR after taking competing risk by death into account.

subgroup analyses were limited due to the lower number of participants, cancer or other comorbidities with high mortality rates did not appear to substantially contribute to this relationship, as risk estimates were only slightly attenuated after excluding patients with active cancer and when competing risk of death was taken into account [29]. Other comorbidities or conditions, including surgery, acute medical conditions (acute myocardial infarction, ischemic stroke and major infectious disease), hypertension, renal function (i.e. eGFR), history of bleeding and anemia appeared to partially explain the relationship, as adjustment for these conditions had a marginal impact on the risk estimates, with an increasing platelet count still being associated with a higher risk of major bleeding. Moreover, a positive association between platelet count and major bleeding risk was found not only in patients with provoked VTE but also in those with unprovoked events, albeit less pronounced in the latter group with the 95% CIs of risk estimates including unity. Thus, transient factors related to provoked VTEs that can induce an increase in platelet count, like major surgery or trauma [18], did not seem to contribute to a great extent to the relationship between platelet count and major bleeding.

In the present study, a platelet count measured on average 7 years prior to the incident VTE still showed a dose-response relationship with major bleeding in analyses adjusted for age, sex, and BMI, although the confidence intervals of risk estimates included unity. When compared to the lowest quartile of platelet count, the highest quartile was associated with a 2.5-fold (95% CI 0.9–6.8) higher risk of major bleeding. Furthermore, the platelet count measured at VTE diagnosis displayed a significant positive correlation with the platelet count measured several years before the thrombotic event. Such findings reinforce the notion that platelet count is a stable phenotype within an individual over time, as previously demonstrated by others [20,22]. This notion is consistent with the high degree of heritability reported for some platelet-related phenotypes, including platelet count and indices of platelet size, such as MPV [19–21]. In our study, platelet count and MPV, measured at Tromsø study enrollment, showed a negative moderate correlation, and the impact of platelet count on major bleeding risk was somehow attenuated after adjusting for MPV. This finding suggests that differences in platelet size could partly explain the association between platelet count and major bleeding. Large and small platelets have been shown to substantially differ in their functional roles in the hemostatic system.

Compared to small platelets, large platelets are associated with increased reactivity, shortened bleeding time, faster adhesion to collagen and aggregation *ex vivo*, and increased expression of glycoproteins on their membranes [30,31]. Reticulated platelets, which are large and hyperreactive platelets, display a prothrombotic profile, as recently revealed in transcriptome analysis [35]. Moreover, results from epidemiological studies, including the Tromsø Study, show that an increased MPV is associated with a higher risk of arterial cardiovascular disease [36] and VTE [24], thereby supporting the higher prothrombotic potential of large platelets [24]. It is of interest that in the presence of substantial thrombocytopenia (<20 x 10^9/L), a low MPV has been shown to be a stronger predictor of bleeding than platelet count [37]. In light of these data, we can speculate that an increasing platelet count, even within a normal range, would be associated with a lower platelet reactivity, as reflected by a decrease in MPV, which could predispose to major bleeding during anticoagulant treatment after a VTE.

Platelet count, as a phenotype that is stable over time within an individual, seems a promising biomarker to improve stratification of major bleeding risk during anticoagulant treatment. However, the use of platelet count in risk assessment models in patients with VTE and atrial fibrillation has yielded controversial results, with studies using different cutoff values of platelet count [38–40]. Whether an elevated platelet count at the appropriate cutoff value can improve discrimination of VTE patients with high and low risk of major bleeding would require further investigation.

The inclusion of subjects derived from the general population, the complete and validated registry of VTE events, and the strict criteria used to define major bleeding based on the ISTH recommendations are among the main strengths of the present study. Additionally, the exclusivity of UNN as the sole healthcare provider enhances the probability to capture all relevant major bleeding events. The study also has some limitations. There were 157 subjects (17% of eligible participants) with missing values on platelet count who were excluded from our analyses (Figure 1). However, there were no substantial differences in the baseline characteristics among subjects with and without missing values of platelet count (data not shown), indicating that missing values was presumably at random. The number of major bleeding events was low in some subgroups, which could have resulted in limited statistical power. Our results should therefore be interpreted with caution, especially in subgroup analysis. Only a few patients (15 out of 744 subjects included in the analyses) had a platelet count <100 x 10^9/L at the time of VTE diagnosis. Therefore, we were not able to evaluate the role of moderate to severe thrombocytopenia on the risk of major bleeding. It is noteworthy that the association between platelet count and major bleeding was less pronounced when platelet count was measured at Tromsø study enrollment as compared to the assessment at VTE diagnosis. Even though platelet count is subject to less intra-individual compared to inter-individual variability [22], individual changes due to advancing age or environmental factors (such as onset of diseases) may occur over time [18,41]. In our cohort study, with a long period of follow-up, values of platelet count might have changed over time in participants with or without a major bleeding event. This could have led to an underestimation of the association between platelet count measured at Tromsø study enrollment and major bleeding, due to regression dilution bias, a phenomenon that occurs in the long-term follow-up of cohort studies [42]. Unfortunately, we did not have information on concomitant use of drugs that might have affected the bleeding risk, such as antiplatelet agents. However, we conducted a sensitivity analysis excluding VTE cases with a known medical history of myocardial infarction or stroke (n = 131), as these would be the most likely users of antiplatelet medication (Supplemental Table IV). The association between an increasing platelet count and major bleeding remained, albeit somewhat attenuated compared to the main analyses, suggesting that the association was not primarily driven by antiplatelet medications. Finally, MPV is not commonly part of the diagnostic work-up for subjects with suspected acute VTE, and information on MPV at VTE diagnosis was therefore not available.

In conclusion, our results suggest that an increasing platelet count, measured several years before and at VTE diagnosis, is associated with a higher risk of major bleeding during the first year after an incident VTE. Our findings imply that a platelet count measured at VTE diagnosis is a stable phenotype within an individual over time that has the potential to improve risk stratification of major bleeding after a VTE.

Acknowledgements

K. G. Jebsen TREC is supported by an independent grant from Stiftelsen K.G. Jebsen.

H. S. Johnsen receives a grant from the North Norwegian regional health authorities (Helse-Nord).

Disclosure Of Conflict Of Interest

The authors report that they have no conflicts of interest.

Supplementary Material

Supplemental data for this article can be accessed on the publisher's website.

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Supplementary material

Supplemental Table 1. Characteristics of major bleeding events in patients with venous thromboembolism

Major bleeding site	% (n)
Gastrointestinal	36 (20)
Intracranial	18 (10)
Intramuscular/compartment syndrome	15 (8)
Urogenital	13 (7)
Retroperitoneal	5 (3)
Other*	13 (7)
ISTH major bleeding criteria	
Fatal bleeding[a]	5 (3)
Critical area or organ[b]	40 (22)
Blood transfusion[c] ± hemoglobin fall[d]	55 (30)

ISTH, International Society on Thrombosis and Haemostasis.

*Pericardial, intra-abdominal and subcutaneous/intramuscular/hematoma.

[a]Fatal outcome within one week after major bleeding, without other specified cause of death.

[b]Intracranial, retroperitoneal, pericardial and intramuscular with compartment syndrome.

[c]Bleeding leading to transfusion of ≥ 2 units of whole blood or red cells.

[d]Bleeding causing a fall in hemoglobin level of ≥20 g/L.

Supplemental Table 2. The one-year risk of major bleeding according to quartiles of platelet count measured at venous thromboembolism diagnosis

Quartiles of Platelet count (10^9/L)	Major bleeding	IR (95% CI)	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	HR (95% CI)[d]
All VTE patients						
≤192	6	3.8 (1.7-8.6)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193-239	15	9.2 (5.6-15.3)	2.7 (1.0-6.9)	2.7 (1.0-7.0)	2.7 (1.0-7.0)	2.6 (1.0-6.8)
240-299	16	11.0 (6.7-17.9)	3.1 (1.2-8.0)	3.2 (1.2-8.2)	3.3 (1.3-8.5)	3.2 (1.2-8.2)
≥300	18	12.8 (8.1-20.3)	4.3 (1.7-10.9)	4.4 (1.7-11.2)	4.7 (1.8-12.0)	4.3 (1.7-11.0)
p for trend			.002	.002	.001	.002
VTE patients without cancer						
≤192	5	4.1 (1.7-9.8)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193-239	9	6.3 (3.3-12.2)	1.9 (0.6-5.6)	2.0 (0.7-5.9)	2.0 (0.7-5.9)	1.9 (0.6-5.7)
240-299	9	7.2 (3.7-13.8)	2.0 (0.7-6.1)	2.1 (0.7-6.4)	2.1 (0.7-6.5)	2.1 (0.7-6.3)
≥300	13	11.6 (6.8-20.0)	4.0 (1.4-11.4)	4.2 (1.5-12.1)	4.3 (1.5-12.3)	3.8 (1.3-11.1)
p for trend			.01	.01	.01	.01

IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval; SHR, sub-distribution hazard ratio.

[a]Adjusted for age and sex.

[b]Adjusted for age, sex, body mass index and planned duration of anticoagulant therapy.

[c]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy and surgery.

[d]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery and acute medical conditions (acute myocardial infarction, ischemic stroke, or major infectious disease).

Continued

HR (95% CI)[e]	HR (95% CI)[f]	HR (95% CI)[g]	HR (95% CI)[h]	SHR (95% CI)[h]
1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
2.4 (0.9-6.3)	2.4 (0.9-6.3)	2.2 (0.8-5.8)	2.4 (0.9-6.3)	2.5 (1.0-6.5)
2.8 (1.1-7.3)	2.8 (1.1-7.3)	2.8 (1.0-7.3)	2.8 (1.1-7.4)	3.1 (1.2-8.0)
3.9 (1.5-10.2)	3.9 (1.5-10.2)	3.6 (1.4-9.5)	3.4 (1.3-8.8)	3.2 (1.2-8.6)
.005	.005	.007	.01	.02
1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
1.8 (0.6-5.4)	1.8 (0.6-5.4)	1.6 (0.5-5.0)	1.8 (0.6-5.4)	1.9 (0.6-5.7)
1.5 (0.5-4.8)	1.5 (0.5-5.0)	1.6 (0.5-5.0)	1.6 (0.5-5.2)	1.8 (0.5-6.1)
3.1 (1.0-9.2)	3.0 (1.0-8.9)	2.9 (1.0-8.6)	2.8 (0.9-8.5)	2.9 (0.9-9.5)
.05	.07	.07	.08	.09

[e]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery, acute medical conditions and estimated glomerular filtration rate (eGFR).

[f]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery, acute medical conditions, eGFR and hypertension (systolic blood pressure > 160 mmHg).

[g]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery, acute medical conditions, eGFR, hypertension and history of bleeding (specifically noted in the medical records of VTE cases).

[h]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery, acute medical conditions, eGFR, hypertension, history of bleeding and anemia (hemoglobin level below 11.5 g/dL for women and below 13.0 g/dL for men at VTE diagnosis).

SHR denotes the HR after taking competing risk by death into account.

Supplemental Table 3. The one-year risk of major bleeding according to quartiles of platelet count measured at venous thromboembolism diagnosis stratified by planned type of anticoagulant

Quartiles of Platelet count (10^9/L)	Major bleeding	IR (95% CI)	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
Heparin and VKA (n=488)						
≤192	3	2.8 (0.9-8.6)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193-239	9	7.4 (3.8-14.2)	3.2 (0.9-12.0)	3.3 (0.9-12.3)	3.5 (0.9-13.0)	3.4 (09-13.0)
240-299	11	10.5 (5.8-19.0)	4.1 (1.1-14.6)	4.2 (1.2-15.0)	3.1 (0.8-11.5)	3.2 (0.8-12.3)
≥300	10	10.0 (5.4-18.6)	5.3 (1.4-19.5)	5.4 (1.5-20.0)	4.5 (1.2-17.1)	4.4 (1.1-17.1)
p for trend			.008	.007	.04	.03
Heparin (n=145)						
≤192	1	4.1 (0.6-28.9)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193-239	4	18.2 (6.8-48.4)	5.2 (0.6-48.5)	5.0 (0.5-47.5)	3.4 (0.3-36.7)	5.5 (0.7-43.3)
240-299	2	10.7 (2.7-42.6)	3.1 (0.3-35.5)	3.2 (0.3-36.8)	6.0 (0.4-92.8)	5.3 (0.5-56.8)
≥300	6	28.1 (12.6-62.6)	6.8 (0.8-57.2)	6.6 (0.8-55.0)	4.1 (0.4-47.3)	4.3 (0.6-31.0)
p for trend			.09	.1	.3	.2

IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval; SHR, sub-distribution hazard ratio; VKA, vitamin k antagonist.

*Information on anticoagulation type was missing in 34 subjects.

[a]Adjusted for age and sex.

[b]Adjusted for age, sex, body mass index and planned duration of anticoagulant therapy.

[c]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery, acute medical conditions (acute myocardial infarction, ischemic stroke, or major infectious disease), estimated glomerular filtration rate, hypertension (systolic blood pressure > 160 mmHg), history of bleeding (specifically noted in the medical records of VTE cases), and anemia (hemoglobin level below 11.5 g/dL for women and below 13.0 g/dL for men at VTE diagnosis).

SHR denotes the HR after taking competing risk by death into account

Supplemental Table 4**.** The one-year risk of major bleeding according to quartiles of platelet count measured at venous thromboembolism diagnosis after excluding subjects with previous myocardial infarction or stroke

Quartiles of Platelet count (10^9/L)	Major bleeding	IR (95% CI)	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	SHR (95% CI)[c]
All VTE patients (n=613)						
≤192	5	4.1 (1.7-10.0)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
193-239	11	7.8 (4.3-14.1)	1.9 (0.7-5.6)	2.0 (0.7-5.7)	1.9 (0.6-5.5)	2.0 (0.7-6.0)
240-299	12	9.9 (5.6-17.4)	2.4 (0.8-6.9)	2.4 (0.9-7.0)	2.4 (0.8-7.1)	2.7 (0.9-8.1)
≥300	16	13.2 (8.1-21.5)	3.8 (1.4-10.5)	3.9 (1.4-10.6)	3.1 (1.1-8.7)	2.9 (1.0-8.7)
p for trend			.006	.005	.03	.04

IR, incidence rate per 100 person-years; HR, hazard ratio; CI, confidence interval; SHR, sub-distribution hazard ratio.

[a]Adjusted for age and sex.

[b]Adjusted for age, sex, body mass index and planned duration of anticoagulant therapy.

[c]Adjusted for age, sex, body mass index, planned duration of anticoagulant therapy, surgery, acute medical conditions (acute myocardial infarction, ischemic stroke, or major infectious disease), estimated glomerular filtration rate, hypertension (systolic blood pressure > 160 mmHg), history of bleeding (specifically noted in the medical records of VTE cases), and anemia (hemoglobin level below 11.5 g/dL for women and below 13.0 g/dL for men at VTE diagnosis).

SHR denotes the HR after taking competing risk by death into account.

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