UiT The Arctic University of Norway

Faculty of Health Sciences

Plasma von Willebrand factor and risk of future venous thromboembolism

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December 2023

A dissertation for the degree of Philosophiae Doctor (PhD)

Table of Contents

Acknowledgments

The work of the present thesis was carried out at the Thrombosis Research and Expertise Center (TREC) at the Department of Clinical Medicine, UiT – The Arctic University of Norway, from January 2020 until December 2023. I was initially part of the Student Research Programme for medical students, and during the last semester I have been working full-time as a PhD student with funding from the Northern Norway Regional Health Authority.

First of all, I would like to express my heartfelt gratitude to my main supervisor, Professor John-Bjarne Hansen, for the opportunity to do this project and for being a brilliant and inspirational captain of our team. Thank you for your expert guidance, rewarding discussions and incredible ability to design cutting edge research projects. I was surprised to encounter a person with these abilities who is simultaneously so easy going and humorous, and for that you are a great inspiration to me. Your moderate demand for sleep, however, gives you an unfair advantage.

To my co-supervisors, Vânia M. Morelli and Professor Sigrid K. Brækkan, thank you for your countless feedbacks on manuscripts and theses, which were always thorough and of the highest quality. The progress of my project was heavily dependent on your willingness and ability to somehow find time in your busy schedules. The majority of what I know about navigating and participating in the complex jungle of epidemiological research I learned from our meetings and discussions. Most importantly you deserve credit for being notoriously compassionate and inclusive while guiding novice researchers on their path.

Further, a special thanks is extended to my co-authors, Ellen-Sofie W. Eilertsen, Kristian D. Hindberg, Line H. Evensen, Thor Ueland and Pål Aukrust. This work would not have been possible without your contributions. In particular, the patient guidance on statistical software and scientific writing given by Kristian D. Hindberg and Line H. Evensen during my first months at TREC was highly appreciated and essential to both the project and my progress as a young researcher. My gratitude is also extended to the staff and participants of the Tromsø Study, without whom this research project would have been impossible. Moreover, my appreciation goes out to Vegard Skogen and the Student Research Programme for allowing me to begin my pursuit of a scientific career while still in medical school.

To all my friends and colleagues in TREC, both past and present (Parwez Ahmad, Christopher Antoun, Carl Arne L. Arnesen, Anna Y. Arntzen, Esben Bjøri, Safak Çaglayan, Celina J. Cathro, Christabel E. Damoah, Nikolai H. Eide, Tobias Frischmuth, Eduarda M. Guerreiro, Erin M. Hald, Maryam Hosseini, Trond Isaksen, Lisa Jakobsen, Håkon S. Johnsen, Helle Jørgensen, Camilla Langholm, Nadezhda Latysheva, Oda G. R. Leknessund, Dana Meknas, Clément Naudin, Asbjørn L. Onsaker, Cathrine Ramberg, Fridtjof B. Rinde, Joakim K. Sejrup, Hanne Skille, Omri Snir, Mathias H. Storvand, Eike C. Struck, Samantha Swamy, Marthe N. Thoresen, Birgitte G. Tøndel, Casper Ullsten-Wahlund, Vårin E. Wikan, Line Wilsgård, Bjarne Østerud and those I apologize to have forgotten), thank you for making these years a pleasant experience. It is with fondness I look back on our clotteries, Tuesday meetings, TRECxercises and all the countless conversations during morning coffee and lunch. Our trips to Ghent and Reykjavik were truly rewarding experiences, and the pandemic-ridden congress in Milan was a pleasure to look forward to. To my office-mates and friends Carl Arne L. Arnesen and Birgitte G. Tøndel, I would like to extend an extra thanks for making my daily life at TREC cheerful and amusing. Your diversions and convincing appeals to indulge in the temptations of the cafeteria have been most welcome, although my current financial balance suggests otherwise.

Finally, I would like to express my sincere gratitude towards my family and the people closest to me. To my parents, Tove and Espen, thank you for your unwavering support and invaluable advice throughout my life. To my brother William, thank you for your good humour, competitiveness and kindness, and most importantly our strong friendship. To my girlfriend Amalie, thank you for your love and support during the challenging period of graduating and writing this thesis. To all my friends, thank you for all the countless memories making the university years the time of my life.

Magnus Sandvik Edvardsen

Tromsø, November 2023

Summary

Venous thromboembolism (VTE), an umbrella term for deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease with serious complications. Meanwhile, the exact underlying mechanisms remain unclear. Von Willebrand factor (VWF) is pivotal in haemostasis, and likely causally involved in VTE. VWF is regulated by ADAMTS13 and can promote haemostasis in two distinct ways; i.e., interaction with platelets and serving as the carrier of coagulation factor (F)VIII. The aims of the present thesis were to investigate the prospective association between VWF and VTE and assess the impact of platelet interaction.

The study population was derived from the fourth survey of the Tromsø Study, where 27,158 individuals above the age of 25 years participated. The participants were followed from baseline in 1994-95 through August 2007, and 462 incident VTE cases were identified by thorough assessment of available registries. For each case, two age- and sex-matched controls were sampled from the parent cohort, forming a nested case-control study. Plasma samples obtained at cohort baseline were then thawed and analysed for VWF, ADAMTS13 and FVIII. Unconditional logistic regression was used to estimate odds ratios (ORs) for VTE.

In paper I, we found a dose-dependent association between plasma levels of VWF and risk of future VTE. Those with plasma VWF levels in the highest quartile had a 45% increased OR for VTE compared with those in the lowest quartile, and the OR for unprovoked DVT was increased 6.7-fold. In paper II, our data suggested that plasma ADAMTS13 in the lowest quartile was also associated with increased OR for VTE. Further, the ratio between plasma VWF and ADAMTS13 had a dose-dependent relationship with OR for future VTE, and those in the highest quartile had 70% higher OR for VTE than those in the lowest quartile. In paper III, we found that plasma VWF had synergistic effects with mean platelet volume (MPV) and platelet count on OR for VTE. Specifically, elevated plasma VWF did not result in increased OR for VTE if platelet measures were not concomitantly high. Paper IV assessed the same interactions, but with FVIII. Here, a more than additive effect was only seen for MPV.

In conclusion, our main findings indicate that elevated VWF represents a long-term risk factor for VTE, and that platelet interaction is involved in the mechanism by which VWF promotes venous thrombus formation.

Sammendrag

Venøs tromboembolisme (VTE) er en samlebetegnelse som omfatter blodproppsykdommene dyp venetrombose (DVT) og lungeemboli. VTE er en vanlig og multifaktoriell sykdom med alvorlige komplikasjoner. Likevel er det mye man ikke vet om mekanismene for sykdommen. Von Willebrand faktor (VWF) er essensiell for vår evne til å stanse blødninger, og er sannsynligvis involvert i prosessen der slike blodpropper dannes. VWF reguleres av ADAMTS13, og virker på to måter; den kan binde seg til blodplater og fungerer som bærer for koagulasjonsfaktor (F)VIII. I denne avhandlingen har vi undersøkt sammenhengen mellom VWF og VTE, samt hvorvidt interaksjon med blodplater spiller inn.

Studiepopulasjonen var deltakere fra den fjerde Tromsøundersøkelsen, som ble avholdt i 1994-95 og inkluderte 27 158 personer over 25 år. I løpet av oppfølgingsperioden fram til 2007 identifiserte vi 462 tilfeller av førstegangs VTE ved å søke i tilgjengelige registre og validere endepunktene. For hver kasus inkluderte vi to deltakere av samme kjønn og alder som ikke fikk VTE i oppfølgingstiden, og opprettet en nøstet kasus-kontroll studie. Blodprøvene som var avlagt ved studiestart ble deretter tint og analysert for VWF, ADAMTS13 og FVIII. Logistisk regresjon ble brukt til å estimere odds ratioer (OR) for VTE.

I artikkel I fant vi en dose-respons-sammenheng mellom nivåer av VWF og VTE. De med VWF i øverste kvartil hadde 45% økt OR for VTE sammenliknet med de i laveste kvartil, og 6,7 ganger økt OR for DVT uten kjent årsak. I artikkel II fant vi at de med ADAMTS13 i laveste kvartil hadde økt risiko for VTE sammenliknet med andre. I tillegg var det en doseavhengig sammenheng mellom forholdstallet VWF/ADAMTS13 og OR for VTE. De som hadde høyest forholdstall (høyeste kvartil) hadde 70% høyere OR for VTE enn de med lavest forholdstall (laveste kvartil). Artikkel III viste at høyt VWF-nivå kombinert med gjennomsnittlig blodplatevolum (MPV) og blodplatetall hadde synergisk effekt på OR for VTE. Høyt VWF-nivå hadde faktisk ingen effekt på risikoen for VTE dersom MPV og blodplatetall var lave. Artikkel IV besto av liknende analyser, men så på FVIII i stedet for VWF. Her fant man en interaksjon mellom FVIII og MPV, men ikke med blodplatetall.

Funnene i denne avhandlingen tyder på at VWF representerer en langvarig risikofaktor for VTE, samt at interaksjon med blodplater er involvert i måten VWF fører til dannelse av blodpropp i venesystemet.

List of Papers

I. **Plasma levels of von Willebrand factor and future risk of incident venous thromboembolism**
Edvardsen MS, Hindberg K, Hansen ES, Morelli VM, Ueland T, Aukrust P, Brækkan SK, Evensen LH, Hansen JB
Blood Advances. 2021 Jan 12;5(1)224-232

II. **Impact of the von Willebrand factor-ADAMTS-13 axis on the risk of future venous thromboembolism**
Edvardsen MS, Hansen ES, Ueland T, Aukrust P, Brækkan SK, Morelli VM, Hansen JB
Journal of Thrombosis and Haemostasis. 2023 May;21(5):1227-1237

III. **Combined effects of plasma von Willebrand factor and platelet measures on the risk of incident venous thromboembolism**
Edvardsen MS, Hansen ES, Hindberg K, Morelli VM, Ueland T, Aukrust P, Brækkan SK, Evensen LH, Hansen JB
Blood. 2021 Dec 2;138(22):2269-2277

IV. **Combined effect of high factor VIII levels and high mean platelet volume on the risk of future incident venous thromboembolism**
Hansen ES, Edvardsen MS, Aukrust P, Ueland T, Hansen JB, Brækkan SK, Morelli VM
Journal of Thrombosis and Haemostasis. 2023 Jun 29:S1538-7836(23)00507-X

Abbreviations

ADAMTS13	A disintegrin and metalloprotease with thrombospondin type 1 motif, member 13
Ag	Antigen
AP	Attributable proportion
APC	Activated Protein C
ARIC	Atherosclerosis risk in communities
BMI	Body mass index
CHS	Cardiovascular health study
CI	Confidence interval
CRP	C-reactive protein
CTEPH	Chronic thromboembolic pulmonary hypertension
CTPA	Computed tomography pulmonary angiogram
CVD	Cardiovascular disease
DOAC	Direct oral anticoagulant
DVT	Deep vein thrombosis
EIA	Enzyme immunoassay
EV	Extracellular vesicle
F	Factor
FVL	Factor V Leiden
GP	Glycoprotein
GWAS	Genome-wide association study
HRT	Hormone replacement therapy
hsCRP	High-sensitivity C-reactive protein
ICD	International classification of diseases
LITE	Longitudinal Investigation of Thromboembolism Etiology
MAR	Missing at random

MCAR	Missing completely at random
MI	Myocardial infarction
MNAR	Missing not at random
MP	Microparticle
MPV	Mean platelet volume
MR	Mendelian randomization
OC	Oral contraceptive
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor-1
PE	Pulmonary embolism
PFP	Platelet-free plasma
PPP	Platelet-poor plasma
PS	Phosphatidyl serine
PTS	Post-thrombotic syndrome
Q	Quartile
RCT	Randomized controlled trial
REK	Regional committee for medical and health research ethics
RERI	Relative excess risk due to interaction
RR	Relative risk
SNP	Single nucleotide polymorphism
T	Tertile
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
tPA	Tissue plasminogen activator
TTP	Thrombotic thrombocytopenic purpura
ULVWF	Ultra-large von Willebrand factor
UNN	University Hospital of North Norway
uPA	Urokinase-type plasminogen activator

VTE	Venous thromboembolism
VWD	von Willebrand disease
VWF	von Willebrand factor

1 Introduction

Venous thromboembolism (VTE) is a disease entity comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), which can present separately or simultaneously (Figure 1).[1,2] DVT occurs when a blood clot, or thrombus, is formed in a deep vein. The most frequent location for DVT is in the lower extremities, while the upper extremities, cerebral veins and splanchnic veins are more rarely affected.[3] As a consequence of thrombosis the blood flow may be compromised, resulting in signs and symptoms such as swelling, pain and redness of the affected limb or organ.[4,5] Subsequently, a part of the thrombus may break away, or embolize, and travel through the blood stream and right side of the heart before it eventually lodges in a pulmonary artery, resulting in PE.[5,6] Moreover, PE can appear spontaneously and is found to occur in the absence of DVT in almost half of the cases.[7-9] As in DVT, PE leads to blockage of blood flow, culminating in symptoms such as pleuritic chest pain, dyspnea, coughing and haemoptysis. Furthermore, circulatory collapse and sudden death may occur in the most severe cases.[1,2,4,5] Due to the mechanistic link and shared pathophysiology, the two diseases are considered jointly under the umbrella term VTE.

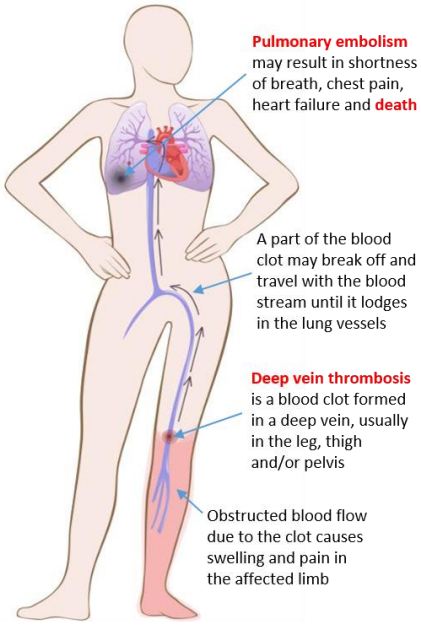

Figure 1. Venous thromboembolism (VTE).

VTE is a common disease with a stable or slightly increasing incidence recorded over the last decades.[10-12] It is a major cause of morbidity and mortality worldwide and demands substantial human and economic resources.[13-16] When diagnosed, VTE is treated with anticoagulant medication, most often using direct oral anticoagulants (DOACs).[17-19] Still, patients suffer considerable short- and long-term complications.[2,17,20] Major research objectives therefore involve improved knowledge on disease mechanism and prevention.[2,6] The underlying pathophysiology of VTE is multicausal and is a result of both inherited and acquired risk factors, which have been increasingly unravelled in recent years. Furthermore, acquired risk factors may be divided into transient (e.g., surgery) or persistent (e.g., cancer), and modifiable (e.g., immobilization) or non-modifiable (e.g., advancing age).[4,21] Around half of all VTE events are attributed to the presence of a provoking risk factor, either transient or

persistent, and are thereby classified as provoked VTEs. Meanwhile, the other half occurs in the absence of any known provoking factors, emphasizing the need for further research into the underlying mechanisms.[1,5,11,22]

Thrombogenesis is inherently akin to haemostasis, as the two processes share essential components and functional pathways.[4,23] Under physiological conditions, the haemostatic system is activated when bleeding occurs due to damage to the blood vessel wall, and it has two main stages; the primary haemostasis (i.e., formation of a platelet plug) and the secondary haemostasis (i.e., stabilization of this plug by fibrin deposition).[24-26] The end product is a blood clot, which stops the bleeding and restores the integrity of the vessel. Although the pathogenesis of VTE is not yet completely understood, it is assumed to involve activation of the haemostatic system in absence of predisposing vascular injury, thus leading to thrombus formation in an intact vessel.[3,4] This process is a complex collaboration involving different cells, proteins and circumstantial factors such as hypoxia and stasis of blood.[3,4] All known components of haemostasis have therefore been subject to extensive research during the past decades, with the aim of identifying aspects specific to thrombosis.[27,28] As all pharmaceutics available for treatment and prevention of thrombosis come with an increased risk of bleeding, a way of targeting thrombosis specifically is considered the Holy Grail in the field.[29-31]

1.1 Epidemiology of venous thromboembolism

The incidence rate of VTE is 1-2 per 1000 adults annually in Western countries, with approximately 1.1 million affected individuals in Europe per year.[13-16] It is thereby the third most common cardiovascular disease (CVD) after myocardial infarction (MI) and stroke.[32] VTE occurs in all age groups, but the risk increases exponentially with age and culminates in a yearly incidence rate of around 1 per 100 individuals over the age of 80 years.[14,15,33,34] Individuals of African descent have the highest risk of VTE, followed by Caucasians and Hispanics, while those of Asian descent are reported to suffer less frequently from VTE.[14,33,35] It is currently under debate whether there is a true sex difference in the overall incidence of VTE. Recent data suggests that females have a higher incidence in their reproductive age while males compensate with higher rates later in life, yielding a fairly similar lifetime risk.[15,36,37]

Multiple studies across different populations have reported an increase in VTE incidence over the past decades, mainly due to increased rates of PE.[10,11,38-41] A proposed explanation is improved diagnostics due to increased awareness and availability of more accurate diagnostic tools such as computed tomography pulmonary angiogram (CTPA).[40,42,43] However, a substantial decrease in the incidence of MI and stroke has been observed over the same period, despite similar improvements in the diagnostic process.[44-47] The favourable trend in MI and stroke incidence has therefore been attributed to successful reduction of important risk factors such as smoking, hypertension and hyperlipidaemia.[46-48] On this basis, it is reasonable to assume that increased prevalence of major VTE risk factors such as obesity, cancer and advanced age have contributed to the persistent trends in VTE incidence.[38,49]

VTE comprises a major health burden both in society and at the individual level. First, VTE is a potentially fatal disease, and studies have found up to 10% all-cause mortality within the first 30 days after the event and 23% the first year.[37,50] Recent studies have reported decreasing in-hospital mortality rates in PE patients over the past decades, suggesting improvements in disease management.[39,51,52] Still, it was estimated that around 540,000 VTE related deaths occur in Europe yearly.[13] Second, serious short- and long-term complications are common in those who suffer a non-fatal VTE event. DVT can lead to persistent impairment of venous circulation in the affected leg, causing around 50% of DVT patients to suffer from post-thrombotic syndrome (PTS). Resulting clinical manifestations are typically a combination of chronic leg pain, fatigue, oedema and ulcers.[53,54] Proximal DVT, recurrent events, venous insufficiency, obesity and advanced age are factors found to increase the risk of developing PTS.[53] Following an acute PE event, around 50% of patients report enduring functional limitations and reduced quality of life, a condition recently defined as the post-PE syndrome.[55,56] A smaller proportion, 1-4% of PE patients, suffer chronic thromboembolic pulmonary hypertension (CTEPH), a condition where unresolved thromboembolic material in the pulmonary circulation can result in right-sided heart failure and occasionally death.[57,58] Further, a Danish nation-wide cohort study found that VTE patients had a substantially increased risk of developing depression, indicating a detrimental psychosocial impact of the disease.[59] As a consequence of the complications mentioned, VTE patients encounter increased risk of work-related disability and reduced quality of life.[60-62] Furthermore, VTE has a recurrence rate of up to 30% within 10 years, causing a substantial proportion of patients to reface the burden of disease and risk of complications.[15,63] Recurrent

events are most frequent in the first year after the incident VTE, and the risk is highest among those with a persistent provoking factor (e.g., cancer).[15,63]

1.2 Pathophysiology of venous thromboembolism

As a general principle, thrombus formation occurs due to one or more of the following three factors: Change in blood composition (hypercoagulability), change in blood flow (stasis), and injury or change in the vessel wall (endothelial dysfunction).[64] These concepts were first described by the German physician Rudolf Virchow in the year 1856 and are therefore collectively referred to as Virchow's Triad (Figure 2).[65,66] The model remains relevant to this day, and all risk factors for VTE can be attributed to one or more of these components. However, VTE is a complex and multifactorial disease, and the exact mechanism for venous thrombogenesis is not yet completely understood.

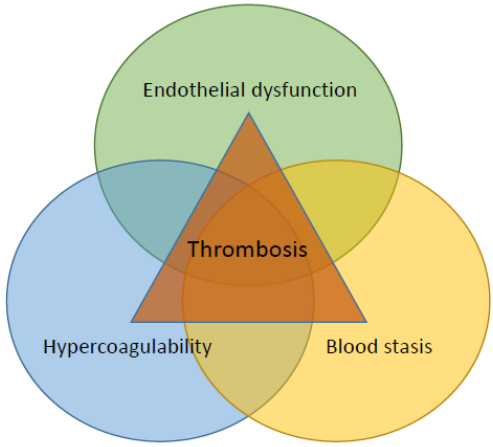

Figure 2. Virchow's Triad.

Venography and post-mortem studies have shown that venous thrombi are commonly formed in the pocket sinuses of venous valves.[4,67,68] These leaflet-shaped valves are essential in assuring venous blood return to the heart and preventing reflux of blood. Specifically, as the skeletal muscles contract, the veins are compressed and the venous blood is forced aside. As the valves close upon retrograde flow, they maintain the circulatory direction towards the heart. Venous insufficiency occurs when the valves are deficient and can cause burdensome symptoms in the affected extremity.[69] Under physiological conditions, a laminar, streamlined flow is present in the venous circulation. Meanwhile, a vortical flow occurs in the pocket sinuses surrounding the valves, making them more prone to local stasis of blood.[70-72] Consequently, red blood cells are found to accumulate in the valve sinuses, and a decreasing oxygen gradient has been observed towards the base of the sinus.[71-74] The combination of low oxygen tension and aggregated blood cells is recognized as a stimulus for endothelial and blood cell activation, which in turn is known to promote haemostasis and inflammation.[74,75] Although VTE is an acute disorder, thrombi may grow for several weeks before eventually

causing clinical symptoms.[76] Several acknowledged risk factors for VTE, such as obesity, surgery, immobilization and cancer, are proposed to mediate parts of their effect by increasing venous resistance and cause stasis.[76]

Hypercoagulability and stasis have traditionally been regarded as the propagators of venous thrombogenesis.[66,77] Meanwhile, vessel wall injury has been considered important mainly in arterial thrombosis (MI and stroke), as VTE occurs in the presence of an intact endothelium.[74,78] However, increasing knowledge regarding the function of endothelial cells has emerged in the last decades, and it is now widely acknowledged that they are heavily involved in haemostasis and thrombogenesis by regulation of the vascular microenvironment.[79] Under normal conditions, the endothelial cell membrane contains heparan sulfate-bound antithrombin, protein C receptor and tissue factor pathway inhibitor (TFPI), all mediating resistance towards thrombogenesis.[74,78] However, they release several prothrombotic components upon activation, including platelet- and monocyte-recruiting proteins such as von Willebrand factor (VWF) and P-selectin, and plasminogen activator inhibitor-1 (PAI-1), an inhibitor of fibrinolysis.[74,78] Activated platelets and monocytes further promote thrombus formation by secretion of microparticles (MPs) containing tissue factor (TF).[4,76] Advanced age, hypoxia, inflammation and thrombin generation are among the known triggers for endothelial cell activation.[74,79] Thus, while further research is needed to disentangle the exact mechanisms, the concept of endothelial dysfunction appears to remain important in VTE.

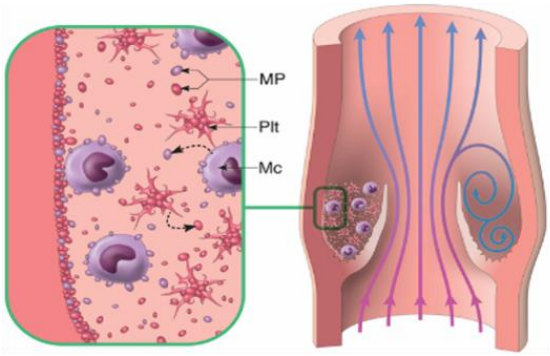

Figure 3. Proposed mechanism for thrombus formation in the pocket sinus of a venous valve. Streamlined flow is replaced by a vortex, leading to hypoxia and blood cell aggregation with subsequent activation of endothelial cells. Platelets (Plt) and monocytes (Mc) are recruited and activated, promoting thrombus growth by secretion of microparticles (MPs) containing tissue factor.

1.3 Risk factors for incident venous thromboembolism

A risk factor is defined as any element which increases the probability of an outcome.[80] VTE is a multicausal disease, and an event is regarded as the culmination of an interplay between several risk factors.[77,81,82] Further, biomarkers (biological markers) is a broad term used to

define all biological features which can be objectively measured, e.g., blood pressure or weight.[83] Biomarkers can serve different purposes, as they can be used in diagnostic, predictive, prognostic and therapeutic settings, depending on their association with a particular outcome.[84] In VTE, major research objectives are to identify causal risk factors which can be targeted in prevention and treatment, as well as risk factors which can serve as biomarkers for risk prediction.[28,85] All risk factors are commonly divided into two categories; hereditary and acquired (Table 1). Acquired risk factors often accumulate over time, and the total burden of risk factors thereby normally coincide with the increased VTE risk seen by advancing age.[34,37,86] Our current understanding of the risk factors' effect on VTE occurrence is based on the thrombosis potential model (Figure 4) first described in 1999 by Professor Rosendaal.[77] This model states that any individual is at a certain risk of VTE, illustrated by the thrombosis threshold. Further, the total burden of risk factors is reflected by the thrombosis potential, which is subject to change during life. A VTE is thought to occur when the burden of risk factors, i.e., the thrombosis potential, crosses the thrombosis threshold. An individual may, for example, undergo surgery (a transient risk factor) early in life without suffering from a VTE. Meanwhile, after acquiring more risk factors (e.g., obesity and advanced age), the same surgery may result in a VTE later in life. The model also provides distinctions between risk factors; Transient versus persistent, hereditary versus acquired, and constant versus progressive.

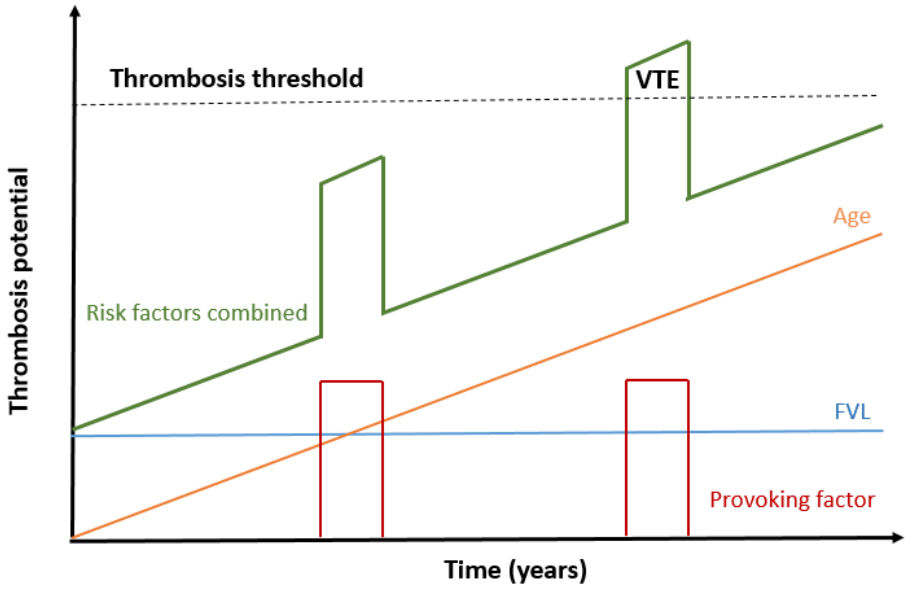

Figure 4. The thrombosis potential model. This graph depicts a hypothetical scenario where an individual has an inherited risk factor, Factor V Leiden (FVL), which is constantly present. Acquired risk factors, here represented by age and provoking factors (e.g., surgery), appear as time progresses. The green line illustrates the thrombosis potential obtained by the risk factors combined. In the example, a provoking factor appears early in life without any consequence, while the increasing burden of risk factors results in a venous thromboembolism (VTE) when facing a similar provoking factor later in life. The assumption is that the thrombosis potential crosses a certain threshold, leading to thrombosis. (Adapted from Rosendaal, *Lancet*, 1999).

VTE events are categorized as provoked or unprovoked based on whether the patient was exposed to certain environmental risk factors in the time closely preceding the event (often 2-3 months for transient risk factors).[21] Acknowledged provoking factors are associated with an immediate increase in VTE risk, and include surgery, immobilization, hospital admission, cancer and pregnancy.[21] Conversely, VTE events occurring in the absence of provoking factors are categorized as unprovoked. The applied criteria for provoking factors vary slightly across published research, but existing data from large populations suggests that nearly half of all VTE events are considered unprovoked.[11,16,87] The underlying causes of disease are therefore largely unknown, emphasizing the need for further research with the aim of disentangling the pathophysiology.[22,28] Further, the risk of recurrent VTE depends on the presence of provoking factors at the incident event. Recurrence risk is highest in those with a persistent provoking factor (e.g., cancer) and lowest in those with a transient provoking factor (e.g., surgery), while those with a first unprovoked VTE have an intermediate risk of recurrence.[21] Consequently, the recommended duration of anticoagulant treatment following

an event varies according to provoked/unprovoked status.[1,88] Thus, the categorization holds major clinical implications while also shaping our understanding of the underlying causal mechanisms.[28,88,89]

Table 1. Established risk factors for venous thromboembolism.[82,90]

Hereditary risk factors	Acquired risk factors
▪ Antithrombin deficiency [91,92]	▪ Increasing age [37]
▪ Protein C deficiency [91,92]	▪ Surgery and trauma [97]
▪ Protein S deficiency [91,92]	▪ Immobilization [98-100]
▪ Factor V Leiden [93,94]	▪ Cancer [101]
▪ Prothrombin G20210A [95]	▪ Antiphospholipid syndrome [102]
▪ Non-O blood group [96]	▪ Pregnancy and puerperium [103,104]
	▪ Oestrogen therapy [105,106]
	▪ Obesity [107]
	▪ Acute medical conditions [108-110] *

* Infection, myocardial infarction and ischemic stroke.

1.3.1 Hereditary risk factors

Inheritance is a major determinant of VTE risk, and family studies and population-based studies have shown that up to 60% of the variance in VTE incidence can be attributed to genetic factors.[111,112] Interestingly, the impact of genes on VTE risk is reduced with increasing age, as older individuals to a larger degree suffer from VTE in the absence of known prothrombotic genotypes, likely due to accumulation of acquired risk factors and depletion of susceptible individuals.[113] Further, siblings of VTE patients are found to have a 3-fold increased VTE risk compared with the general population.[114] The hereditary risk factors for VTE mainly comprise mutations in genes encoding coagulation factors, and result in a hypercoagulable phenotype.[115,116] While all these mutations result in thrombophilia, they are frequently divided into loss-of-function (deficiencies) and gain-of-function, depending on how the encoded proteins are affected.[116] Antithrombin, Protein C and Protein S are natural anticoagulants with well-known deficiencies of genetic origin. Deficiencies in these proteins

are associated with a substantially increased risk of VTE, but the mutations are rare in the general population (0.2%, <1% and <1%, respectively).[111,117] Contrarily, the most known gain-of-function mutations are more common and are associated with a relatively smaller increase in VTE risk. Such genotypes include Factor V Leiden (FVL), prothrombin G20210A and non-O blood type, which in Caucasians have a prevalence of 5%, 2% and >50%, respectively.[94,95,116,118]

Recent years have provided considerable technical advances resulting in a new era in the field of genetics. While some of the established culprit genes were identified using the candidate gene approach, this technology required that genes of interest had to be defined beforehand and was mainly applied to study patients with an apparent familial accumulation of thrombosis.[94,119,120] High-throughput microarray-based genotyping has since emerged, allowing analysis of millions of single nucleotide polymorphisms (SNPs) simultaneously and providing the possibility of genome-wide association studies (GWAS).[119,121] GWAS meta-analyses have confirmed the effects of the previously discovered risk alleles and provided hundreds of novel variants associated with VTE risk.[122,123] However, most recently discovered SNPs have a relatively small effect on the overall risk of VTE and are therefore found to be clinically irrelevant at the individual level.[119,124-126] Still, as several of these risk alleles are common, they comprise a substantial population attributable fraction (PAF) of VTE events in the population and may also be essential in unravelling causal mechanisms in the pathogenesis of VTE.[119] As one single genetic biomarker is insufficient in predicting VTE occurrence, de Haan and colleagues designed a risk prediction model using the 5 SNPs most strongly associated with VTE risk: FVL (rs6025 in *F5* gene), prothrombin G20210A (rs1799963 in *F2* gene), coagulation factor XI (rs2036914 in *F11* gene), fibrinogen gamma (rs2066865 in *fibrinogen gamma gene*) and non-O blood group (rs8176719 in *ABO* gene).[127] Interestingly, this 5-SNP risk score had similar discriminative precision as a risk score comprising 31 SNPs. Further, the accuracy was improved when combined with other risk factors, suggesting a greater value of genetic testing in high-risk populations.[127]

The emergence of GWAS also allowed the development of Mendelian randomization (MR) studies. This study design uses genetics to reflect randomization used in clinical trials, and is the gold standard to infer causal associations in epidemiology.[128,129] Namely, GWAS can be used to identify SNPs associated with exposures of interest, as virtually all modifiable exposures also have genetic variants which account for parts of their variability.[129] Further, as

genes are inherited randomly, this study design can use these SNPs to allocate individuals into different categories of exposure, and then estimate the risk of VTE according to the genetic exposure category. As genes are not subject to change during life, MR studies assume that the exposure status was present before the outcome. Reverse causation can therefore be disregarded, and any association can be recognized as potentially causal and unbiased.[130] Importantly, this is given under the assumption of certain conditions, including that the SNP is associated with the exposure, that the SNP only affects the outcome through the exposure of interest and that it is not related to confounding factors.[129,131] The phenomenon where the SNP affects the outcome through pathways other than the one related to the exposure is known as pleiotropy.[129]

1.3.2 Acquired risk factors

An exponential rise in VTE incidence is seen across age groups, and **increasing age** is presumably the most acknowledged risk factor for VTE.[15,37] The yearly incidence rates roughly follow a rule of 10s according to age; 1 in 100,000 in children, 1 in 10,000 in reproductive age, 1 in 1,000 in middle aged and 1 in 100 in elderly.[33,37,50,81,132] The association is assumed to be mediated through an increase in other risk factors, and older individuals suffer more often from known VTE triggers such as cancer, immobilization and medical conditions.[34,74] Furthermore, individuals of advanced age are more prone to encounter all aspects of Virchow's Triad. First, a clear association between increasing age and venous stasis is reported, and contrast media used in venography is observed to linger for up to 60 minutes in the veins of elderly individuals due to fibrous valve leaflets and reduced tone in the vascular smooth muscles.[74,133,134] Second, increasing age is associated with elevated levels of VWF, prothrombin, fibrinogen and coagulation factors VIII, IX and XI, suggesting a hypercoagulable state.[135-137] Third, biomarkers for endothelial cell activation are found to be elevated in a similar manner, and endothelial senescence is assumed to be essential in the pathogenesis of several CVDs.[135,138]

Around half of all VTE events occur during **hospitalization** or shortly after, and hospitalized patients have a more than 100-fold increased age- and sex-adjusted VTE incidence when compared with non-hospitalized community residents.[139,140] However, the risk varies greatly depending on the reason for hospital admission. Major **surgery** and **trauma** have long been recognized as important triggers for VTE, and are associated with 11-

to 40-fold increased odds for VTE depending on the injury or procedure.[49,97,141,142] Further, **acute medical conditions** (MI, ischaemic stroke and infection) are associated with a substantial short-term increase in VTE risk.[108-110] While the association between hospitalization and VTE risk may emerge through biological aspects in the underlying conditions, **immobilization** is *per se* recognized as a highly relevant transient risk factor.[100] Studies have found that immobility due to bed rest, orthopaedic conditions and neurological conditions yields increased risk of VTE, and the leading hypothesis is that thrombosis occurs due to venous stasis with subsequent hypercoagulability.[143,144] Furthermore, the association between hospitalization and VTE is found to be partly mediated by nosocomial infections, which is a known trigger for VTE regardless of immobility and the reason for hospital admission.[145,146]

Cancer patients have for two centuries been noted to suffer frequently from VTE, and the French physician Armand Trosseau first described the association in the middle of the 19th century.[101,147] Cancer has since been recognized as a major risk factor for VTE, and studies have reported a 4- to 7-fold increased VTE risk in cancer patients compared with the general population.[49,148,149] Altogether, around 1 in 5 patients are found to have cancer at the time of VTE diagnosis.[11,140,150] The risk of VTE varies substantially according to the type, stage, localization and treatment of the underlying cancer.[151] A multifactorial pathophysiology is assumed, as cancer cells may produce excessive procoagulant proteins or inadequate amounts of anticoagulants, promote inflammation, and cause stasis due to vein compression.[152,153] Furthermore, both chemotherapy and immunotherapy are known to promote thrombosis independent of other factors, and cancer patients are often subjects to immobilization and hospitalization.[152,154,155]

Although the lifetime risk is reported to be fairly similar in women and men, women suffer from VTE twice as often as men during reproductive age.[36,37,156] This is partly explained by **pregnancy** and **puerperium**, which are hypercoagulable states observed to yield a 5-fold and 60-fold increased VTE risk, respectively, compared with non-pregnant women of the same age.[157,158] Another reason behind the sex-dependent patterns of VTE incidence is the use of exogenous **oestrogen supplements**. Oral contraceptives result in a clinically relevant increased risk of VTE which is highest during the first months of use, and similar findings are reported in postmenopausal women taking hormonal replacement therapy.[159,160]

Overweight and **obesity** demand a substantial amount of health care resources as they increase the risk of several illnesses, including VTE.[161,162] They form a major source of public health concern as the number of obese individuals worldwide has tripled over the last 50 years and is expected to increase further despite preventive efforts.[161] Compared with subjects of normal weight, obese individuals have a 2- to 3-fold increased risk of suffering from VTE, and recent data from the Tromsø Study suggests that almost 25% of all VTEs in the general population can be attributed to overweight and obesity.[162-164] Furthermore, the association is linear, and risk estimates are higher for morbidly obese individuals.[162,165] Studies have also found that weight gain is associated with an increase in the risk of VTE, especially in individuals who are already obese.[166] Body mass index (BMI), defined as weight divided by height squared, is the most established metric to define overweight and obesity, and it is indeed found to be linearly associated with VTE risk.[167] Meanwhile, other anthropometric measures of obesity show the same pattern, and waist circumference appears to most accurately predict risk of VTE.[168] Obesity is strongly associated with several established causal risk factors for arterial CVD, including hypercholesterolemia, hypertension and type 2 diabetes mellitus.[169,170] However, most studies, including population-based cohorts, do not report an association between these factors and VTE, and the underlying mechanisms of obesity-related VTE are therefore not completely understood.[171,172] Meanwhile, MR studies have shown that genetically predicted elevated BMI is associated with VTE risk, implying a causal relationship.[173,174]A key role of venous stasis is assumed, as visceral obesity is known to raise the intra-abdominal pressure, which in turn may increase resistance in the venous circulation.[162,175,176] Further, a potential role of adipokines and proinflammatory cytokines secreted by adipocytes is under investigation.[177,178] Though inflammation is known to promote coagulation,[179,180] research on inflammatory markers and VTE risk have provided somewhat inconsistent results.[27,181-183]

1.4 Role of haemostasis in venous thromboembolism

Haemostasis, the physiological process which stops bleeding, is essential for survival in humans and other mammals.[23,24,184] Upon injury of a blood vessel and subsequent leakage of blood, several processes occur simultaneously to ensure the formation and stabilization of a thrombus at the site of injury, thus stopping the bleed and restoring the integrity of the vessel.[24-26] For the sake of simplicity, this process is often divided into two stages, i.e.,

primary and secondary haemostasis. Primary haemostasis refers to the formation of a platelet plug, which involves platelet recruitment, adhesion, activation, aggregation and secretion at the site of injury.[185,186] This will be described with further detail in the next subchapter of the thesis (1.4.1 Platelets). Secondary haemostasis involves the stabilization of the platelet plug by cross-linked fibrin and is the culmination of a series of enzymatic reactions, i.e., the coagulation cascade.[23] The serine proteases involved, termed coagulation factors, are present in the circulation as inactive zymogens under physiological conditions.[23,184] Upon vascular injury, an abundance of subendothelial TF is exposed to the circulation, which is bound by coagulation factor (F)VII. TF serves as a cofactor and activator for FVII, allowing the TF-FVIIa complex to proteolytically cleave and activate FIX and FX.[187] This is referred to as the extrinsic pathway, as it depends on extrinsic TF to be exposed to the circulation with subsequent activation of FIX and FX.[24]. FXa (activated FX) subsequently forms a complex with FVa (prothrombinase complex), which in turn converts prothrombin (FII) into thrombin (FIIa), with subsequent activation of FXIII.[188] The cascade culminates in thrombin cleaving fibrinogen to make insoluble fibrin, the end product which cross-links and stabilizes the platelet plug with the help of FXIIIa.[188,189] Further, the presence of a negatively charged cell membrane (e.g., on activated platelets) is known to activate FXII and thereby provide another route for activation of FX, termed the intrinsic or contact pathway.[190,191] Specifically, FXIIa leads to cleavage and activation of FXI, which subsequently activates FIX. With FVIIIa (activated by thrombin) as a cofactor, FIXa completes the intrinsic pathway by activating FX.[191] Furthermore, thrombin is known to provide a positive feedback loop through the intrinsic pathway, as it activates FV, FVIII and FXI.[189] The route from FXa to fibrin generation is identical regardless of the trigger, and is therefore termed the common pathway.[23] Although the extrinsic and intrinsic pathways have been regarded as different systems, it has become increasingly clear that haemostasis *in vivo* acts as an integrated pathway triggered by TF.[189] In thrombosis, however, research indicates that activation of the intrinsic pathway has a crucial role. Specifically, preclinical studies have found that targeting components of the intrinsic pathway reduces thrombosis without significantly affecting physiological haemostasis.[192] The coagulation cascade is illustrated in Figure 5.

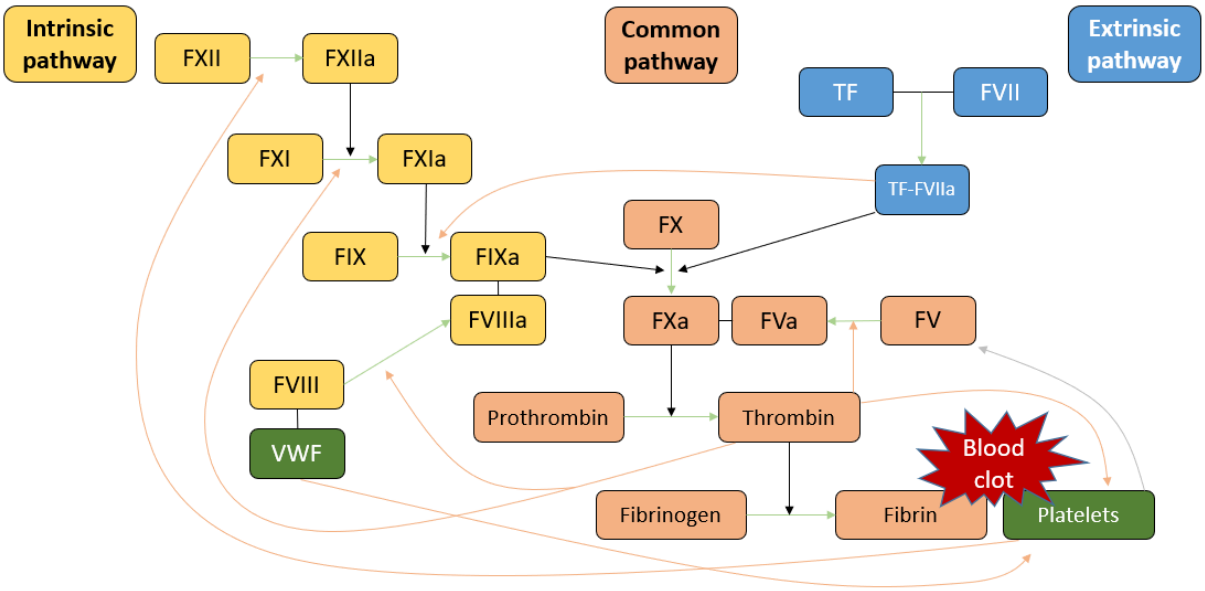

Figure 5. The coagulation cascade. Black arrows indicate enzymatic activity, green arrows represent activation of coagulation factors and orange arrows portray important additional pathways for facilitation and augmentation of the cascade. When tissue factor (TF) is exposed, it forms a complex with FVII which activates FX (extrinsic pathway, blue boxes). FXa co-localizes with FVa and cleaves prothrombin into active thrombin, which subsequently turns fibrinogen to fibrin (common pathway, red boxes). FXII is activated by negatively charged cell membranes, for instance on activated platelets, and provides another route to activation of FX through activation of FXI and FIX (intrinsic pathway, yellow boxes). Activated platelets also contribute by secretion of FV (grey arrow). The end product is cross-linked fibrin which reinforces the platelet plug, resulting in a stable blood clot.

In order to avoid inappropriate activation of the coagulation cascade, a series of anticoagulants exist to regulate its activity. First, endothelial cells create a local anticoagulant environment by constitutive expression of TFPI and antithrombin-binding heparan sulfate.[78] TFPI blocks and prevents formation of the TF-FVII complex, while antithrombin serves by inactivating several coagulation factors, including thrombin (FIIa) and FXa.[189,193] Further, thrombin provides a negative feedback mechanism on the coagulation cascade as it activates Protein C upon binding the endothelial cell receptor thrombomodulin. Activated Protein C (APC) proceeds to inactivate FVa and FVIIIa with the help of its cofactor, Protein S.[189] Finally, plasmin is the main enzyme responsible for fibrinolysis, the cleavage of cross-linked fibrin to degradation products such as D-dimer, which is essential in the diagnostic work-up for VTE.[1,194] Circulating as inactive plasminogen, plasmin is formed upon activation by tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), mainly synthesized and secreted by endothelial cells and monocytes, respectively.[188] These agents

display strong antithrombotic effects, and are utilized therapeutically in some thromboembolic events.[193] Under normal circumstances, tPA and uPA are counteracted mainly by PAI-1, preventing untimely plasmin formation.[188] Several of the anticoagulant properties in the circulation are disrupted upon endothelial cell activation, causing a skew towards a procoagulant state. Specifically, heparan sulfate and TFPI are less expressed on the endothelial cell surface, and PAI-1 is secreted in larger amounts.[78] Further, expression of P-selectin and secretion of Weibel-Palade bodies containing VWF contribute to platelet recruitment and transformation to a prothrombotic microenvironment.[74]

The importance of balance between pro- and anticoagulant forces is clearly demonstrated by the hereditary conditions affecting the mentioned proteins. Individuals with an imbalance due to gain-of-function mutations in coagulation factors (e.g., FVL) or deficiencies in anticoagulants (e.g., antithrombin deficiency) suffer a significantly increased risk of thrombosis,[90] while deficiencies of coagulation factors, e.g., FVIII deficiency (Haemophilia A), result in susceptibility to pathological bleeding episodes.[195] A pivotal role of hypercoagulability in VTE has been assumed due to the morphology and components of venous thrombi, so called “red clots”, which are rich in fibrin, contain large amounts of entrapped red blood cells and relatively few platelets.[76,196] Subsequently, extensive research has shown in the past decades that plasma levels of several coagulation factors are associated with the risk of VTE.[27] Of note, Koster and colleagues in 1995 were the first to report higher levels of FVIII and its carrier protein VWF in VTE cases compared with healthy controls.[197] The leading hypothesis on the underlying mechanism is that elevated levels of coagulation factors lead to increased endogenous thrombin generation, reflected by reduced lag time, increased peak thrombin formation and endogenous thrombin potential in the thrombin generation assay.[198-200]

1.4.1 Platelets

Platelets are circulating, non-nucleated cells derived from megakaryocytes, which serve as the primary cellular regulator of haemostasis. Moreover, they interact with immune cells and endothelial cells and are consequently involved in immunity and inflammation.[201] In physiological haemostasis, platelets are recruited to the site of vascular damage mainly through the exposure of subendothelial collagen.[202] In conditions with low shear stress, the

collagen can capture platelets directly by reversible binding to the transmembrane glycoprotein (GP)VI, while high shear stress conditions require VWF to provide tighter anchorage by acting as a bridge between collagen and the platelet GP1b/IX/V complex.[185,201] Adhesion to the glycoprotein receptors triggers intricate intracellular signalling resulting in activation of the platelets, which involves; (I) Ca^{2+}-signalling and exposure of negatively charged phosphatidyl serine (PS), which is essential for coagulation to occur on the platelet surface, (II) secretion of cytoplasmic granules containing ADP, serotonin, thromboxane A2, FV and VWF, which stimulate local vasoconstriction, coagulation and recruitment and activation of other platelets, (III) conformational change in the α2bβ3 integrin, which allows platelet aggregation by binding to VWF and fibrinogen, and (IV) change of platelet shape, as it develops several pseudopodia.[185,201] Altogether, after adherence and activation, platelets aggregate to form a plug at the site of injury, while simultaneously acting as facilitators of secondary haemostasis.[185] Further, extracellular vesicles (EVs) derived from activated platelets and monocytes are assumed to have a role in haemostasis and thrombosis.[203,204] While expression of TF was hypothesized as an important platelet-dependent mediator of haemostasis, research has found that platelet-derived EVs primarily appear to acquire TF through interaction with immune cells in the circulation.[184,203,205] The current opinion is that the association between EVs and VTE is mainly a result of negatively charged phospholipids such as PS on the EV surface, which are known to facilitate assembly of several coagulation factors and accelerate the activity of the TF-FVII complex substantially.[206,207] While platelet-derived EVs are small (~200 nm in diameter), their combined surface area can be massive and represent a significant increase in the potential for coagulation and thrombus formation.[206]

Arterial thrombosis arises from rupture of atherosclerotic plaque, exposing its lipid-laden, highly platelet-adhesive core to the circulation.[48] Consequently, the typical culprit thrombus in MI and ischaemic stroke, the “white clot”, is rich in platelets.[76,196] Due to the central role of platelets in arterial thrombosis, antiplatelet medications form the backbone of secondary prevention in MI and stroke.[46,208] In contrast, venous thrombi contain less platelets, and are normally targeted with anticoagulant therapy.[196] Still, platelets are considered important mediators in venous thrombogenesis, as they are known to facilitate coagulation upon recruitment by activated endothelial cells.[201] Randomized controlled trials have explored the effect of aspirin, an inhibitor of platelet activation, on the risk of recurrence after a first unprovoked VTE, and found that the recurrence risk was nearly halved in the group receiving

aspirin compared with those who received placebo.[209,210] Similar results have been found in research on aspirin as primary VTE prevention in orthopaedic patients.[211] Meanwhile, conflicting results have been reported when exploring platelet measures as biomarkers for VTE risk.[212] Platelet count has been found to predict VTE occurrence and prognosis in cancer patients, but no association is observed in cancer-free subjects.[164,213,214] Further, it is not regarded as a useful predictor of other thromboembolic events, and both low and high platelet count is associated with increased mortality in the general population.[215-217]

Platelets are known to vary in size according to the state of the parent megakaryocyte at the time of discharge, as platelets formed in the presence of inflammation, type 2 diabetes and hypercholesterolaemia are found to have a larger volume.[218,219] Moreover, large platelets contain larger amounts of thromboxane and other signalling substances, and are thus found to be more reactive and haemostatically active.[220,221] Mean platelet volume (MPV) is consequently regarded as an indirect marker of platelet reactivity, and potential associations with various diseases have been explored. Indeed, MPV was found to be associated with risk of future VTE, and unprovoked events in particular.[222] Hence, research indicates a role of platelets in VTE pathogenesis, which remains to be disentangled.

1.4.2 Von Willebrand factor

VWF is a large multimer glycoprotein which is synthesized by megakaryocytes and endothelial cells.[223,224] Platelets contain VWF stored in α-granules, which are secreted upon platelet activation. Meanwhile, endothelial cells carry VWF in Weibel-Palade bodies, and provide both constitutive and regulated secretion. Endothelial cells are the main source of VWF in the circulation, and VWF is hence regarded as a reliable marker of endothelial cell activation.[225,226] The discovery of VWF was initiated in 1926, when the Finnish physician Erik von Willebrand encountered a family from Föglö island, with an inherited bleeding tendency.[227,228] The family was lacking an unknown protein, which was later identified and named after von Willebrand.[229] Consequently, VWF is still most recognized as the deficient factor in von Willebrand Disease (VWD), the most common inherited bleeding disorder.[229-231] VWF serves as a facilitator of haemostasis by two distinct mechanisms; (I) it acts as a ligand for platelet GP1b and α2bβ3 integrin, thereby promoting adhesion and aggregation of

platelets, and (II) it is the plasma carrier and protector of FVIII, ensuring prolonged half-life and transport of FVIII in the circulation, thus facilitating coagulation.[232,233]

The *VWF* gene is located on chromosome 12 and encodes a precursor protein which undergoes several processes before storage and secretion.[234] First, disulphide bridging results in dimerization and multimer formation in the endoplasmic reticulum and Golgi apparatus, resulting in formation of VWF multimers of various size. Next, the molecules are glycosylated with 23 glycans per subunit, which in turn can carry ABO oligosaccharides according to blood type.[225,234] Finally, the mature VWF multimers are assigned to their storage organelles, namely Weibel-Palade bodies in endothelial cells and α-granules in platelets.[225] When the parent cell is activated, the organelles fuse with the cell membrane and VWF is released into the circulation. At this point, a portion of the endothelial cell-derived VWF remains anchored to the parent cell in ultra large VWF (ULVWF) strings, which consist of several thousand subunits and are highly platelet-adhesive.[234,235] When eventually cleaved into smaller multimers, VWF can perform its functions in the circulation. In the liver, VWF binds FVIII to its D'D3 domain, thus assuming the role as a protective chaperone protein for FVIII.[229] Upon initiation of the coagulation cascade, thrombin cleaves FVIII and liberates it from VWF. Subsequently, FVIII can exert its haemostatic function by accelerating the activation of FX by FIXa.[236,237] This process occurs on the surface of activated platelets and is more potent in the presence of negatively charged phospholipids which effectively bind FVIIIa.[237] Further, when vascular injury occurs, VWF operates as a bridge between platelets and the site of injury, as it binds to the exposed subendothelial collagen with its A3 domain and recruit platelets with the A1 domain.[235] Finally, the VWF C4 domain is found to contribute in platelet aggregation by linking with their α2bβ3 integrin.[235] The domain structure of VWF is illustrated in Figure 6. Importantly, VWF is found to circulate in a globular conformation, but unfolds and elongates upon increased shear stress (e.g., due to stenosis or vessel rupture), resulting in increased availability of the platelet-adhesive domains.[238,239] The lifespan of plasma VWF is limited, and degradation is found to occur largely by uptake in macrophages. In the population, the half-life of VWF in plasma is ~16 hours on average, but varies substantially between individuals.[240] Different patterns of glycosylation is found to account for parts of the variability, and individuals with a non-O blood group are found to have longer VWF half-life and around 25% higher plasma levels of VWF, compared with those with blood group O.[223,241] In total, genetic variants are found to

account for up to 65% of the variability in plasma levels of VWF, while the remaining proportion can be attributed to acquired factors such as aging, pregnancy, exercise and cigarette smoke.[242]

The main regulator of VWF is ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13), an enzyme mainly synthesized and secreted by hepatic stellate cells.[243] While other proteolytic metalloproteases have several substrates, the only known function of ADAMTS13 is the cleavage of VWF at the Tyr^{1605}-Met^{1606} site of the A2 domain.[244,245] Cleavage by ADAMTS13 results in reduced haemostatic activity of VWF due to the fact that the platelet affinity of VWF depends on its multimeric size. The importance of ADAMTS13 is best illustrated by the condition in which it is lacking, thrombotic thrombocytopenic purpura (TTP), a potentially fatal disease characterized by an excess of ULVWF and platelet-rich thrombi disseminated in the microvasculature.[246-248] While severe deficiency of ADAMTS13 is inherited or acquired due to the formation of autoantibodies, a significant decrease in plasma levels is found to occur due to various physiological and pathological conditions, including advanced age, pregnancy and inflammation.[249,250]

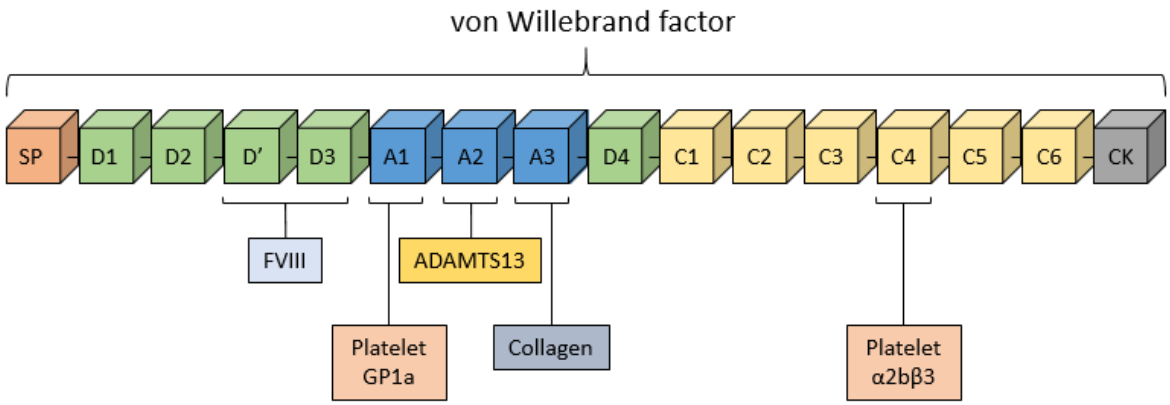

Figure 6. Schematic representation of VWF domains and main sites of interaction. The organization of domains is presented according to the findings of Zhao and colleagues.[251] In the liver, circulating VWF binds to FVIII at the D'D3 domain, where it remains until degradation or activation of FVIII. Upon increased shear stress, the A1 and A2 sites are revealed, allowing interaction with platelet GP1a and cleavage by ADAMTS13. When subendothelial collagen is exposed, it is bound by the A3 domain of VWF. The C4 domain has affinity to the α2bβ3 integrin of platelets, promoting platelet aggregation. Adapted from Rauch and colleagues.[252]

During the past decades it has become increasingly clear that the VWF-ADAMTS13 axis is pivotal in haemostasis and thrombogenesis. Following this, observational research has reported that plasma levels of VWF is linearly associated with risk of arterial thrombosis, and results from MR studies indicate that the association is causal.[253,254] Further, a similar role is asserted for reduced plasma levels of ADAMTS13.[253,255] As VWF and ADAMTS13 are functionally linked and assumed to have opposite effects, researchers have hypothesized that an increase in the ratio between plasma levels of VWF and ADAMTS13 would also be associated with increased risk of arterial thrombosis.[256,257]

The evidence for a potential association between VWF levels and VTE risk is scarcer and of lesser quality. Since Koster and colleagues first reported elevated VWF and FVIII levels in DVT patients in the Leiden Thrombophilia study,[197] the majority of subsequent research exploring VWF and incident VTE have also been case-control studies.[258-264] This study design comes with inherent susceptibility to bias due to reverse causation and selection of control subjects.[265] Meanwhile, few have investigated the association using a prospective design. In the Longitudinal Investigation of Thromboembolism Etiology (LITE) cohort study, consisting of over 19,000 participants and 159 VTE events, plasma levels of VWF were linearly associated with risk of future VTE.[27] Further, the association was found to remain after 13 years of follow-up.[266] In the Women's Health Initiative nested case-control study, plasma VWF measured at baseline was associated with future VTE when considered as a continuous variable, but no statistical significant association was found when plasma VWF was dichotomized at the 75th percentile.[267] Furthermore, a recent MR study from the INVENT consortium found that increased plasma VWF levels were causally associated with VTE, but whether the entire effect was mediated by FVIII could not be asserted due to shared genetic determinants.[254] In the first case-control study exploring the relationship between plasma VWF and VTE, the association disappeared after adjustments for FVIII levels, and it was therefore assumed that FVIII mediates the entire association.[197] Since then, several epidemiologic studies have confirmed that elevated FVIII levels are associated with increased risk for VTE.[27,198,258,262,263,267,268] However, in LITE, the association between VWF and VTE was found to be independent of FVIII.[27] This finding has been replicated by others, and suggests a role of the platelet-adhesive properties of VWF in VTE.[262] However, as FVIII and VWF circulates in a complex and hence are tightly correlated in blood,[269] multivariable adjustment is unsuitable for dissecting their respective effects on VTE risk.[270,271]

For ADAMTS13, existing evidence on a potential association with VTE comprises only small case-control studies and studies on cancer patients, and it is suggestive of an inverse relationship where decreased plasma levels of ADAMTS13 are seen in VTE patients.[260,272-275] Overall, there is a need for more research on the role of the VWF-ADAMTS13 axis on VTE risk using prospective data from unselected populations. Further, as VWF has a dual role in haemostasis, the independent impacts of FVIII and the platelet-binding properties of VWF need to be unravelled.

2 Aim of the thesis

The overall aim of the present thesis is to unravel the role of the VWF-ADAMTS13 axis in VTE, using prospective data from an unselected population.

Specific aims:

I. To investigate the association between plasma levels of VWF and risk of future incident VTE in a population-based nested case-control study.

II. To investigate whether plasma ADAMTS13 levels and an imbalance with VWF levels, assessed by the VWF/ADAMTS13 ratio, are associated with risk of future incident VTE in a population-based nested case-control study.

III. To investigate the combined effects of plasma VWF and platelet measures (platelet count and mean platelet volume) on the risk of future incident VTE in a population-based nested case-control study.

IV. To investigate the combined effects of plasma FVIII and mean platelet volume on the risk of future incident VTE in a population-based nested case-control study.

3 Methods

3.1 The Tromsø Study

The Tromsø Study is a single-center prospective cohort study on the general population of Tromsø, the largest municipality in northern Norway (population in 2015: 73,000).[276,277] The population is spread across urban (80%) and rural areas, and mainly comprises individuals of Norwegian origin (85%).[276] The study was inaugurated in 1974 with the aim of combating the high mortality of cardiovascular diseases, which was particularly prominent in Northern Norway.[278,279] The approach was later expanded to cover a wide range of diseases and other parameters of health.[280] The Tromsø Study has been conducted as repeated health surveys, which have included a variable proportion of the population and collected a various set of data on the participants. A total of seven surveys have been conducted to this date, and more than 45,000 unique individuals have attended at least one of the surveys. Importantly, the University Hospital of North Norway (UNN) is the only hospital in the study area, which increases the probability of a complete follow-up of study participants by linkage to hospital discharge registries.

All four papers in the present thesis included data from Tromsø 4, the fourth and largest survey of the Tromsø Study.[280] It was conducted in 1994-1995, and all inhabitants above the age of 25 years were invited to participate. In total, 27,158 individuals took part, resulting in a participation rate of 77%. Non-attendees were younger on average, more often single and comprised a larger proportion of males, compared with those who attended the survey. The survey involved broad data collection by self-administered questionnaires, blood sampling and various physical examinations.

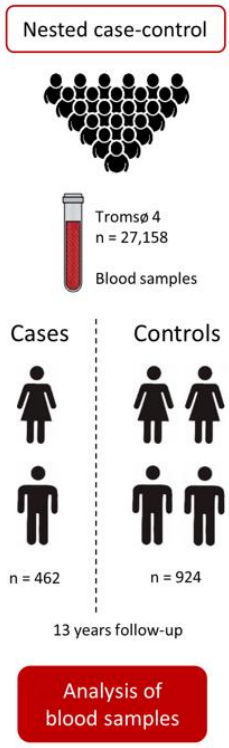

Figure 7. Nested case-control design. Two controls were selected from the source cohort each time a case occurred. Blood samples collected at baseline were thawed and analyzed after the study period.

3.2 Study design

A **nested case-control** design was applied in all four papers of the present thesis (Figure 7). The study participants of Tromsø 4 (n = 27,158) were followed from the enrolment date until the date of

incident VTE, death, migration or end of follow-up on September 1, 2007. During the study period (1994-2007), 462 participants suffered an incident VTE event, and were included as cases. For each VTE case, two age- and sex-matched controls who were alive at the index date of the VTE event were randomly sampled among the remaining participants of Tromsø 4 (n = 924). The studied exposure levels were based on data and blood samples collected at cohort baseline, ensuring that the temporal sequence between exposure and outcome was preserved. A variable amount of participants were excluded from the final analytic sample due to plasma samples being unavailable or of insufficient quality for laboratory analyses. In papers I-IV the numbers of excluded cases/controls were 48/81, 79/144, 59/108 and 97/214, respectively.

3.3 Assessment of exposures

3.3.1 Baseline measurements

Baseline information was obtained from self-administered questionnaires, blood samples and physical examinations. The questionnaires were used to gather detailed information on smoking habits, oestrogen therapy and disease history, including current or former cancer and CVD (i.e., stroke, MI and angina pectoris).[277] In the physical examination, body weight (to the nearest 0.5 kilogram) and height (to the nearest centimetre) were measured with participants in light clothing and without shoes. BMI was subsequently calculated as body weight in kilograms divided by the square of height in metres (kg/m^2). Blood pressure was measured three times using an automatic device (Dinamap Vital Signs Monitor). Participants rested in a sitting position for 2 minutes before and between the measurements, and the mean systolic and diastolic blood pressure was calculated based on the last two measurements.

3.3.2 Blood sampling, storage and laboratory analysis

Non-fasting blood samples collected from an antecubital vein were obtained from all participants at cohort baseline. Blood was sampled into 5-mL vacutainers (Becton Dickinson, Le Pont-de-Claix, France) containing EDTA (K_3-EDTA 40 μL, 0.37 mol/L per tube) as anticoagulant. Measurement of MPV and platelet count was performed within 12 hours of blood sampling using an automated blood cell counter (CoulterCounter, Coulter Electronics,

Luton, UK) at the Department of Clinical Chemistry at the UNN.[222] Centrifugation at 3000*g* was performed for 10 minutes at room temperature to prepare platelet-poor plasma (PPP). Then, the supernatant was transferred into cryovials (Greiner Laboratechnik, Nürtingen, Germany) in 1-mL aliquots and stored at -80°C until further analysis, as previously described.[281]

After the study period, the stored plasma samples were analysed for antigen levels of VWF, FVIII, ADAMTS13 and high-sensitivity C-reactive protein (hsCRP) at the Research Institute of Internal Medicine at Oslo University Hospital, Rikshospitalet. Frozen PPP samples were thawed for 5 minutes in a 37°C water bath and subsequently centrifuged at 13,500*g* for 2 minutes to obtain platelet-free plasma. Antigen levels were measured by enzyme immunoassay (EIA). Commercially available reagents were obtained from Dako (Glostrup, Denmark) for VWF, Affinity Biologicals (Ontario, Canada) for FVIII and R&D Systems (Minneapolis, MN) for ADAMTS13 and hsCRP. For VWF and FVIII, human plasma from 20 healthy individuals was diluted in parallel and used as standard, and measurements were expressed as percentage of the control population mean (100%). Similarly, the mean value of plasma ADAMTS13 in the control population was set to 100%, and all values were adjusted accordingly and expressed as percentage of the control population mean. EIAs were performed in a 384-format with a pipetting robot and a BioTek dispenser/washer (Synergy H1 Hybrid). Absorption was read at 340 nm using an EIA plate reader with wavelength correction set to 540 nm. The intra- and inter-assay coefficients of variation were 2.6% and 10.8% for VWF, <10% and <10% for FVIII, 9.8% and 8.8% for ADAMTS13, and 2.6 and 9.1% for hsCRP, respectively. The laboratory analyses have been described in further detail in papers I-IV.

3.4 Assessment of outcome

The discharge diagnosis registry, radiology procedure registry and autopsy registry of the UNN, the only hospital in the study region, were used to identify all incident VTE events. International Classification of Diseases (ICD) diagnosis codes were used to retrieve cases from the registries. ICD-9 was used for events occurring in 1994 to 1998, and relevant codes were 325, 415.1, 451, 452, 453, 671.3, 671.4 and 671.9. Cases occurring after 1998 were identified by ICD-10 codes I80, I81, I82, I67.6, O22.3, O22.5, O87.1, O87.3 and I26. To

identify cases with a missing diagnosis code, description of all relevant diagnostic procedures (i.e., spiral computed tomography, perfusion-ventilation scan, pulmonary angiography, compression ultrasonography and venography) were retrieved from the radiology procedure registry. Subsequently, the medical record of each potential VTE case was extensively reviewed by trained personnel, and a VTE was only registered when signs and symptoms were followed by objective radiological confirmation and resulted in a VTE diagnosis requiring treatment (unless there were specified contraindications). For events identified in the autopsy registry, a VTE was only recorded when PE was defined as the main cause of death or a significant contributing cause of death. All events were then categorized as DVT or PE, with simultaneous evidence of both conditions recorded as PE. Further, the events were classified as provoked or unprovoked based on presence of acknowledged provoking factors closely preceding the VTE, as recommended by the International Society on Thrombosis and Hemostasis.[21] The event was categorized as provoked if the patient had one or more of the following: Surgery, trauma or acute medical conditions (stroke, MI or acute infection) within 8 weeks before the event; active cancer at the time of VTE diagnosis; or immobilization (wheel chair confinement within the last 8 weeks or >3 days bed rest). Other factors were recognized as provoking if specified by a physician (e.g., venous catheters).

3.5 Statistical analyses

STATA version 16.0 (Stata Corporation, College Station, Texas, USA) and R version 3.6.3 and 4.0.5 (The R Foundation for Statistical Computing, Vienna, Austria) were used to perform the statistical analyses.

For paper I-IV, baseline characteristics of study participants across exposure levels of VWF, ADAMTS13 or FVIII antigen (Ag) were expressed as percentage (number of participants) for categorical variables and mean (± standard deviation) or median (25^{th} – 75^{th} percentile) for continuous variables.

All participants were categorized according to quartiles of VWF:Ag, ADAMTS13:Ag and VWF:Ag/ADAMTS13:Ag ratio, and cutoffs were determined from the distribution in the control population. The same approach was used to derive tertiles of FVIII:Ag and VWF:Ag Three exposure categories (low, medium and high) were also defined for MPV and platelet

count. Cutoffs were set at 8.5 and 9.5 fL for MPV and 230 and 300·L^{-1} for platelet count, to conform with our previous study on platelet measures and risk of VTE.[222]

3.5.1 Logistic regression

In papers I and II, logistic regression was used to estimate odds ratios (ORs) with 95% confidence intervals (CIs) for VTE across quartiles of plasma VWF, ADAMTS13 and VWF/ADAMTS13 ratio. The lowest quartile served as reference category for VWF and VWF/ADAMTS13 ratio, while the reference category for ADAMTS13 was the highest quartile. In paper IV, the same approach was applied in regression analyses for FVIII, with the lowest tertile used as reference. Regression analyses were performed for both overall VTE and according to VTE location (DVT or PE). Subgroup analyses also included analyses restricted to provoked or unprovoked events. *P* value for linear trend was estimated across increasing quartiles of VWF and VWF/ADAMTS13 ratio, decreasing quartiles of ADAMTS13 and increasing tertiles of FVIII. All associations were adjusted for the matching factors, i.e., age and sex, according to recommendations.[282] Further adjustments were performed by including BMI, hsCRP and history of CVD at cohort baseline as covariates.

As the investigated exposures are modifiable, it is likely that the levels measured in samples obtained at cohort baseline had a decreasing accuracy during follow-up, thus introducing a possibility of underestimating true associations due to regression dilution bias.[283] To take this into account, the associations between exposure variables (e.g., plasma levels of VWF and FVIII) and VTE were modelled as a function of time from baseline. For instance, in paper I, ORs for VTE for the highest versus lowest exposure category were estimated at every 0.1-year increase in time from baseline until the end of follow-up. Available VTE cases were restricted based on the date of the event, while keeping all controls in the analyses. Estimates were generated from the time when 10 VTE events had occurred, and statistical significance was defined as $P < 0.05$.

3.5.2 Interaction analysis

In paper III, the combined effects of VWF and platelet measures (MPV and platelet count) were assessed, while paper IV included analyses on the combined effect of FVIII and MPV. Three exposure categories (low, medium, high) were made for each variable, and subsequently combined to form a nine-level variable. Subjects in the category with low levels of both exposures served as the reference group, and unconditional logistic regression was performed to estimate ORs for VTE. All analyses were adjusted for age and sex in Model 1. In paper III, Model 2 included additional adjustments for BMI, hsCRP, smoking, hypertension, oestrogen use, self-reported history of cancer at baseline and MPV/platelet count. In paper IV, Model 2 was adjusted for age, sex, BMI, hsCRP and platelet count.

Presence of biological interaction on an additive scale was evaluated using the relative excess risk due to interaction (RERI), which was calculated as $(OR_{AB} - 1) - (OR_A + OR_B - 2)$.[284] Here, $_A$ represented high plasma VWF:Ag or FVIII:Ag, while $_B$ indicated high MPV or platelet count. Further, the proportion of events in the joint exposure group which could be attributed to the biological interaction, i.e., the attributable proportion (AP), was calculated as RERI divided by OR_{AB}. All parameters of interaction were presented with their 95% CI. A RERI and AP > 0 suggest the presence of biological interaction, as this indicates that the effect of the two exposures combined exceeded the sum of the separate effects.

3.6 Ethics

Written informed consent was obtained from all participants in the Tromsø Study prior to study inclusion. Further, all participants were free to withdraw their consent at any time. The Regional Committee for Medical and Health Research Ethics (REK) approved the present study project (REK nr. 113706).

4 Main results

4.1 Paper I: Plasma levels of von Willebrand factor and future risk of incident venous thromboembolism

Several case-control studies have reported elevated plasma VWF levels in patients with VTE compared with controls. However, because few studies have investigated the association in a prospective design, it is unclear whether elevated plasma VWF is a predictive biomarker or a consequence of the VTE event. Therefore, we aimed to investigate the association between plasma VWF levels and risk of future VTE. In addition, we set out to perform subgroup analyses of DVT, PE, provoked and unprovoked events.

The fourth survey of the Tromsø Study cohort was used to establish a population-based nested case-control study of 414 VTE cases and 843 age- and sex-matched controls. Blood samples were collected at cohort baseline (1994-1995), and the follow-up period ended in 2007. ORs with 95% CIs for VTE were estimated across quartiles of VWF levels. Further, analyses were performed with stratification by disease subgroups.

We found that the risk of VTE increased linearly across quartiles of VWF levels (*P* for trend = 0.023). Participants with VWF in the highest quartile had an OR of 1.45 (95% CI, 1.03-2.03) for VTE compared with those in the lowest quartile. The association was strongest for unprovoked VTE (OR, 2.74; 95% CI, 1.66-4.54) and unprovoked DVT in particular (OR, 6.73; 95% CI, 3.07-14.76). Further adjustment for BMI, CRP, hypertension, oestrogen use, and smoking had a negligible effect on the risk estimates.

In conclusion, we found a dose-dependent association between VWF levels and future incident VTE. Subgroup analyses indicated that the overall association was mainly driven by high risk estimates for unprovoked DVT. Our findings suggest that VWF may represent a promising biomarker for risk of future incident VTE.

4.2 Paper II: Impact of the von Willebrand factor-ADAMTS13 axis on the risk of future venous thromboembolism

VWF is established as a pivotal component in haemostasis. Its haemostatic activity is regulated by ADAMTS13, which reduces the potency of VWF by cleavage into smaller multimers. While VWF has been explored as a risk factor for VTE, prospective data on ADAMTS13 and the balance between the two proteins is lacking. The role of the VWF-ADAMTS13 axis for the risk of future VTE is thereby unknown. We set out to investigate whether plasma ADAMTS13 levels and an imbalance with VWF levels, reflected by the VWF/ADAMTS13 ratio, were associated with the risk of future VTE.

The Tromsø Study cohort was used to derive a population-based nested case-control study, comprising 383 incident VTE cases and 780 age- and sex-matched controls. Antigen levels of ADAMTS13 and VWF were measured in plasma samples obtained at cohort baseline (1994-1995) and participants were followed until 2007. Quartiles of plasma ADAMTS13 and VWF/ADAMTS13 ratio were defined from levels measured in the control population. Subsequently, ORs with 95% CIs were estimated according to quartiles of ADAMTS13 and VWF/ADAMTS13 ratio.

In analyses adjusted for age and sex, ADAMTS13 levels were inversely associated with the VTE risk, and participants with ADAMTS13 in the lowest quartile had an OR of 1.40 (95% CI, 0.99-1.99) for VTE compared with those in the highest quartile. The VWF/ADAMTS13 ratio was linearly associated with the VTE risk (*P* for trend = .001). Here, participants in the highest quartile had an OR of 1.70 (95% CI, 1.19-2.43) for VTE compared with those with VWF/ADAMTS13 ratio in the lowest quartile. The risk estimates were highest for unprovoked VTE (OR, 2.81; 95% CI, 1.65-4.81). Further adjustment for BMI and CRP only resulted in a slight attenuation of the risk estimates.

To conclude, we observed that low plasma levels of ADAMTS13 and an imbalance between ADAMTS13 and VWF levels, reflected by an increased VWF/ADAMTS13 ratio, were associated with an increased risk of future VTE. Our findings suggest that the VWF-ADAMTS13 axis is involved in the pathogenesis of VTE.

4.3 Paper III: Combined effects of plasma von Willebrand factor and platelet measures on the risk of incident venous thromboembolism

Increased plasma levels of VWF and high platelet reactivity are established risk factors for VTE. While VWF is known to promote haemostasis by interaction with platelets, the impact of this mechanism in the pathogenesis of VTE is not clear. In this study, we explored the combined effects of plasma VWF and platelet measures on the risk of incident VTE.

A population-based nested case-control study with 403 cases and 816 controls was derived from the fourth survey of the Tromsø Study cohort. Plasma VWF, platelet count and MPV were measured in blood samples drawn at baseline. VTEs were recorded during a follow-up period of 13 years. ORs with 95% CIs for VTE were estimated across VWF tertiles, within predefined MPV (<8.5, 8.5-9.5, ≥9.5 fL) and platelet count (<230, 230-299, $\geq 300 \cdot 10^9$ L^{-1}) strata.

Participants with VWF levels in the highest tertile and MPV ≥9.5 fL had an age- and sex-adjusted OR of 1.98 (95% CI 1.17-3.36) for VTE compared with those in the lowest exposure category of VWF and MPV. In the joint exposure group, 48% (95% CI 15% to 96%) of VTE events were attributable to the biological interaction between VWF and MPV. Similarly, individuals with VWF in the highest tertile and platelet count ≥300·109 L-1 had an OR of 2.91 (95% CI 1.49-5.67) for VTE compared with those with concomitant low VWF and platelet count <230. Further, 39% (95% CI -2% to 97%) of VTEs in the joint exposure group could be attributed to the interaction. Multivariable adjustment had a negligible effect on the risk estimates.

In conclusion, our results suggest that both platelet reactivity, assessed by MPV, and platelet count interact biologically with high plasma VWF, resulting in an increased risk of incident VTE. As elevated VWF did not yield significantly increased risk estimates for VTE in absence of elevated platelet measures, a platelet-dependant role of VWF in VTE was implied.

4.4 Paper IV: Combined effect of high factor VIII levels and high mean platelet volume on the risk of future incident venous thromboembolism

High plasma levels of FVIII and large platelets, as reflected by a high MPV, are separately found to be associated with increased risk for VTE. However, it is unknown whether the combination of high FVIII levels and large platelets has a supra-additive effect on VTE risk. We set out to investigate the joint effect of high FVIII levels and high MPV on the risk of future incident VTE.

A nested case-control study with 365 incident VTE cases and 710 age- and sex-matched controls was derived from the Tromsø Study, a population-based prospective cohort study. Blood samples drawn at baseline were used to measure FVIII antigen levels and MPV, and levels in the control population were used to define tertile cutoffs for plasma FVIII. ORs with 95% CIs were estimated across tertiles of plasma FVIII (<85%, 85%-108%, and ≥108%) and within predefined MPV strata (<8.5, 8.5-9.5, and ≥9.5 fL).

The risk for overall VTE increased linearly across FVIII tertiles (*P* for trend < 0.001) in models adjusted for age, sex, BMI and CRP. Those with plasma FVIII in the highest tertile had an OR of 1.97 (95% CI, 1.07-2.46) for VTE compared with those in the lowest tertile. In the combined analysis, participants with FVIII levels in the highest tertile and an MPV of ≥9.5 fL (i.e., joint exposure) had an odds ratio for VTE of 2.71 (95% CI, 1.44-5.11) compared with those with FVIII levels in the lowest tertile and an MPV of <8.5 fL (reference). In the joint exposure group, 52% (95% CI, 17%-88%) of VTEs could be attributed to the biological interaction between FVIII and MPV. No biological interaction was found between FVIII levels and platelet count on the VTE risk.

To conclude, we found that elevated plasma levels of FVIII and high MPV interacted to yield a more than additive effect on the risk of future VTE. Our results suggest that large platelets, as reflected by high MPV, might be involved in the mechanism by which high FVIII level increases the risk of incident VTE.

5 Discussion

5.1 Methodological considerations

Epidemiology is defined as the study of distributions and determinants of health-related events in specified populations, and it is applied to describe and control diseases.[271] Several methods exist within this broad study category, and can be regarded as tools designed to investigate different aspects of disease occurrence. There are two main categories of epidemiologic research, namely studies using groups as units of observation (i.e., ecological studies) and studies using individuals as observation units.[271] The latter category includes cross-sectional studies, cohort studies, case-control studies and case-crossover studies, which are observational by nature.[285] In contrast, experimental studies include randomized controlled trials (RCTs), where an exposure is actively modified by researchers, which are considered the gold standard to assess causal associations in epidemiology.[285] All these study designs have some inherent limitations which are substantially impacted by the research methodology. As only a part of the population is observed, it is impossible to be certain whether the patterns identified pertains to individuals outside the study population. Further, several methodological factors can induce bias and provide deceitful results.[271]

5.1.1 Study design

Papers I-IV were conducted as **nested case-control** studies, derived from the Tromsø Study cohort. This study design can be described as a combination between a cohort and a case-control study. Specifically, the selection of participants and statistical analyses mimic a case-control study, while the source population is a cohort, and the temporal sequence between exposure and outcome conforms with a cohort study design.[271]

The Tromsø Study is a prospective cohort following the general population in a Northern Norwegian municipality. Exposures are assessed at cohort baseline, and each individual is followed until an outcome of interest (e.g., VTE) or a censoring event (e.g., death or migration) occurs, or when the study period is over. An important benefit with the cohort design is the possibility to explore several exposures and outcomes. Further, a temporal sequence between the exposure and outcome can be established, which is a prerequisite (but not sufficient) for inferring causality in the observed association. As time-to-

event data is available and incidence rates can be estimated, it is possible to calculate both absolute and relative risks.[271] However, cohort studies are resource-demanding and poor at investigating rare outcomes.[285] Other limitations which threaten the validity of the findings in epidemiological studies, including confounding and different types of bias, will be considered in the next section of the thesis (5.1.2 Validity).

Case-control studies involve a group of individuals with an outcome of interest (i.e., cases) who are compared with individuals without the outcome (i.e., controls) with regards to exposures of interest. This design has several benefits, including a possibility to investigate several exposures, beneficial overall cost-efficiency and no required follow-up.[285] Meanwhile, case-controls have some important disadvantages. Only one outcome can be studied, and measures of absolute risk cannot be calculated as incidence rates are not available. Moreover, "classical" case-control studies determine the exposures of interest after recruiting study participants, and investigators can therefore most often not know whether the exposure was present before the outcome or emerged as a result of it. This phenomenon is referred to as reverse causation and is a major obstacle when contemplating whether the observed associations are causal. Furthermore, an important challenge when conducting a case-control study is the selection of control subjects. Their purpose is to describe the distribution of the exposure in the same population from which the cases are derived from, and it is therefore crucial that the selection of controls is independent of the exposure.[271] Finally, case-control studies may be susceptible to suffer from information bias; a situation where the precision of exposure registration differs between cases and controls. An example of this is recall bias, where cases and controls recall and report exposures differently, which can be detrimental when gathering information by interviews or questionnaires.

By applying a nested case-control design, we were able to overcome some of the important drawbacks in case-control and cohort studies.[271] First, cases and randomly sampled controls were drawn from the same parent cohort when an incident VTE event occurred, which reduced the probability of selection bias. Second, "classical" case-control studies often require cases to survive for a certain period in order to be included, making the study population susceptible to survivor bias.[285] This was likely not a significant problem in the papers of this thesis as cases were included irrespective of survival, and the autopsy registry was searched to identify additional cases which were undiagnosed prior to the occurrence of death. Third, as the Tromsø Study is a population-based cohort with a good attendance rate, it

is reasonable to assume that the results are generalizable to the general population. The nested case-control design was more cost-efficient than a cohort study as only a subset of the participants is included in the analytic sample. As only the baseline blood samples of the included participants had to be thawed and analysed, the demand of resources was reduced substantially compared with a scenario where all cohort participants would be included. In contrast to a "classical" case-control study, the exposures were measured in samples obtained at baseline and the temporal sequence was thereby preserved. However, time-to-event analyses were not applicable as controls were sampled at the time of the event, and logistic regression was therefore applied to obtain ORs as approximations of the relative risk (RR). While ORs have a tendency of overestimating the magnitude of associations compared with the RR, this effect is found to be negligible when the studied outcome is rare in the population.[286]

5.1.2 Validity

To assess whether studies provide valid results is an important task in research. In epidemiology it is common to consider validity from two perspectives; internal and external. Internal validity refers to the degree which the study was able to test the hypothesis, i.e., *did the study accurately measure what it set out to?* Consequently, systematic research errors are antagonists of internal validity.[287] External validity is preconditioned by internal validity, and refers to whether the findings are generalizable to other settings, populations or time-periods than those of the conducted study.[288]

5.1.2.1 External validity

The general population serves as both the source population and target population of the Tromsø Study. The degree of generalizability therefore predominantly relies upon the recruitment efficacy. All papers in the present thesis used data from Tromsø 4, where the entire adult population aged ≥25 years was invited to participate. Importantly, the attendance rate was 77%, which is among the highest rates recorded in Norwegian cohort studies.[277] Non-attendees were more likely to be young, single and of male sex. There was also a notable decline in the participation rate in the elderly (aged ≥80 years at time of invitation). Caution is therefore warranted when extrapolating our findings to individuals in these categories.

While external validity is a key component in research aiming to describe the target population, it can be counterproductive when exploring causal associations. According to Rothman and colleagues, the pursuit of representativeness may breed misleading results due to an increase in unmeasured confounders and can hinder the extrapolation of true causal associations.[80] In other words, restricting the study population to individuals within a narrow range of characteristics may provide a more valid foundation for comparison, which is typically seen in RCTs. The present thesis aims to explore risk factors which have the potential to be causally associated with VTE, and the value of the results therefore mainly depend on internal validity.

5.1.2.2 Bias

All research findings in epidemiology can be distorted by either random or systematic errors which can occur at any stage of the research process. While the effect of random errors can be minimized by increasing the sample size, this does not improve estimates calculated in a context of systematic errors. Another term for systematic error is bias, which is the opposite of validity and describes a tendency where the results are skewed from the true association.[287]

Selection bias refers to distortions which arise due to erroneous recruitment of study participants.[271] This is best exemplified by case-control studies where the control population does not reflect the population from which the cases are derived from. A systematic difference in the exposure variable may therefore occur, which will be interpreted in the analysis of the results as an association between the exposure and outcome even though there may be no true association. Internal validity may therefore be compromised. Further, non-responder bias is an important concept that affects both external and internal validity in epidemiological studies.[285] Specifically, if participation is associated with the exposure or outcome, the findings will be less generalizable and may be biased. As the entire adult population of the municipality were invited to participate in the Tromsø Study and the attendance rate was high, our study population was to a large degree unselected. Meanwhile, as previously mentioned, participation varied according to age and sex, and older age is associated with both the investigated exposures and risk of VTE. Taking this into account, cases and controls were matched on age and sex in all papers in the present thesis, and

analyses were adjusted for the matching factors. It is therefore highly unlikely that our results were biased due to systematic difference in exposure between cases and controls.

Information bias occurs as a result of systematic errors in the estimation of a variable, i.e., exposure or outcome.[271] This leads to a misclassification, as study participants are allocated to categories which do not reflect the true value of the studied variable. A classic example from case-control studies is recall bias, a subtype of information bias; when asked about specific exposures, cases and controls remember and report past exposures differently. This can lead to a misclassification of exposure which is differential with regards to the outcome. In other words, exposed controls may for instance have been wrongfully registered as unexposed, and analyses may consequently show a spurious or exaggerated association between the exposure and outcome. This type of bias can be safely excluded from papers I-IV as they all used a nested case-control design, with exposure data being collected prior to the occurrence of the outcome. Further, differential misclassification of VTE events would not be plausible, as investigators responsible for assessing the outcome were not aware of the participant's exposure status. Further, VTE diagnosis was based on objective criteria. In addition, the investigators who performed the laboratory analyses were blinded with regards to VTE status of the study participants. Of note, misclassification can also be nondifferential, i.e., independent of all other variables. While differential misclassification may lead to either over- or underestimation of associations, nondifferential misclassification typically underestimates the risk.[271]

5.1.2.3 Regression dilution bias and long-time storage of plasma samples

All exposure variables investigated in the present thesis are susceptible to change over time due to acquired environmental factors. It is therefore reasonable to assume that the levels of exposure fluctuated during the 13 years of follow-up, and that the values established at cohort baseline often did not reflect the true values at the time points of VTE occurrence. However, as the study did not involve repeated measurements of exposures, this could not be taken into account in the analyses. Due to the concept of regression to the mean, it is most likely that individuals with extreme values at baseline, either high or low, would later approach the mean value in the population.[283] Consequently, the exposure groups most likely developed increasingly similar exposure levels during follow-up. Further, if an individual with high

plasma levels of VWF at baseline proceeded to have even higher levels, the person would still fall into the correct quartile (Q4), while a change in the opposite direction would result in a misclassification. In other words, our data were likely to include individuals in Q1 who did not have low exposure levels and individuals in Q4 who did not have high exposure levels at the time point of interest. As the exposure assessments were similar for all study participants it is highly unlikely that changes in exposure levels would occur differently in VTE cases and controls or that exposures would be systematically over- or underestimated, and the potential misclassification was thereby expected to be arbitrary and nondifferential. The resulting effect is addressed as regression dilution bias, which leads to an underestimation of true associations.[283,289]

We assessed the effect of regression dilution bias on the associations of VWF, ADAMTS13 and FVIII with VTE by performing regression analyses with restricted follow-up time, as the accuracy of registered exposure valuables were expected to be most valid shortly after baseline. Specifically, the ORs for VTE were estimated considering the time elapsed between blood sampling at baseline and the occurrence of VTE events, and generally showed that the associations persisted throughout the study period. Meanwhile, the magnitudes of the risk estimates were larger with a shorter time between blood sampling and the VTE event, and we concluded that the risk estimates were likely biased to a modest degree due to regression dilution.

Long-time storage of blood samples is known to result in gradual degradation of plasma proteins, resulting in a reduction in measured levels.[290-292] This phenomenon was indeed observed in the plasma samples used in our studies, where the average levels of VWF and FVIII in controls were ~25% of the levels measured in fresh pooled plasma from 20 healthy individuals. ADAMTS13 was found to decompose to a smaller degree, as measured levels in study participants were closer to that of the fresh pooled plasma. Prior to regression analyses, we carried out standardization by redefining the mean value of the control population as 100%, and adjusted plasma values of all participants accordingly. There was no reason to suspect that the effect of long-term storage would differ between the plasma samples of VTE cases and controls, or that it would be associated with any other exposure. Any potential misclassification in the allocation to tertiles/quartiles of exposure levels was therefore assumed to be non-differential with regards to future VTE status, thus introducing the possibility of underestimation of the true associations.

5.1.2.4 Confounding

Associations identified in epidemiological research have varying implications based on whether the association is causal or not. While a modifiable causal risk factor may be used as a target to reduce the risk of the outcome, a non-causal risk factor may primarily be useful in prediction. Non-causal associations occur due to the influence of one or more confounders, which are; (I) associated with the exposure, (II) associated with the outcome, and (III) not in the causal pathway between the exposure and outcome (Figure 8).[287] In simple terms, confounding leads to a confusion between the effects of different variables. When the effects of the exposure and a confounder are mixed, the estimated association may be strengthened, weakened or reversed, depending on the underlying mechanisms.[271,285] Importantly, if a factor is in the causal pathway between exposure and outcome it is denoted as an intermediate variable or mediator of the association, as illustrated in Figure 8.[271]

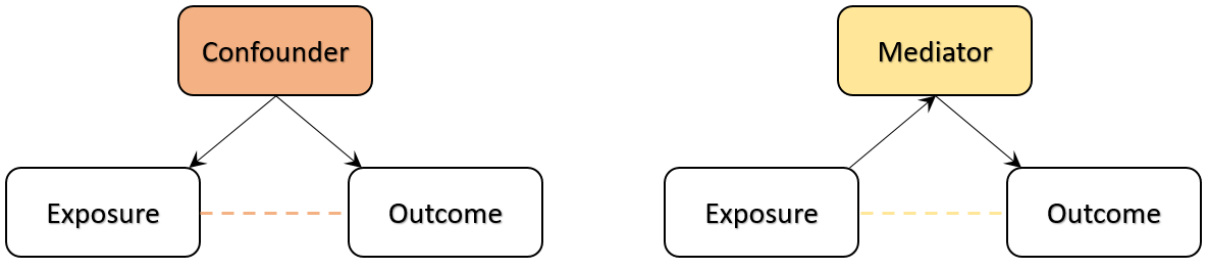

Figure 8. Confounding and mediation. If a confounder is present, an association between the exposure and outcome may appear in the analyses, which occurs due to the association of the confounder with both the exposure and outcome. A mediator leads to an association between exposure and outcome by being a factor in the causal pathway.

Several strategies exist to remove the effect of confounders. In RCTs, participants are randomized into exposure groups to obtain an even distribution of confounders. Another method is restriction or stratification, where either study participants or specific analyses are restricted to participants with a specific value or within narrow range of values for a potential confounder. If smoking was hypothesized to be a confounder in a study, one could for instance include only non-smokers or perform analyses stratified by smoking status. In case-control studies, a common strategy is matching, which can be regarded as a type of stratification. In all papers in the present thesis, the selection of controls was restricted to individuals with, i.e., matched on, the same sex and year of birth as the index VTE event, thus

ascertaining that these potential confounders were equally distributed in the two groups. Further, various strategies exist to overcome confounding in the analytic process. In addition to stratification, researchers may apply standardization or multivariable adjustment, which was employed in paper I-IV. In matched case-control studies, one may perform either conditional or unconditional regression, i.e., either analyse each stratum (1 case, 2 controls) separately or consider the entire study population. As the matching factors in our studies were not specific on the individual level, we performed unconditional regression with multivariable adjustment for both the matching factors and other potential confounders. Specifically, by including these factors as covariates in the analyses, we were able to retrieve ORs for VTE which took the potential confounders into account. A common rule of thumb when applying this strategy is that confounding is likely if a risk estimate is altered >10% upon inclusion of a covariate. Potential confounders should be defined before conducting a study and depend on insights into the disease pathophysiology and determinants of the exposures. Still, presence of unknown or unmeasured confounders is always possible despite elaborate consideration, and residual confounding can consequently never be dismissed in any observational study.[271]

As increasing age, sex-specific factors and inflammation are acknowledged risk factors for VTE and determinants of plasma levels of VWF and FVIII,[90,242] these factors were relevant as potential confounders in all papers in the present thesis. Age and sex were included as covariates in Model 1 of all analyses due to their status as matching factors, while plasma hsCRP at baseline, as a proxy for inflammation, was included in multivariable adjusted analyses. Further, as obesity is a strong risk factor for VTE and associated with a wide variety of hormonal and proinflammatory alterations,[178] the multivariable analyses were adjusted for BMI. In papers III and IV we explored MPV and platelet count as exposures of interest and subsequently performed adjustments for the other platelet measure. This was carried out to remove the effect of compensatory mechanisms which may have affected the total burden of platelet thrombogenicity. Moreover, paper III included adjustments for smoking, hypertension, oestrogen use and self-reported history of cancer in order to take into account potentially skewed distributions of these factors. While a key point of the present thesis was to disentangle the impacts of the FVIII-dependent and platelet-interacting properties of VWF on risk for VTE, this could not be determined by including both VWF and FVIII as covariates in multivariable analyses. As previously mentioned, this would be an

inappropriate method due to the fact that the two proteins are tightly correlated and circulate in a complex, causing collinearity.[271]

5.1.2.5 Biological interaction

If the effect of an exposure on an outcome varies across values of another exposure, this is defined as an interaction between the two exposures.[287] A distinction is made between biological and statistical interaction, depending on whether the combined effect of the two exposures departs from the sum or the product of the effects seen by the single exposures, respectively.[271] In other words, the proposed approach to assess biological interaction is to explore the effects of the exposures on an additive scale. While risk estimates provided by logistic regression, i.e., ORs, are on a multiplicative scale, the RERI and AP are designed to assess departure from additivity using multiplicative risk estimates.[80,293] Interaction analyses were performed in papers III and IV, and findings were presented with both separate and combined effects of the exposures of interest. Thus, transparency was preserved and readers could assess interaction on both additive and multiplicative scales, conforming to recommendations.[294] According to Rothman, the concept of two risk factors interacting to cause a disease forms the basis of biological interaction.[80] As both VWF and FVIII are known to interact with platelets on the molecular level to promote haemostasis, papers III and IV focused on biological interaction to address potentially causal mechanisms.

Analyses on biological interaction are accompanied by some important inherent limitations. When study participants are divided into smaller subgroups, the power of the performed analyses are weakened and the estimates are more likely to lose precision. This was evident in the results from our interaction analyses, where the confidence intervals were markedly wide although statistical significance was seen in most cases. Attention to the biological relevance, magnitude and patterns of the interactions is thereby warranted, rather than a focus on P-values and CIs.[295,296] Further, it is probable that potential nuances in the nature of the associations were masked when continuous variables were strictly categorized as either low, medium or high.[287]

5.1.2.6 Missing data

When conducting an epidemiological study, it is improbable that researchers will be able to avoid missing observations entirely. This introduces a major concern of whether the absence of data introduces bias in the remaining observations.[297] This mainly depends on whether the data is randomly missing or there is an underlying pattern in the unobserved data.[298] Specifically, a variable is deemed missing completely at random (MCAR) if the missingness is neither associated with the value of the missing variable nor other variables. Further, a variable is considered missing at random (MAR) if it is more frequently missing for participants in a specific category (e.g., sex or older individuals), but is not associated with the true value of the missing variable. Lastly, when data is missing not at random (MNAR), it is associated with the true value of the missing observation. A typical example of observations which are MNAR is if smokers are less likely to respond to a question regarding smoking status.[298] Different methods exist to handle missing data in analyses, including imputation methods and omission of either variables or participants. When omission is carried out, the analytic sample decreases and the study power is consequently reduced. The risk estimates may therefore lose precision and the CIs will get wider.

The papers in the present thesis contained a various number of missing observations for plasma levels of VWF, ADAMTS13 and FVIII, which was due to haemolysis and inadequate amounts of plasma in the samples. Importantly, the proportion of missing values was similar in cases and controls and there was no reason to suspect that the probability of this occurring would differ according to the levels of the missing variable or potential confounders. We therefore assumed that the data was MCAR, and proceeded to perform analyses with omission of the participants with missing data, i.e., complete case-analysis.[297] The risk estimates were considered valid as we did not find any reason to suspect bias in the analytic sample, but likely suffered from reduced precision due to decreased study power.

5.2 Discussion of main results

5.2.1 VWF and VTE

In paper I, we reported a dose-dependent association between plasma levels of VWF and odds for future incident VTE in a nested case-control study derived from a population-based cohort. Participants with VWF in the highest quartile had a 1.5-fold increased OR for VTE

compared with those in the lowest quartile in age- and sex-adjusted analyses. The risk estimate remained similar after multivariable adjustment. Further, the impact of elevated VWF levels on VTE risk was similar across age categories (data not shown), and the association remained after 13 years of follow-up. Subgroup analyses revealed that the association was particularly strong for unprovoked DVT, where the plasma VWF in the highest versus lowest quartile yielded a 6.7-fold increased OR. In contrast, elevated plasma VWF had no effect on the odds for PE or provoked VTE.

An association between plasma levels of VWF and VTE was first reported by Koster and colleagues in a paper from the Leiden Thrombophilia case-control study.[197] Here, VWF levels measured several months after the event were significantly higher in 301 DVT patients compared with 301 healthy control subjects. Since this study, many case-control studies have confirmed the association,[258-263] while prospective data have been scarcely reported. A nested case-control study from the Women's Health Initiative reported that a 1 standard deviation increase in plasma VWF was associated with a 1.3-fold increased OR for future VTE.[267] Still, the most comprehensive evidence on a prospective association was gathered from the LITE study and reported by Tsai and colleagues in 2002.[27] Here, a linear relationship was seen between plasma VWF levels and risk of future VTE in the Atherosclerosis Risk in Communities (ARIC) cohort, which included participants aged 45-64 years at baseline. The participants were followed for a median of 7.8 years, and the association was later found to persist after 13 years of follow-up.[27,266] Meanwhile, data from a subpopulation of the Cardiovascular Health Study (CHS) cohort, consisting of individuals aged 65 years or older at entry, provided inconclusive results. Specifically, there was a trend of increasing VTE risk across quartiles of plasma VWF, but no association was found between plasma VWF above the 95th percentile and VTE.[27] Consequently, it remained a question whether elevated plasma VWF mainly results in increased VTE risk in younger individuals or the findings from CHS were spurious or due to lack of study power. Our findings in paper I suggested an age-independent impact of plasma VWF on VTE risk, which was reinforced by recent data from the British Regional Heart Study cohort consisting of 3,000 males, where the average age at baseline was 68 years.[299]

While genetic variation accounts for approximately 65% of the variability of plasma VWF levels in the population, environmental factors may substantially contribute to variations at the individual level.[242] Increasing age, pregnancy, menstrual cycle, exercise,

cigarette smoke, air pollution and circadian rhythm are among the factors known to cause short- and long-term fluctuations in VWF levels.[242] It would therefore be reasonable to assume that occurrence of regression dilution bias would cause plasma VWF to be a poor predictive biomarker. Meanwhile, our results indicated that the association persisted throughout the 13-year study period, with a mean follow-up of 7 years. Further, the Women's Health Initiative study assessed the effect of short-term fluctuations by measuring plasma VWF both at baseline and 1 year later, and found that the difference between these measurements was not associated with VTE risk.[267] It may therefore be speculated that short-term fluctuations, e.g., due to acute-phase reactions, have a relatively small impact on VTE risk compared with high basal levels. Taken together, current knowledge suggests that plasma VWF represents a long-term risk factor for VTE.

A growing body of evidence has suggested a causal role of VWF in the pathogenesis of VTE. In a MR study based on GWAS data from 46.354 individuals in the INVENT consortium, Sabater-Lleal and colleagues reported that a 1 SD increase in genetically predicted VWF levels was associated with a 2.4-fold increased OR for VTE, supporting a causal association.[254] This finding was recently replicated in an MR study based on GWAS data from the Million Veteran Program and UK Biobank, containing over 26,000 cases and 624,000 controls.[300] However, whether the platelet-binding capacities of VWF explained parts of the effect or it was entirely due to increased FVIII levels could not be accurately addressed due to the fact that no genetic loci are found to regulate VWF independently of FVIII.[254,300] Using a variety of experimental approaches, several animal studies have in recent years provided increasing insight into the differential roles of VWF and FVIII in thrombus formation, and suggest that both VWF and FVIII are required to form a stable thrombus.[301,302] Subsequently, both VWF and FVIII have been proposed as reasonable targets for prevention and treatment of thrombosis, and the effects of specific antagonism using antibodies, nanobodies and aptamers are currently under investigation.[239,303] Still, a vast majority of experiments and trials have so far been conducted with a focus on arterial thrombosis.[304]

To our knowledge, paper I was the first to assess the prospective association between plasma VWF and subgroups of VTE. Our analyses revealed that the association between VWF and VTE was primarily explained by high plasma VWF in those who later suffered from unprovoked DVTs. It has previously been reported that FVIII levels were slightly higher in unprovoked versus provoked events,[305] and that elevated FVIII levels were more common

in patients with DVT than PE.[306] The exclusive association with unprovoked events suggests that the pathways by which VWF leads to VTE are independent of acknowledged provoking factors, which may strengthen the arguments for causality.[22] While DVT and PE are largely considered to be manifestations of the same disease, several risk factors are found to have a disparate impact on the two. An example is FVL which has been reported to only be a risk factor for DVT, a phenomenon termed the FVL paradox.[307] Findings from experiments on mice has led researchers to hypothesize that thrombi formed in carriers of the FVL mutation develop under conditions favouring enhanced thrombin generation and are subsequently more stable and less likely to embolize.[307,308] A similar mechanism may be involved in the association between VWF and VTE, as concomitantly increased FVIII levels in those with elevated plasma VWF may lead to increased endogenous thrombin potential.[198-200] Another possible explanation is that a thrombus formed in the presence of an abundance of VWF may contain platelets, which are more tightly anchored to other platelets and the vessel wall due to the interaction between VWF and platelet glycoproteins, and is therefore less likely to embolize. Interestingly, plasma VWF was found to be correlated with the severity and prognosis of COVID-19, and histological and immunohistochemical analyses on autopsies from diseased individuals revealed a larger presence of VWF in those who suffered from PE.[309] While the association between VWF and PE may have been confounded by inflammatory pathways in this situation, researchers suggested an important causal role of VWF in the pathogenesis of *de novo* PE.[309,310]

5.2.2 The VWF-ADAMTS13 axis

In paper II, an inverse association was observed between plasma levels of ADAMTS13 and future incident VTE. Our data were suggestive of a threshold effect, where a clear impact was mainly seen in those with plasma ADAMTS13 in the lowest quartile. Participants with ADAMTS13 levels in the lowest quartile had a 1.4-fold increased age- and sex-adjusted OR for VTE compared with those in the highest quartile. The association was similar across subtypes of VTE, and additional adjustment for BMI and CRP had a minor effect on the risk estimates, indicating that confounding by obesity-related mechanisms or inflammation was unlikely. The effect of regression dilution appeared to be modest, as analyses showed similar risk estimates with increasing time between blood sampling and VTE events.

Previous data on the association between plasma ADAMTS13 and VTE was scarce and with conflicting results. Four case-control studies assessed the association in selected populations,[260,272,273,311] of which three reported results in accordance with those in paper II. Of note, Pagliari and colleagues recently found that ADAMTS13 activity levels were lower in 365 patients with unprovoked DVT compared with controls.[311] Prospective data was restricted to cancer patients, with studies also yielding conflicting results, and no clear association between ADAMTS13 levels and VTE could be established.[274,275,312]

ADAMTS13 cleaves VWF into smaller multimers, which results in a reduction of the haemostatic, and likely prothrombotic, activity of VWF. ADAMTS13 and VWF thereby form an axis where both proteins can compensate for the effect of each other, and it is reasonable to assume that an imbalance between them would reflect a prothrombotic state more accurately than altered levels of one of the proteins. In other words, low levels of ADAMTS13 may not have an impact on VTE risk if plasma VWF is concomitantly low, and the effect of elevated plasma VWF may be cancelled out by increased proteolytic activity resulting from elevated levels of ADAMTS13. A high ratio between plasma VWF and ADAMTS13 and the combination of low ADAMTS13 and high VWF levels have subsequently been established as significant risk factors for arterial thrombosis.[313-315] For VTE, however, this is scarcely investigated. In the aforementioned case-control study by Pagliari and colleagues, the combination of ADAMTS13 activity in the lowest quartile and VWF antigen in the highest quartile displayed a synergistic effect on the OR for unprovoked DVT.[311] Setiawan and colleagues reported that VWF/ADAMTS13 ratio was associated with future DVT in a cohort consisting of 40 cancer patients.[312] Further, a prospective study on 79 patients with compensated cirrhosis revealed that a disturbance in the VWF/ADAMTS13 ratio was associated with increased risk of portal vein thrombosis.[316]

Based on previous literature we explored the effect of the VWF/ADAMTS13-ratio on risk for future VTE in paper II and found a dose-dependent association. Here, those with ratios in the highest quartile had a 1.7-fold increased age- and sex-adjusted OR for VTE compared with those in the lowest quartile, which was only slightly attenuated by further adjustment for BMI and CRP. The effect of increased VWF/ADAMTS13 ratio was strongest for DVT and unprovoked VTE, where the OR for the highest versus lowest quartiles were 1.8 and 2.8, respectively. These findings were largely comparable to those observed for plasma VWF in paper I, although the impacts on DVT and PE were more similar for the

VWF/ADAMTS13 ratio. Importantly, the association was not explained by extreme levels in one variable, as adjustment for VWF and ADAMTS13 only slightly altered the risk estimates. Furthermore, the association remained after 13 years of follow-up, and the VWF-ADAMTS13 axis thereby displayed potential for serving as a reliable predictive biomarker. Our findings suggest that imbalance between plasma levels of ADAMTS13 and VWF represents a long-term risk factor for VTE and may have a role in VTE pathogenesis.

Both increased VWF levels and decreased ADAMTS13 levels may emerge due to hereditary factors, and genes are found to account for up to 65% and 80% of the variability in plasma levels of VWF and ADAMTS13, respectively.[242,317] Meanwhile, several acquired factors are known to promote disruption of ADAMTS13 levels and release of VWF from its intracellular storage vesicles. Increasing age, inflammation, cancer and pregnancy are examples of conditions where plasma levels of both proteins are skewed towards the high-risk category.[242,318-322] Interestingly, these conditions are also major risk factors for VTE.[90] The acquired factors may therefore be relevant for the VWF/ADAMTS13 axis as a causal culprit in the pathogenesis of provoked VTE due to the fact that extreme levels are most likely to occur in the presence of the acknowledged VTE triggers. Of note, as the effect of regression dilution was modest in our data, our findings indicated that one baseline measurement was representative throughout the study period. Meanwhile, irrespective of the cause of imbalance in the axis, it is reasonable to assume that inadequate amounts of ADAMTS13 in plasma may result in excessive circulating amounts of ULVWF with high platelet affinity.[246] Recruitment and activation of platelets may subsequently contribute to activation of the coagulation cascade and induce formation of a thrombus.[196] It is unclear whether the affinity for FVIII varies according to the multimeric size of VWF,[234,323,324] and therefore whether inadequate levels of ADAMTS13 may indirectly lead to increased circulating levels of FVIII. On the other hand, it may be speculated that low levels of ADAMTS13 may result in a larger proportion of VWF remaining tethered to the parent cell, and thereby that a smaller proportion can bind and protect FVIII in the circulation. However, most research suggests a stable 50:1 ratio between VWF and FVIII molecules in plasma irrespective of multimeric size, implying that the effect of ADAMTS13 levels on VTE is independent of FVIII levels.[325,326] This theory is endorsed by the finding of normal FVIII levels in individuals with VWD type 2A, who have a genetic defect preventing multimerization of VWF.[327]

As the VTE risk appears to increase linearly according to the extent of disruption in the VWF-ADAMTS13 axis, it is likely that short-term fluctuations due to acquired factors may be a significant contributor to the pathogenesis of both provoked and unprovoked VTE. However, it is challenging to obtain comprehensive data on plasma VWF and ADAMTS13 in the time closely preceding VTE events in observational studies. Available prospective evidence of the association therefore entails disrupted VWF/ADAMTS13-axis as a long-term risk factor for VTE, while it is fair to assume that several patients developed an unmeasured skew in the axis between baseline and the time of disease manifestation. On this note, the association between increased VWF/ADAMTS13 ratio and VTE observed in paper II was explained by unprovoked events, which implied a role of activated endothelial cells, and perhaps platelets, years before idiopathic VTE events. However, we were not able to assess the role of the VWF/ADAMTS13 axis as a short-term mechanistic trigger of VTE due to the long follow-up and lack of repeated measurements. As the axis is likely causally involved in the pathogenesis of VTE, there is a need for further research to disentangle the specific mechanisms involved.

While MR analyses have confirmed a causal role for the VWF/FVIII complex in VTE,[254,300] such evidence has been elusive for ADAMTS13. SNPs associated with antigen and activity levels of ADAMTS13 were only found to obtain borderline statistical significant associations with VTE in an MR analysis using data from the UK Biobank.[328] However, analyses still indicated a causal negative correlation between ADAMTS13 antigen and VTE.[328] Importantly, the SNPs were derived from studies with a relatively small sample size and the majority did not account for a substantial proportion of the variability in ADAMTS13 levels.[317,329] More comprehensive and unambiguous data is therefore warranted before a causal role of ADAMTS13 in VTE can be established or ruled out.

5.2.3 VWF and FVIII

The current understanding of the role of VWF in VTE is insufficient and subject to dispute among researchers. The association with VTE has been thought to be mediated entirely by FVIII, and the platelet-binding properties of VWF have primarily been considered relevant in arterial thrombosis. However, as the role of platelets in VTE is unclear, it is crucial to disentangle the contribution of the VWF-platelet interaction.[225,254] As for other proteins

involved in coagulation, it is known that elevated plasma levels of FVIII are associated with reduced TF-threshold and enhanced thrombin generation, which in turn is associated with increased risk of VTE.[199,330] This way, FVIII has been assumed to mediate the association between VWF and VTE.[330] Several studies have confirmed the association between FVIII and VTE have in the past few decades,[27,198] and various arguments have been made against a FVIII-independent effect of VWF. However, some of these have recently been challenged by other findings.

First, there is an argument arising from the statistical approach used in epidemiological studies. In the first study to assess the association between VWF and VTE, Koster and colleagues compared VWF levels in DVT cases and controls and found that the association vanished when they performed regression analyses with adjustment for FVIII.[197] Meanwhile, subsequent data has indicated the opposite; that VWF and FVIII are independently associated with VTE.[27,262,264] While multivariable adjustment is often an appropriate method to remove the effect of confounding factors, it is unfit to dissect the effect of tightly correlated variables, such as VWF and FVIII, which circulate in a complex.[270] Specifically, adjustment for FVIII levels is likely to remove not only the effect of FVIII, but also the potential independent effect of VWF.

Further, it has been considered biologically implausible that VWF multimers can achieve a significant interaction with platelets in the venous circulation. VWF is found to circulate in a globular conformation under conditions of low shear, which is typical for veins, making the binding sites for platelets inaccessible.[235] Meanwhile, under conditions of increased shear, e.g., due to vascular injury or stenosis, VWF is elongated and the platelet-binding domains are exposed.[235] Collagen- or endothelial cell-bound VWF is thereafter able to slow down the flow of platelets through reversible binding to their GPIb receptor, allowing other ligands to contribute to irreversible attachment to the (sub)endothelial surface.[331] Experimental research has suggested that VWF can mediate platelet adhesion and aggregation also at venous shear rates, and it appears unreasonable to dichotomize the platelet-capturing role of VWF based on venous and arterial flow conditions.[239,332-334] Moreover, a turbulent flow is characteristic of the sinus pockets of venous valves, which are the most common sites for venous thrombus formation, indicating that the microenvironment where a venous thrombus develops deviates from the typical venous flow.[4,70] Furthermore, the ability of VWF to bind platelets depends not only on flow conditions, but on the multimeric size. Specifically,

longer strings containing more VWF molecules require less hydrodynamic force to unravel.[235] As a proportion of newly secreted platelet-hyperadhesive ULVWF tends to tether on the activated endothelial cells, elongation and platelet recruitment is possible also at low shear rates.[238] It is therefore clear that endothelial cell activation not only leads to increased plasma levels of VWF, but also increased platelet-affinity for the available VWF molecules.

Another argument against a direct effect of VWF on VTE is that venous thrombi, or "red clots", are found to contain less platelets and more fibrin than arterial thrombi, or "white clots".[76,196] Meanwhile, both platelets and VWF are also present in venous thrombi,[335] and it is hypothesized that their mechanistic role might still be highly relevant. On this basis, several studies during recent years using animal models have provided useful insights into the underlying mechanisms of venous thrombus formation. Interestingly, both in stasis models and upon intravenous ferric chloride injection, VWF-deficient mice had impaired venous thrombus growth compared with wild-type mice.[301,302] Furthermore, injection of FVIII did not restore the thrombus growth, indicating that the interaction between VWF and platelets was necessary for venous thrombus formation.[301,302] Using a rabbit model, where a polyethylene tube was inserted into the iliac vein, Takahashi and colleagues found that an antibody preventing interaction between VWF and platelet GP1b significantly reduced the formation of both DVT and PE.[335] This finding was reproduced by Michels and colleagues using anti-VWF in an inferior vena cava model on obese mice.[336] Anti-VWF has also been explored as a therapeutic agent in humans, with potentially promising results for arterial thromboembolic outcomes and TTP.[337-339] Additionally, aspirin, an antiplatelet agent most frequently used for secondary prevention of arterial thrombosis, has been found to reduce the recurrence rates after a first unprovoked VTE in RCTs.[209,210]

In paper III, we found that high plasma VWF and platelet measures, assessed by MPV and platelet count, had supra-additive effects on odds for VTE. Those with a combination of high plasma VWF and high MPV had a 2-fold increased OR for overall VTE compared with those with low levels in both parameters, while those with high VWF and high platelet count had a 2.9-fold increased OR. The risk estimates were adjusted for age and sex, and additional adjustment for BMI, CRP, hypertension, oestrogen use and platelet count/MPV had a modest effect on the ORs. The associations were particularly strong for unprovoked VTE, where the risk estimates were increased more than 6-fold for both exposure combinations. Further, our data suggested that high plasma VWF did not yield an increased OR for overall VTE when

the platelet measures were in the lowest category, suggesting that highly reactive platelets or high platelet count was required for VWF to mediate its effect on VTE risk.

Similarly, paper IV not only confirmed the linear association between plasma FVIII and VTE risk, but also assessed the combined effects of plasma FVIII and platelet measures. Here, those with plasma FVIII in the highest tertile and high MPV had a 2.8-fold increased age- and sex-adjusted OR for VTE, compared with those with both exposures in the lowest category. This was higher than the sum of the individual effects of plasma FVIII and MPV, and interaction analyses suggested that half of all VTEs in the combined high-risk category could be attributed to the interaction. Further adjustment for BMI, CRP and platelet count had a minor effect on the risk estimates. Interestingly, FVIII levels did not interact with platelet count in a similar manner as VWF, as the effect of both exposures combined was similar to the sum of the individual effects. It is formerly known that the activation of FX by FIXa and FVIIIa occurs on the surface of activated platelets and is more potent in the presence of negatively charged phospholipids which effectively binds to FVIIIa.[237] Our findings may indicate that this process is important in the pathogenesis of VTE, as large platelets expose larger surfaces and are found to be more reactive and haemostatically active.[220,221]

Meanwhile, as VWF and FVIII are non-covalently bound to each other in plasma and thereby tightly correlated, it is not possible to deduct which mechanism underlies the biological interactions with MPV on VTE risk based only on statistical analyses. In other words, the combined effect of plasma VWF and MPV may simply reflect the effect of concomitantly increased FVIII levels, and *vice versa*. However, only elevated plasma VWF interacted with high platelet count to yield a supra-additive effect on VTE, suggesting that the underlying mechanism involved was independent of FVIII. Further, as confirmed in paper II, ADAMTS13 was inversely associated with risk of VTE. As plasma FVIII does not appear to vary according to ADAMTS13 levels,[325-327] it is reasonable to assume that these risk estimates also reflected a FVIII-independent mechanism.

Taken together, an increasing amount of evidence suggests that the interaction between VWF and platelets plays an essential role in the pathogenesis of VTE, in addition to the established effect of FVIII. Experimental data suggest that the VWF-platelet interaction represents an important early step in venous thrombus formation and as a facilitator for disease development, while the present thesis and other epidemiological data suggest that

plasma levels of VWF, FVIII and ADAMTS13 represent stable long-term risk factors for VTE.

6 Conclusions

> We found that plasma VWF levels were associated with odds for future VTE in a dose-dependent manner. Subgroup analyses suggested that the association was exclusively explained by DVTs and unprovoked events. The association persisted after 13 years of follow-up, indicating a role of early endothelial cell and/or platelet activation in VTE pathogenesis.

> Low plasma levels of ADAMTS13 and an imbalance between VWF and ADAMTS13, reflected by the VWF/ADAMTS13 ratio, were associated with odds for future VTE. A threshold effect was found for ADAMTS13, while VWF/ADAMTS13 ratio was linearly associated with VTE.

> The combination of high plasma VWF and platelet measures, assessed by MPV and platelet count, interacted to provide supra-additive effects on the odds for future VTE. Around half of all VTE events in those with combined high VWF and MPV/platelet count could be attributed to the biological interaction between the two exposures.

> Elevated plasma FVIII interacted with MPV in a similar manner, as those with both risk factors had a larger risk estimate for VTE than the sum of the effects of the single exposures. In those with both exposures present at baseline, half of the VTE events were attributable to the interaction. Our results indicated a role of platelet reactivity, assessed by MPV, in the mechanism by which FVIII increases the VTE risk.

7 Final remarks and future perspectives

Since its discovery nearly a century ago, the role of VWF in haemostasis and thrombosis has been gradually unravelled. Still, however, its contribution in VTE, a complex and multicausal disease, is not yet completely understood. The association between VWF and VTE has been attributed entirely to the procoagulant activity of FVIII, and VWF is assumed to be an innocent bystander. Meanwhile, an increasing amount of evidence indicates that the platelet-binding attributes of VWF play an important role in VTE pathogenesis.

A major challenge in VTE treatment and prevention is the disadvantage that all available therapeutic agents come with a simultaneous increased risk of bleeding. This poses an ethical dilemma, as the "First, do no harm" rule conceived by Hippocrates is essential in medicine.[340] Meanwhile, an increasingly utilitarian approach is seen due to improving insights and prediction models, and guideline recommendations accept the harm of few patients as long as the benefit is overwhelming.[341-343] It is pivotal that researchers, clinicians and decision-makers have this dilemma in mind. As the burden of VTE is expected to increase due to rising prevalence of major risk factors, it is imperative to unravel causal mechanisms specific to pathological thrombosis.

The aim of the present thesis was to explore the prospective role of VWF in VTE using data from a population-based study. We found that plasma levels of VWF, FVIII and ADAMTS13 were associated with risk of future VTE, and that platelet measures interacted differently with VWF and FVIII. Future epidemiological studies should build on the available research and obtain more prospective data on the associations. This may reveal characteristics of the VTE events in which VWF is likely to play a major role. Additionally, it is necessary to extend the current knowledge on genetic predictors of ADAMTS13 levels and the VWF/ADAMTS13 ratio. High-quality GWAS are likely to provide useful insights and form the basis for MR studies which may provide unambiguous results regarding the causal role of the VWF-ADAMTS13 axis in VTE.

Further, experimental studies are indicated to shed more light on the molecular mechanisms of venous thrombogenesis, and it is expected that VWF will be explored further as a therapeutic target. While several trials have been conducted with arterial thrombosis as the outcome of interest, current experiences on VWF-antagonism in VTE arise from

preclinical studies. It is reasonable to assume that commercially available VWF-antagonists may be explored in treatment and prevention of VTE in high-risk populations, once the knowledge from animal experiments is expanded. Although outside the scope of the present thesis, our findings also imply a potential for utilizing the proteins of the VWF/ADAMTS13 axis as predictive biomarkers. Future research should therefore aim to further explore their predictive abilities in prospective studies.

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Paper I

REGULAR ARTICLE

blood advances

Plasma levels of von Willebrand factor and future risk of incident venous thromboembolism

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Key Points

- High VWF plasma levels are associated with increased risk of venous thromboembolism.
- The association is strongest for unprovoked events.

Several case-control studies have reported elevated plasma von Willebrand factor (VWF) levels in patients with venous thromboembolism (VTE) compared with controls. However, because few studies have investigated the association in a prospective design, it is unclear whether elevated plasma VWF is a risk factor or a consequence of the VTE event. Therefore, we aimed to investigate the prospective association between plasma VWF levels and risk of VTE, as well as to perform subgroup analyses of deep vein thrombosis (DVT) and pulmonary embolism. We established a population-based nested case-control study of 414 VTE cases and 843 age- and sex-matched controls based on the Tromsø study cohort (1994-2007). Blood samples were collected at cohort baseline (1994-1995). Odds ratios (ORs) with 95% confidence intervals (CIs) for VTE were estimated across quartiles of VWF levels. We found that the risk of VTE increased linearly across quartiles of VWF levels (P for trend = .023). Participants with VWF in the highest quartile had an OR of 1.45 (95% CI, 1.03-2.03) for VTE compared with those in the lowest quartile. The association was strongest for unprovoked VTE (OR, 2.74; 95% CI, 1.66-4.54) and unprovoked DVT in particular (OR, 6.73; 95% CI, 3.07-14.76). Further adjustment for body mass index, C-reactive protein, hypertension, estrogen use, and smoking had a modest effect on the risk estimates. To conclude, we found a dose-dependent relationship between plasma VWF levels and future risk of incident VTE, and unprovoked events in particular. Our findings suggest that VWF may represent a promising biomarker for future risk of incident VTE.

Introduction

Venous thromboembolism (VTE), a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease with important short- and long-term complications, such as recurrence, postthrombotic syndrome, chronic thromboembolic pulmonary hypertension, and death.[1-6] In contrast to the declining incidence of arterial cardiovascular disease (CVD), a slight increase in VTE incidence has been observed over the last decades.[7-10] This trend is expected to persist as a result of the increasing prevalence of major VTE risk factors, such as advancing age, cancer, and obesity.[11,12] To reduce the burden of VTE in the population, it is imperative to identify individuals at high risk and provide targeted prevention. Hence, there is an urgent need to identify novel biomarkers and increase the understanding of causal pathogenic pathways of VTE.

von Willebrand factor (VWF) is a multimer glycoprotein that is synthesized by endothelial cells and megakaryocytes. Endothelial cells are the major cellular source of circulating VWF; therefore, VWF is

Submitted 5 August 2020; accepted 25 November 2020; published online 7 January 2021. DOI 10.1182/bloodadvances.2020003135.

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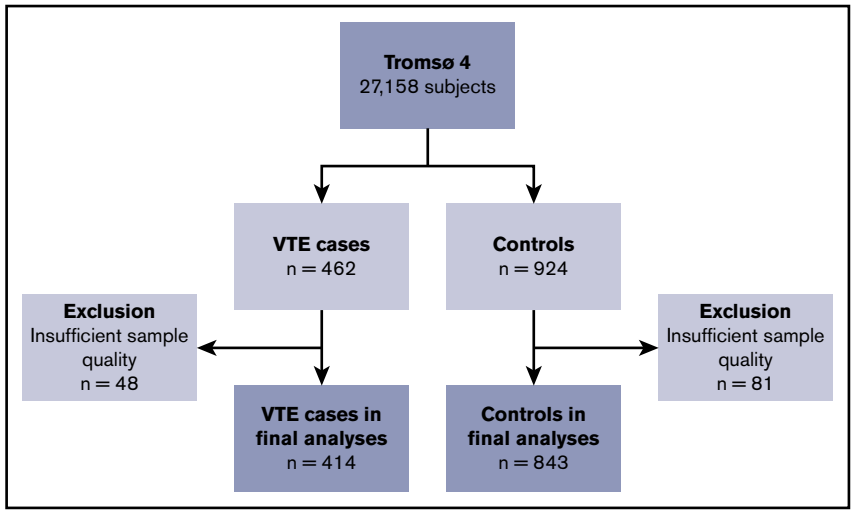

Figure 1. Flowchart of the study population. The chart illustrates the nested case-control design. Subjects were sampled from the general population, aged ≥25 years. Controls were age- and sex-matched with the cases.

regarded as a reliable marker of endothelial cell activation.[13] Platelets release α-granules containing VWF when activated, whereas endothelial cells combine constitutive secretion and release from Weibel-Palade bodies upon stimulation.[14-16] Thrombin generation and acute-phase reactions are among the stimuli that induce release of VWF.[17,18] VWF has a dual role in hemostasis: it promotes adhesion and cohesion of platelets by acting as a ligand for their glycoproteins and serves as the carrier and protector for coagulation factor VIII (FVIII).[19] Plasma levels of VWF and FVIII are strongly interrelated, and both are associated with the risk of VTE.[20-23] However, the majority of data on VWF and VTE risk arises from case-control studies,[24-30] which may be susceptible to bias because of reverse causation and selection of controls. Few studies have investigated the relationship between VWF and VTE using a prospective design.[21] In the Longitudinal Investigation of Thromboembolism Etiology (LITE) study, a cohort of 19 231 participants with 159 VTE events, VWF and FVIII were linearly associated with increased VTE risk.[21] To confirm a temporal relationship between increased levels of VWF and VTE risk, there is a need for more prospective data from unselected populations. Insight into the nature of the association may increase our understanding of the VTE pathophysiology and improve strategies for prevention.

In the present study, we sought to investigate the prospective association between plasma VWF levels and risk of incident VTE, including the subtypes DVT and PE, in a nested case-control study derived from a general population. We hypothesized that individuals with elevated VWF levels would be at higher risk for VTE.

Methods

Study population

The Tromsø Study is a single-center population-based cohort with repeated health surveys of the inhabitants of Tromsø, Norway.[31] The present nested case-control study is derived from the fourth survey (Tromsø 4), which was conducted in 1994-1995. All inhabitants aged ≥25 years were invited to participate in this survey; 27 158 individuals (77% of those invited) took part. Participants were followed from the date of inclusion until an incident VTE, migration, death, or end of follow-up (1 September 2007). During follow-up, 462 participants experienced a VTE event. For each case, 2 age- and sex-matched controls were randomly sampled from the parent cohort, and these had to be alive at the index date of the corresponding VTE event. Insufficient quality of plasma samples led to exclusion of 48 cases and 81 controls, resulting in 414 cases and 843 controls in the final analytical sample. A flowchart of study participants is depicted in Figure 1. All participants gave their informed written consent to participate, and the regional committee for medical and health research ethics approved the study.

Identification and adjudication of VTE events

All first lifetime VTE events were identified using the hospital discharge diagnosis registry, the radiology procedure registry, and the autopsy registry at the University Hospital of North Norway, which is the only hospital in the study region. Trained personnel reviewed the medical record of each potential case, and VTE was only registered when clinical signs and symptoms were combined with objective radiological confirmation and resulted in a diagnosis of VTE requiring treatment (unless contraindications were specified). VTE identification and adjudication have been described in detail.[32] All events were classified as a DVT or PE and as provoked or unprovoked. In case of simultaneous evidence of DVT and PE, the event was classified as a PE. An event was classified as provoked if it occurred in the presence of ≥1 provoking factor. Surgery or trauma within 8 weeks prior to the event, an acute medical condition (myocardial infarction, ischemic stroke, or infectious disease), active cancer, and marked immobilization (wheelchair confinement or >3 days bed rest) were regarded as provoking factors. Other factors, such as venous catheters, were also recognized as provoking when specifically accentuated by a physician in the medical record.

Baseline measurements

Baseline information was collected through physical examinations, blood samples, and questionnaires. Height (to the nearest centimeter) and weight (to the nearest 0.5 kg) were measured with participants in light clothing and no shoes. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Specially trained personnel used an automatic device (Dinamap Vital Signs Monitor) to perform 3 consecutive

Table 1. Distribution of baseline characteristics according to quartiles of plasma levels of VWF

	Plasma VWF, %			
	Quartile 1 (<88.5)	Quartile 2 (88.5-94.6)	Quartile 3 (94.7-104.7)	Quartile 4 (≥104.7)
n	298	305	315	339
Age, y	56.4 ± 14.1	58.9 ± 14.1	61.3 ± 12.8	63.9 ± 13.0
Males	49.7 (148)	47.9 (146)	46.7 (147)	44.5 (151)
BMI, kg/m^2	25.5 ± 3.8	26.8 ± 4.5	26.1 ± 3.7	27.0 ± 4.8
CRP, mg/L	1.29 (0.65-2.35)	1.05 (0.59-1.90)	1.14 (0.65-1.88)	1.36 (0.72-2.36)
Hypertension*	49.8 (148)	55.6 (169)	54.9 (173)	62.2 (211)
Cancer†	4.8 (12)	6.2 (15)	8.1 (20)	4.3 (11)
CVD†	10.1 (30)	17.4 (53)	18.7 (59)	16.8 (57)
Estrogen use‡	4.4 (13)	4.3 (13)	5.7 (18)	4.7 (16)
Smoking‡	45.3 (135)	28.2 (86)	23.5 (74)	26.3 (89)

Continuous variables are shown as mean (± standard deviation) or median (25th-75th percentile). Categorical variables are shown as percentages (n).
*Defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg.
†Self-reported history of cancer or myocardial infarction, angina, or stroke (CVD) at baseline.
‡Self-reported daily use of oral contraceptives or hormonal replacement therapy (estrogen use) or smoking of cigarettes or cigars (smoking).

blood pressure measurements on the upper right arm of all participants. Participants rested in a sitting position for 2 minutes before and between the measurements, and the last 2 measurements were used to calculate the mean systolic and diastolic blood pressure. History of CVD (myocardial infarction, stroke, or angina pectoris) and cancer, as well as estrogen use and smoking, was obtained through self-administered questionnaires.

At baseline, nonfasting blood was collected from an antecubital vein into 5-mL Vacutainers (Becton Dickinson, Le Pont-de-Claix, France) containing EDTA (K_3-EDTA 40 μL, 0.37 mol/L per tube) as an anticoagulant. Centrifugation at 3000*g* at room temperature was done for 10 minutes to prepare platelet-poor plasma. Thereafter, the supernatant was transferred into cryovials (Greiner Laboratechnik, Nürtringen, Germany) in 1-mL aliquots and stored at −80°C until further analysis.

Laboratory analyses

Measurement of VWF and C-reactive protein (CRP) was performed at the Research Institute of Internal Medicine at Oslo University Hospital, Rikshospitalet. Plasma samples were thawed in a water bath for 5 minutes at 37°C before platelet-free plasma was obtained by centrifugation at 13 500*g* for 2 minutes. Commercially available reagents (R&D Systems, Minneapolis, MN) by enzyme immunoassay (EIA) in a 384 format were used with a SELMA pipetting robot (Jena, Germany) and a BioTek (Winooski, VT) dispenser/washer (EL406) to measure high-sensitivity CRP. Absorption was read at 450 nm using an EIA plate reader (Synergy H1 Hybrid; BioTek) with a wavelength correction set to 540 nm. The intra- and interassay coefficients of variation were 2.6% and 9.1%, respectively.

Plasma VWF levels were measured by EIAs with antibodies (A0082, P02256) obtained from Dako (Glostrup, Denmark) using a polyclonal antibody for coat (A0082) and a horseradish peroxidase–conjugated polyclonal antibody for detection (P02256). Parallel-diluted pooled human plasma from 20 healthy individuals was used as standard, and the mean value in the control population was set to 100%. All other values were adjusted accordingly. The intra- and interassay coefficients of variation were 2.6% and 10.8%, respectively.

Statistical analyses

Statistical analyses were carried out with STATA version 16.0 (Stata Corporation, College Station, TX) and R version 3.6.3 (The R Foundation for Statistical Computing, Vienna, Austria).

Participants were categorized according to quartiles of plasma VWF levels, with cutoffs determined in the control population. Baseline characteristics according to quartiles of VWF were expressed as mean (± standard deviation) or median (25th-75th percentile) for continuous variables and as percentages (quantity) for categorical variables.

Unconditional logistic regression was used to estimate odds ratios (ORs) with 95% confidence intervals (CIs) for VTE according to quartiles of VWF plasma levels. The lowest quartile was used as the reference category. We performed analyses for overall VTE and in subgroups according to VTE location (DVT or PE) and presence of provoking factors (provoked or unprovoked). The *P* value for linear trend of VTE risk was estimated across increasing quartiles of VWF. The associations were adjusted for age and sex in model 1, with the addition of BMI in model 2 and the further addition of CRP in model 3.

VWF is a modifiable risk factor, and a long follow-up (average follow-up was 7 years) could introduce regression dilution bias and result in underestimated associations.[33] To address this, we performed logistic regression analyses with restriction of time from baseline to the VTE event, while keeping all controls in the analyses. These analyses generated ORs at every 0.1-year increase in time from baseline and plotted them as a function of this maximum time. Analyses were set to require ≥10 VTE events.

Results

The distribution of baseline characteristics across quartiles of plasma VWF levels is shown in Table 1. The mean age, proportion of women, and participants with hypertension increased across the quartiles. In the lowest quartile, history of CVD was less frequent, whereas smoking was more common compared with the other quartiles. Mean BMI, median CRP, history of cancer, and daily estrogen use were similar across VWF quartiles.

Table 2. Characteristics of VTE events (n = 414)

Characteristics	Data
Age at VTE, y	67.5 ± 13.6*
Males	48.3 (200)
DVT	62.6 (259)
PE	37.4 (155)
Unprovoked VTE	41.8 (173)
Provoked VTE	58.2 (241)

Unless otherwise noted, data are % (n).
*Mean ± standard deviation.

Characteristics of the VTE events are reported in Table 2. Mean age at the VTE event was 67.5 ± 13.6 years, and the proportion of men was 48.3%. DVTs constituted 62.6% of all VTE events, and 58.2% were classified as provoked VTEs.

The ORs for VTE by quartiles of plasma VWF levels are shown in Table 3. For overall VTE, the ORs increased across quartiles of VWF in the age- and sex-adjusted model (model 1: *P* for trend = .023). Subjects with plasma VWF levels in the highest quartile had a 1.5-fold higher OR for VTE (OR, 1.45; 95% CI, 1.03-2.03) compared with subjects with plasma VWF levels in the lowest quartile. Further adjustment for BMI and CRP (model 2) and hypertension, estrogen use, and smoking (model 3) attenuated the ORs only slightly.

Subgroup analyses revealed that elevated plasma levels of VWF were associated with an increased risk for unprovoked VTE (Table 3) and DVT (Table 4), whereas no association was observed for provoked VTE and PE. Compared with the lowest quartile, plasma VWF levels in the highest quartile yielded age- and sex-adjusted ORs of 2.74 (95% CI, 1.66-4.54; *P* for trend < .001) for unprovoked VTE, 1.63 (95% CI, 1.09-2.43; *P* for trend = .011) for DVT, and 6.73 (95% CI, 3.07-14.76; *P* for trend < .001) for unprovoked DVT. Similar to overall VTE, additional adjustment for BMI, CRP, hypertension, estrogen use, and smoking did not influence the risk estimates substantially.

To assess whether the risk estimates were subjected to regression dilution bias, we estimated ORs for VTE in those with high (quartile 4) vs low (quartile 1) plasma VWF as a function of time between baseline blood sampling and the VTE events. As depicted in Figure 2, the association between plasma levels of VWF and VTE risk was not substantially diluted during follow-up.

Discussion

In this population-based nested case-control study, we observed a dose-dependent association between plasma levels of VWF and future risk of incident VTE. Participants in the highest quartile had a 1.5-fold higher OR for VTE than did those in the lowest quartile after adjustment for age and sex. The association was strongest for unprovoked DVT, with an OR of 6.7 in the highest quartile. The risk estimates were only slightly attenuated after adjustment for BMI, CRP, hypertension, estrogen use, and smoking. Analyses with restricted follow-up time showed that the association remained throughout the 13-year study period. Our findings suggest that VWF may serve as a biomarker to predict first lifetime VTE and implies a role for early platelet and endothelial cell activation in the pathogenesis of VTE.

The association between VWF and VTE risk was first recognized in 1995, when Koster and colleagues reported a higher OR for DVT among participants with elevated levels of VWF in the Leiden Thrombophilia case-control study.[28] The association was replicated

Table 3. ORs with 95% CIs for overall VTE and subgroups according to quartiles of VWF plasma levels

VTE subgroup	Controls (n = 843)	Cases	Model 1	Model 2	Model 3
Overall VTE		n = 414			
VWF Q1	210	88	Ref.	Ref.	Ref.
VWF Q2	211	94	1.07 (0.75-1.51)	1.03 (0.72-1.46)	1.03 (0.72-1.48)
VWF Q3	210	105	1.20 (0.85-1.70)	1.20 (0.85-1.69)	1.20 (0.84-1.71)
VWF Q4	212	127	1.45 (1.03-2.03)	1.35 (0.96-1.90)	1.36 (0.96-1.92)
P value for trend			.02	.05	.05
Provoked VTE		n = 241			
VWF Q1	210	62	Ref.	Ref.	Ref.
VWF Q2	211	56	0.89 (0.59-1.34)	0.86 (0.56-1.30)	0.87 (0.57-1.33)
VWF Q3	210	62	0.97 (0.65-1.46)	0.96 (0.64-1.45)	0.98 (0.65-1.49)
VWF Q4	212	61	0.94 (0.62-1.41)	0.88 (0.58-1.34)	0.90 (0.59-1.37)
P value for trend			.9	.7	.8
Unprovoked VTE		n = 173			
VWF Q1	210	26	Ref.	Ref.	Ref.
VWF Q2	211	38	1.50 (0.88-2.57)	1.49 (0.86-2.57)	1.46 (0.84-2.52)
VWF Q3	210	43	1.75 (1.03-2.97)	1.78 (1.04-3.03)	1.72 (1.00-2.94)
VWF Q4	212	66	2.74 (1.66-4.54)	2.59 (1.55-4.32)	2.52 (1.51-4.22)
P value for trend			<.001	<.001	<.001

Model 1: adjusted for age and sex. Model 2: model 1 + BMI and CRP. Model 3: model 2 + smoking, hypertension, and estrogen use.
Q, quartile; Ref., reference.

Table 4. ORs with 95% CIs for overall, provoked, and unprovoked DVT and PE, according to quartiles of plasma VWF level

VTE subgroup	Controls (n = 843)	Cases	Model 1	Model 2	Model 3
Overall DVT		n = 259			
VWF Q1	210	53	Ref.	Ref.	Ref.
VWF Q2	211	57	1.08 (0.71-1.65)	1.07 (0.70-1.64)	1.06 (0.69-1.63)
VWF Q3	210	65	1.26 (0.83-1.90)	1.26 (0.83-1.90)	1.23 (0.81-1.87)
VWF Q4	212	84	1.63 (1.09-2.43)	1.52 (1.01-2.29)	1.49 (0.99-2.25)
P value for trend			0.01	0.03	0.04
Provoked DVT		n = 156			
VWF Q1	210	45	Ref.	Ref.	Ref.
VWF Q2	211	36	0.79 (0.49-1.27)	0.78 (0.48-1.27)	0.78 (0.48-1.27)
VWF Q3	210	38	0.82 (0.51-1.33)	0.83 (0.51-1.33)	0.83 (0.51-1.34)
VWF Q4	212	37	0.78 (0-48-1.28)	0.74 (0.45-1.21)	0.74 (0.45-1.22)
P value for trend			.4	.3	.3
Unprovoked DVT		n = 103			
VWF Q1	210	8	Ref.	Ref.	Ref.
VWF Q2	211	21	2.73 (1.18-6.33)	2.78 (1.19-6.47)	2.73 (1.17-6.40)
VWF Q3	210	27	3.74 (1.65-8.47)	3.79 (1.66-8.62)	3.59 (1.57-8.23)
VWF Q4	212	47	6.73 (3.07-14.76)	6.40 (2.90-14.12)	6.18 (2.78-13.70)
P value for trend			<.001	<.001	<.001
Overall PE		n = 155			
VWF Q1	210	35	Ref.	Ref.	Ref.
VWF Q2	211	37	1.04 (0.63-1.72)	0.96 (0.58-1.61)	0.99 (0.59-1.67)
VWF Q3	210	40	1.12 (0.68-1.84)	1.10 (0.67-1.83)	1.15 (0.69-1.92)
VWF Q4	212	43	1.18 (0.72-1.93)	1.11 (0.67-1.84)	1.14 (0.69-1.91)
P value for trend			.5	.6	.5
Provoked PE		n = 85			
VWF Q1	210	17	Ref.	Ref.	Ref.
VWF Q2	211	20	1.15 (0.59-2.27)	1.05 (0.53-2.10)	1.14 (0.56-2.31)
VWF Q3	210	24	1.36 (0.70-2.63)	1.32 (0.68-2.56)	1.43 (0.72-2.83)
VWF Q4	212	24	1.33 (0.69-2.57)	1.24 (0.63-2.42)	1.33 (0.67-2.64)
P value for trend			.4	.4	.3
Unprovoked PE		n = 70			
VWF Q1	210	18	Ref.	Ref.	Ref.
VWF Q2	211	17	0.94 (0.47-1.88)	0.90 (0.44-1.83)	0.88 (0.43-1.80)
VWF Q3	210	16	0.89 (0.44-1.80)	0.89 (0.43-1.82)	0.89 (0.43-1.84)
VWF Q4	212	19	1.04 (0.52-2.08)	0.98 (0.49-1.98)	0.97 (0.48-1.96)
P value for trend			.9	.9	.9

Model 1: adjusted for age and sex. Model 2: model 1 + BMI and CRP. Model 3: model 2 + smoking, hypertension, and estrogen use.

in several case-control studies,[24,26,27,29,34] most recently in the MEGA study, in which a dose-dependent relationship was also demonstrated at extreme plasma levels of VWF.[30] On a general basis, case-control studies have the important drawback that plasma samples are collected after the VTE event. Although data from the MEGA study suggested that plasma VWF levels remain stable after the initial treatment period,[30] it is still debated whether these VWF levels reflect a higher risk for VTE or merely a consequence of the acute event itself.[28,30] Few prospective studies have investigated the association between VWF and VTE risk. In the LITE study, a dose-dependent relationship between plasma VWF levels and future risk of VTE was observed in middle-aged participants (age 45-64 years at baseline) from the Atherosclerosis Risk in the Community cohort across 8 years of follow-up, whereas results from the Cardiovascular Health Study cohort with older individuals (age > 65 years at baseline) were inconclusive.[21] Although there were few events (n = 65) in the Cardiovascular Health Study, it may be speculated that elevated VWF levels better reflect VTE risk in the younger population. Notably, the association between VWF and VTE risk in the Atherosclerosis Risk in the Community also remained after 13 years of follow-up.[35] In a prospective nested case-control study from the Women's Health

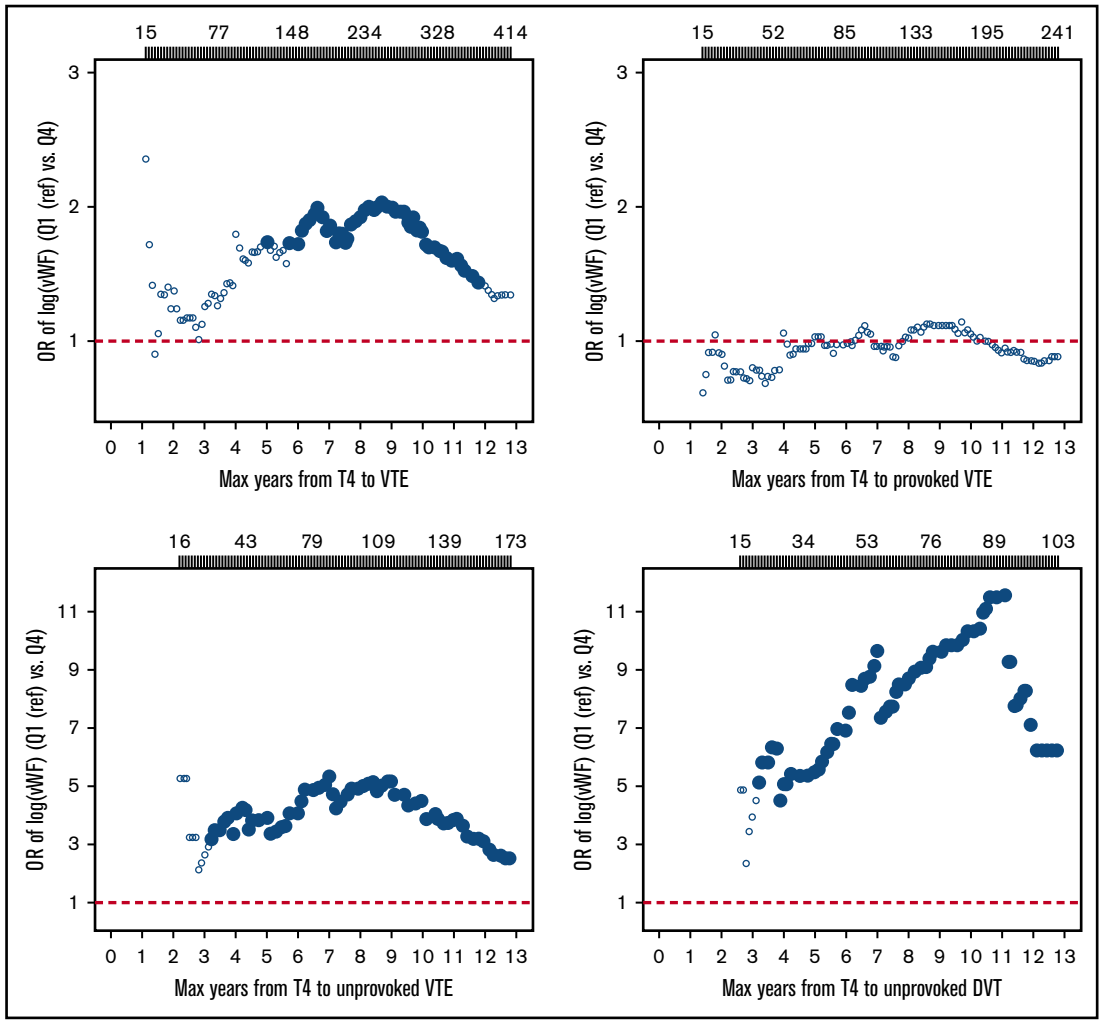

Figure 2. Plots of estimated ORs for overall, provoked, and unprovoked VTE and unprovoked deep vein thrombosis as a function of time from baseline (1994-1995) to VTE events. Participants with plasma VWF levels in the highest quartile were compared with those with levels in the lowest quartile (Q4 vs Q1). Analyses were adjusted for age and sex. Filled symbols indicate ORs with $P < .05$. Number of VTE events according to time of follow-up are provided above the plots. Max, maximum; T4, Tromsø 4 survey.

Initiative trials, an association with VTE was observed when VWF was modeled continuously but not when it was dichotomized at the 75th percentile.[36] In the present study, we add important prospective evidence on the association between plasma VWF and the risk of incident VTE in men and women of a wider age range and extend previous knowledge by showing that the association appears to be mainly driven by unprovoked events and DVT in particular.

Although plasma levels of VWF are influenced by environmental factors, it is estimated that genetic variation accounts for up to 65% of the interindividual variability.[37] Genome-wide association studies have shown that VWF levels are regulated by several genetic loci; 19 loci have been identified so far.[38,39] In recent years, VWF has been explored as a causal risk factor for VTE. Mendelian randomization analyses of a recent INVENT consortium meta-analysis of genome-wide association study results from 46 354 individuals reported that a 1 standard deviation increase in genetically predicted VWF levels was associated with a 2.4-fold increased OR for VTE, supporting a causal association.[38,40] At the individual level, plasma VWF levels are highly modifiable and influenced by several factors, such as menstrual cycle, pregnancy, exercise, aging, circadian rhythm, cigarette smoke, and air pollution.[37] Because of the fluctuating nature of VWF, prospective studies with a long follow-up period may be subject to regression dilution and underestimation of the true association.[33] However, in the present study we found that the association between plasma VWF levels and VTE risk persisted throughout the entire follow-up period (average duration was 7.1 years). Interestingly, the Women's Health Initiative study found that a larger 1-year increase in VWF levels was not associated with VTE risk.[36] This may imply that basal plasma levels, rather than short-term fluctuations in plasma levels, of VWF (eg, due to acute-phase reactions) are important for the VTE risk. Taken together, this underscores that VWF is a reliable biomarker and suggests that genetic predisposition likely outweighs the modification through environmental and acquired factors. Notably, recent findings imply that VWF may represent a promising therapeutic target,[13,41] as well as a biomarker that may be part of a comprehensive risk-assessment model.

VWF is an unspecific biomarker, and elevated levels indicate platelet activation and, in particular, endothelial cell activation.[13] In recent years,

VWF has been recognized as a risk factor for several conditions, in the presence and absence of inflammation.[42-45] Although the role of VWF in hemostasis is well described, its etiological role in thrombogenesis remains to be fully understood. In their case-control study, Koster and colleagues reported that the univariate association between VWF and VTE risk disappeared after adjustment for FVIII.[28] On this basis, it was concluded that the association between VWF and VTE probably is biologically mediated through increased FVIII levels.[28] Currently, no locus has been identified to influence VWF levels independent of FVIII; therefore, this hypothesis could not be tested in a Mendelian randomization design.[38] Because circulating FVIII is primarily determined by VWF, FVIII and VWF have been proposed as reasonable targets for prevention and treatment of VTE.[41] On the contrary, the VWF-platelet interaction has been suggested to be of importance primarily in arterial thromboses, and there are ongoing trials investigating the effect of selective VWF antagonists on coronary artery disease and stroke.[46] Interestingly, mouse studies have recently suggested a critical role for VWF in venous thrombus formation also in the absence of FVIII,[47] suggesting that VWF may represent a promising therapeutic target also in venous thrombosis.[13]

In subgroup analyses, we observed that VWF was primarily associated with the risk of DVT and unprovoked VTE events. Higher levels of FVIII have been observed in patients with unprovoked VTE,[34] and elevated FVIII levels were more prevalent in patients with DVT compared with PE.[48] However, we are not aware of any previous studies investigating VWF and VTE risk according to these subgroups. The association with unprovoked events suggests that elevated plasma VWF levels relate to VTE risk in the absence of known provoking factors, which may strengthen the interpretation of a causal association.[49] Although PE and DVT are regarded as 2 entities of the same disease, there are several examples of risk factors with a differential impact on the presenting location of VTE.[50] Of these, the most prominent is the factor V Leiden (FVL) paradox; the FVL mutation increases the risk of DVT but not PE.[51] A proposed mechanism hypothesized from experiments in mice is that FVL is associated with enhanced thrombin generation, resulting in larger and more stable thrombi that are less prone to embolization.[51,52] This hypothesis may also pertain to VWF, because individuals with elevated plasma VWF levels are likely to have a higher endogenous thrombin potential because of the concomitantly elevated FVIII levels.[53] Another potential mechanism is that elevated VWF levels facilitate more stable thrombi through the adhesion to integrin αIIbβ3 on activated platelets,[54] resulting in a tighter anchorage of the platelets to each other and to the endothelial wall.[13]

The strengths of this study include the nested case-control design with a large sample of VTE patients and age- and sex-matched controls recruited from the same population-based cohort. The prospective design provided the possibility to gain insight into the temporal sequence between VWF and VTE risk. Because there is only 1 hospital in the study area providing VTE diagnostics and treatment, it is likely that a negligible amount of VTE cases were missed. This study also has some limitations that need mentioning. The vast majority of study participants were white; therefore, we encourage caution when extrapolating these findings to other ethnicities. Blood samples were drawn in 1994-1995 and stored for >20 years. This long storage time may have affected the plasma VWF levels, but it is likely that such an effect would be similar in cases and controls and not influence the relative differences between them. Moreover, because the intraindividual plasma VWF levels may have changed during the long follow-up (mean 7.1 years), a single baseline measurement of VWF at baseline could yield results subjected to regression dilution bias and underestimated associations. However, we did not observe evidence of regression dilution in our analyses, and the ORs for VTE according to quartiles of plasma VWF levels remained significant throughout the entire study period.

In conclusion, we found that higher plasma levels of VWF were associated with increased risks of incident VTE. The association was particularly strong for unprovoked DVT, and the increased VTE risk remained throughout the study period. Our findings suggest that VWF may serve as a reliable biomarker for future risk of VTE.

Acknowledgment

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

Authorship

Contribution: M.S.E. performed statistical analyses, interpreted data, and wrote the manuscript; K.H. and L.H.E. performed statistical analyses, interpreted data, and revised the manuscript; E.-S.H., V.M.M., and S.K.B. revised the manuscript; T.U. and P.A. performed laboratory analyses and revised the manuscript; and J.-B.H. conceived and designed the study, interpreted data, and revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Paper II

Received: 10 August 2022 | Revised: 30 December 2022 | Accepted: 23 January 2023

https://doi.org/10.1016/j.jtha.2023.01.024

ORIGINAL ARTICLE

Impact of the von Willebrand factor-ADAMTS-13 axis on the risk of future venous thromboembolism

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Funding information
Stiftelsen Kristian Gerhard Jebsen

Abstract

Background: von Willebrand factor (VWF) and its cleaving protease, ADAMTS-13, form a pivotal axis that regulates hemostasis. However, the role of the VWF-ADAMTS-13 axis in the risk of future venous thromboembolism (VTE) is unknown.

Objectives: To investigate whether plasma ADAMTS-13 levels and an imbalance with VWF levels, assessed as the VWF/ADAMTS-13 ratio, are associated with the risk of future VTE.

Patients/Methods: A population-based nested case-control study, comprising 383 incident VTE cases and 780 age- and sex-matched controls, was derived from the Tromsø study cohort (1994-2007). Antigen levels of ADAMTS-13 and VWF were measured in plasma samples obtained at cohort baseline. Odds ratios (ORs) with 95% CIs were estimated according to quartile cutoffs of ADAMTS-13 and VWF/ADAMTS-13 ratio determined in controls.

Results: In age- and sex-adjusted analysis, ADAMTS-13 levels were inversely associated with the VTE risk, with an OR of 1.40 (95% CI, 0.99-1.99) for the lowest vs highest quartiles. The VWF/ADAMTS-13 ratio was linearly associated with the VTE risk (*P* for trend = .001), with an OR of 1.70 (95% CI, 1.19-2.43) for the highest vs lowest quartiles, and the association was particularly pronounced for unprovoked VTE (OR, 2.81; 95% CI, 1.65-4.81). The ORs were only slightly attenuated after additional adjustments for body mass index and C-reactive protein.

Conclusions: Lowered ADAMTS-13 levels and an imbalance between ADAMTS-13 and VWF levels, reflected by an increased VWF/ADAMTS-13 ratio, were associated with an increased risk of future VTE. Our findings suggest that the VWF-ADAMTS-13 axis is involved in the pathogenesis of VTE.

KEYWORDS
ADAMTS-13 protein, human, venous thrombosis, venous thromboembolism, von Willebrand factor

Manuscript handled by: David Lillicrap

Final decision: David Lillicrap, 23 January 2023

1 | INTRODUCTION

Venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease and a major cause of morbidity and mortality worldwide [1,2]. Despite intensified efforts to improve awareness and prevention of VTE, time-trend studies have shown a slight increase in the incidence of the disease over the last decades [3,4]. Major knowledge gaps still exist regarding the risk factors and mechanisms that drive venous thrombus formation. Understanding the underpinnings of the pathophysiological mechanisms of VTE may reveal novel biomarkers that could help identify individuals at an increased VTE risk and provide potential targets for disease prevention and treatment.

Growing evidence suggests that von Willebrand factor (VWF) is involved in the pathogenesis of VTE [5–7]. VWF is a large multimeric glycoprotein that plays critical roles in hemostasis by interacting with platelets to promote platelet plug formation [8] and by serving as a plasma carrier for coagulation factor VIII [9]. Deficiency of VWF is the cause of von Willebrand disease, which is characterized by a bleeding tendency [10], whereas elevated VWF levels are associated with an increased risk of arterial cardiovascular disease (CVD) [11,12] and VTE [13,14].

The multimeric size and subsequent hemostatic function of VWF are regulated by a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13 (ADAMTS-13) through the cleavage of platelet-hyperadhesive ultralarge VWF (ULVWF) multimers [15,16]. Severe deficiency of ADAMTS-13, either congenital or acquired, can result in an excess of ULVWF and cause thrombotic thrombocytopenic purpura (TTP), a rare and potentially fatal disease characterized by VWF-mediated platelet-rich thrombi in the microcirculation [16,17]. Notably, it has been shown that the contribution of ADAMTS-13 to thrombogenesis expands beyond the microcirculation, as even slightly to moderately reduced ADAMTS-13 levels were found to be associated with an increased risk of arterial CVD (eg, myocardial infarction and ischemic stroke) [18–20]. For VTE, the existing data on ADAMTS-13 is limited to patients with cancer [21–23], or to relatively small case-control studies [24–27], which have an inherent susceptibility to reverse causation due to the lack of temporality between exposure and outcome in the case-control design. Of note, because ADAMTS-13 and VWF are functionally linked to each other, forming a pivotal axis that regulates hemostasis, the assessment of an imbalance of their plasma levels may provide further insights into VTE pathogenesis. A useful metric to evaluate such imbalance is the VWF/ADAMTS-13 ratio, where an increased ratio may reflect a prothrombotic state. Indeed, an increased VWF/ADAMTS-13 ratio in patients with arterial CVD has been consistently associated with cardiovascular complications, including recurrent thrombosis and death [28–31]. However, it remains unknown whether plasma ADAMTS-13 and an imbalance with VWF affect the risk of a future VTE.

We hypothesized that reduced ADAMTS-13 levels and high VWF/ADAMTS-13 ratio are associated with an increased VTE risk. Therefore, we set out to investigate the association of plasma ADAMTS-13 levels and VWF/ADAMTS-13 ratio with the risk of future incident VTE in a population-based nested case-control study.

Essentials

- Von Willebrand factor (VWF) and ADAMTS-13 form a pivotal axis that regulates hemostasis.
- The role of the VWF-ADAMTS-13 axis in the risk of future venous thromboembolism (VTE) is unknown.
- Low ADAMTS-13 levels and increased VWF/ADAMTS-13 ratio are associated with an increased risk of VTE.
- The VWF-ADAMTS-13 axis may be involved in the pathogenesis of VTE.

2 | METHODS

2.1 | Study population and design

The Tromsø study is a cohort consisting of repeated health surveys of the inhabitants in Tromsø, Norway [32]. The present study was derived from the fourth survey of the Tromsø study (Tromsø 4) conducted during 1994-1995. All inhabitants aged ≥25 years were invited, and 77% (27,158) took part. Participants were followed until incident VTE, death, migration, or the end of follow-up (September 1, 2007). A total of 462 study participants suffered a VTE event during follow-up. To conceive the nested case-control study, for each VTE case, two age- and sex-matched controls (n = 924), who were alive at the index date of the corresponding VTE case, were selected randomly from the parent cohort, as previously described [14]. Seventy-nine cases and 144 controls were excluded from the analyses because plasma samples were not available (64 cases and 115 controls) or were of insufficient quality due to hemolysis (15 cases and 29 controls), leaving 383 cases and 780 controls in the final analytical sample (Figure 1). It is important to address that in the nested case-control design, the temporal sequence between exposure and outcome is preserved, as blood samples to measure ADAMTS-13 and VWF were collected at cohort baseline. The regional committee for medical and health research ethics approved the study, and written informed consent was obtained from all participants.

2.2 | VTE registry

As previously described [33], we identified all first lifetime VTE events by searching the discharge diagnosis registry, radiology procedure registry, and autopsy registry from the University Hospital of North Norway, which is the only hospital in the study area. Events were adjudicated and recorded when 1) a VTE diagnosis was stated in the medical record, 2) signs and symptoms consistent with DVT and/or PE were present, 3) objective radiological confirmation was

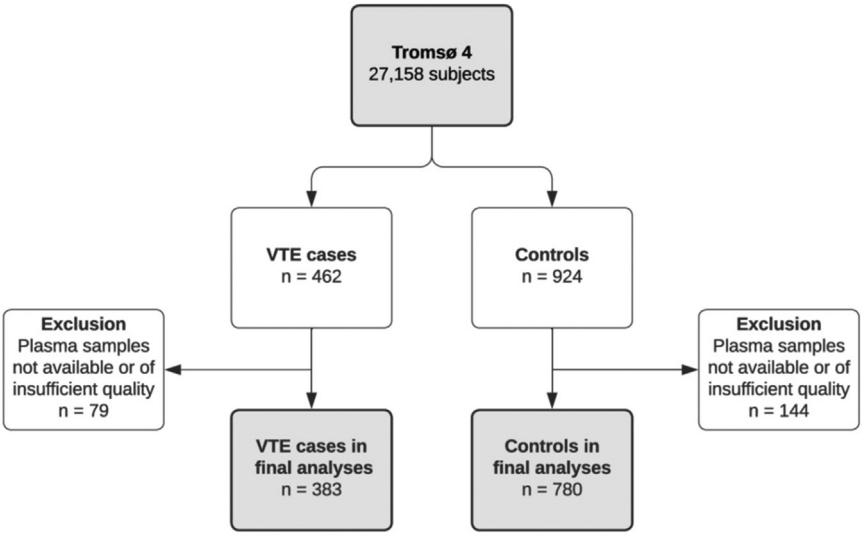

FIGURE 1 Flowchart of the study population. The chart illustrates the nested case-control design. Subjects (aged ≥25 years) were derived from the Tromsø 4 cohort. Cases and controls were age- and sex-matched. VTE, venous thromboembolism.

attained, and 4) treatment was implemented (unless contraindications were specified). For cases obtained from the autopsy registry, an event was recorded when PE was indicated as the cause of death or a condition significantly contributing to death. All VTE events were further classified as DVT or PE and provoked or unprovoked. If DVT and PE were present simultaneously, the event was classified as a PE. An event was considered provoked if it was closely preceded by one or more of the following provoking factors: trauma, surgery, or acute medical conditions (infectious diseases, myocardial infarction, or ischemic stroke) within 8 weeks prior to the event; marked immobilization (wheelchair confinement, >3 days of bed rest within the last 8 weeks, or long-distance travel for ≥4 hours within the last 14 days); and active cancer. If a treating physician specified another cause for VTE (eg, venous catheters), the event was also classified as provoked.

2.3 | Baseline measurements and blood sampling

Questionnaires, physical examination, and blood samples were used to acquire baseline information from all participants at inclusion in the parent cohort (1994/1995). Weight and height were measured with participants wearing light clothing and no shoes, and body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Self-administered questionnaires were used to obtain information on the history of cancer and arterial CVD (including stroke, angina pectoris, or myocardial infarction).

Nonfasting blood samples were collected from an antecubital vein into 5-mL vacutainers (Becton Dickinson) containing EDTA as an anticoagulant (40-μL K_3-EDTA, 0.37 mol/L per tube). Platelet-poor plasma was prepared by centrifugation at 3000 *g* for 10 minutes at room temperature. The supernatant was transferred into cryovials (Greiner Laboratechnik) in 1-mL aliquots, and then stored at −80 °C until further analysis.

2.4 | Laboratory analyses

Measurement of ADAMTS-13, VWF, and C-reactive protein (CRP) was performed at the Research Institute of Internal Medicine at Oslo University Hospital, Rikshospitalet. Before laboratory analyses, platelet-poor plasma samples were thawed for 5 minutes in a water bath at 37 °C and centrifuged at 13,500 *g* for 2 minutes to obtain platelet-free plasma.

Plasma ADAMTS-13 antigen (ADAMTS-13:Ag) was measured in duplicates by an enzyme immunoassay (EIA) with matched antibodies from R&D Systems in a 384 format using a combination of a SELMA pipetting robot and a Biotek dispenser/washer. Absorption was read at 450 nm with wavelength correction set to 540 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad). ADAMTS-13:Ag intra- and interassay coefficients of variation were 9.8% and 8.8%, respectively. The mean value of ADAMTS-13:Ag in the control population was set to 100%, and all other values were adjusted accordingly and expressed in percentage. EIAs with antibodies from Dako were applied to measure plasma VWF antigen (VWF:Ag) in duplicates [14]. A polyclonal antibody (A0082) was used for coat, and a horseradish peroxidase-conjugated antibody (P02256) was used for detection. Parallel-diluted pooled human plasma from 20 healthy individuals was used as a standard, and the intra- and interassay coefficients of variation were 2.6% and 10.8%, respectively. As for ADATMTS13, the mean value of VWF:Ag in the control population was set to 100% and all other values were adjusted accordingly and expressed in percentage. CRP was measured with a high sensitive technique ("hsCRP") by EIA, as previously described in detail [34].

2.5 | Statistical analyses

STATA version 16.0 (Stata Corporation) and R version 4 (R Foundation for Statistical Computing) were used to perform the statistical analyses. Among controls, ADAMTS-13:Ag raw values had a mean of

242 ng/mL (SD ± 84 ng/mL) and a median of 228 ng/mL (percentile 25th, 186 ng/mL; percentile 75th, 278 ng/mL). After expressing the raw values as a percentage of the control population mean (set to 100%), ADAMTS-13:Ag levels were categorized according to the following quartiles cutoffs determined in controls: <77%, 77%-94%, 94%-115%, and ≥115%. The VWF/ADAMTS-13 ratio was calculated for each participant by dividing VWF:Ag by ADAMTS-13:Ag, and then, quartiles cutoffs were determined among controls (<0.42, 0.42-0.74, 0.74-1.40, ≥1.40). Baseline characteristics stratified on ADAMTS-13 and VWF/ADAMTS-13 ratio quartiles were analyzed using descriptive statistics and expressed as percentages (frequency) for categorical variables and as mean (± SD) or median (IQR) for continuous variables.

Unconditional logistic regression was performed to estimate odds ratios (ORs) with 95% CIs for VTE according to quartiles of ADAMTS-13 levels and VWF/ADAMTS-13 ratio. The highest quartile was set as the reference category for ADAMTS-13 levels, whereas the lowest quartile served as the reference for the VWF/ADAMTS-13 ratio. The *P* value for the linear trend of the VTE risk was estimated across quartiles of ADAMTS-13 levels and VWF/ADAMTS-13 ratio. Associations were adjusted for the matching factors (ie, age and sex) [35] in model 1, with the addition of BMI and CRP to model 2 in order to assess potential confounding by obesity-related mechanisms or inflammation. Analyses were performed for overall VTE and VTE subtypes (DVT, PE, provoked, unprovoked) as the outcomes of interest. As ADAMTS-13 levels have been shown to be associated with arterial CVD [18–20], which in turn is found to increase the risk of VTE [36,37], we performed sensitivity analyses for overall VTE excluding participants with a history of arterial CVD at baseline. In all analyses, plasma ADAMTS-13 levels and VWF/ADAMTS-13 ratio were also entered into the logistic regression models as continuous variables. To this end, we calculated the mean and SD of ADAMTS-13 levels and VWF/ADAMTS-13 ratio based on the distribution of the control population. The ORs for VTE were investigated by 1SD decrease for ADAMTS-13 levels and by 1SD increase for the VWF/ADAMTS-13 ratio. Because the VWF/ADAMTS-13 ratio values were not normally distributed, natural logarithm transformation was applied to calculate the SD.

As the follow-up time in the source cohort was long, the results based on baseline ADAMTS-13 and VWF measurements could be influenced by regression dilution [38]. To investigate this, we took into account the time elapsed between blood sampling at cohort baseline in Tromsø 4 (ie, when samples to measure ADAMTS-13 and VWF were drawn) and the occurrence of VTE events. We performed analyses that restricted the maximum time from blood sampling in Tromsø 4 to the VTE events while keeping all controls in the analyses. The logistic regression analyses on time restrictions were set to require at least 10 VTE events, and ORs were estimated at every time point a new VTE event occurred and were plotted as a function of this maximum time, with adjustment for age, sex, BMI, and CRP.

3 | RESULTS

Baseline characteristics of study participants across quartiles of plasma ADAMTS-13:Ag levels are displayed in Table 1. The mean age and the proportion of subjects with a self-reported history of arterial CVD increased with decreasing levels of ADAMTS-13. Median CRP levels were 1.19 mg/L (IQR, 0.66-2.32) in quartile 4 and increased to

TABLE 1 Distribution of baseline characteristics of study participants according to quartiles of plasma levels of ADAMTS-13

	Plasma ADAMTS-13:Ag (%)			
	Quartile 1 <77	**Quartile 2 77-94**	**Quartile 3 94-115**	**Quartile 4 ≥115**
N	307	283	295	278
Age, y	62.9 ± 14.2	61.2 ± 14.0	59.4 ± 13.1	57.5 ± 13.5
Sex, male	48.5 (149)	43.8 (124)	50.2 (148)	47.1 (131)
BMI, kg/m^2	26.6 ± 4.5	25.8 ± 3.8	26.5 ± 4.2	26.6 ± 4.5
CRP, mg/L	1.46 (0.85-2.67)	1.30 (0.67-3.20)	1.38 (0.73-2.75)	1.19 (0.66-2.32)
Cancer[a]	3.9 (12)	5.3 (15)	4.4 (13)	4.3 (12)
CVD[a]	19.9 (61)	17.3 (49)	13.9 (41)	11.2 (31)
VWF:Ag, %	90 (52-164)	70 (41-128)	75 (44-131)	83 (54-131)
VWF:Ag/ADAMTS-13:Ag ratio	1.42 (0.79-2.57)	0.84 (0.48-1.47)	0.73 (0.41-1.28)	0.55 (0.39-0.99)

Continuous variables are shown as mean (± SD) or median (IQR).

Categorical variables are shown as percentages with numbers in brackets.

ADAMTS-13, a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; VWF, von Willebrand factor.

[a] Self-reported history of cancer or arterial CVD (myocardial infarction, angina, or stroke) at baseline.

TABLE 2 Characteristics of the venous thromboembolism events (n = 383)

Characteristics	
Age at VTE, y	68 ± 14
Sex, male	48.8 (187)
Deep vein thrombosis	63.5 (243)
Pulmonary embolism	36.6 (140)
Unprovoked VTE	41.0 (157)
Provoked VTE	59.0 (226)

VTE, venous thromboembolism.

Age is presented as mean ± SD.

Categorical variables are shown as percentages with numbers in brackets.

1.46 (IQR, 0.85-2.67) in quartile 1. The VWF/ADAMTS-13 ratio, as expected, increased with decreasing ADAMTS-13:Ag levels, whereas no substantial differences were observed for VWF:Ag levels. When baseline characteristics were analyzed across quartiles of the VWF/ADAMTS-13 ratio (Supplementary Table S1), the mean age and the proportion of subjects with arterial CVD increased with an increasing VWF/ADAMTS-13 ratio. Predictably, the plasma levels of VWF:Ag increased, whereas ADAMTS-13:Ag levels decreased across quartiles of the ratio. The characteristics of patients at the VTE event are shown in Table 2. The mean age at the time of VTE was 68 years, 48.8% were men, and 59% of the events were provoked VTEs and 63.5% were DVTs.

The ORs for overall VTE and subtypes (ie, provoked and unprovoked VTEs, DVT, and PE) across quartiles of plasma ADAMTS-13:Ag levels are shown in Table 3. ADAMTS-13 levels were inversely associated with VTE risk, with an OR of 1.40 (95% CI, 0.99-1.99) for the lowest vs highest quartiles in analysis adjusted for age and sex. No significant trend in risk estimates was observed across quartiles (*P* for trend = .12), suggesting a threshold rather than a linear relationship between ADAMTS-13 levels and VTE risk. Further adjustment for BMI and CRP had a negligible effect on the risk estimates. For VTE subtypes, the ORs were essentially similar to those observed for overall VTE, with the highest ORs obtained for the lowest vs highest quartiles of ADAMTS-13, with the exception of provoked VTE. When ADAMTS-13 was analyzed as a continuous variable (Supplementary Table S2), 1SD decrease in ADAMTS-13:Ag levels was associated with a 16% higher OR of VTE (OR, 1.16; 95% CI, 1.03-1.30) in the age- and sex-adjusted model, with virtually no change in the risk estimate with additional adjustment for BMI and CRP (OR, 1.17; 95% CI, 1.04-1.32). In line with the quartile-based analysis, estimates for VTE subtypes did not substantially differ from those of overall VTE.

The ORs for overall VTE and subtypes according to quartiles of plasma VWF/ADAMTS-13 ratio are shown in Table 4. A linear association was demonstrated for overall VTE, unprovoked VTE, and DVT (*P* for trend ≤.001). An increasing VWF/ADAMTS-13 ratio was associated with an increased risk of VTE, with an OR of 1.70 (95% CI, 1.19-2.43) for the highest vs lowest quartiles in age- and sex-adjusted analysis. The association was most pronounced for DVT (OR, 1.77; 95% CI, 1.17-2.67) and especially for unprovoked VTE (OR, 2.81; 95% CI, 1.65-4.81). Further subgroup analysis for unprovoked DVT was conducted as the association between VWF/ADAMTS-13 ratio and thrombosis risk was strongest for unprovoked VTE and DVT, which revealed an OR of 4.75 (95% CI, 2.27-9.95) for the highest quartile when compared to the lowest quartile. Additional adjustment for BMI and CRP had a minor impact on the risk estimates for overall VTE and subtypes. The thrombosis risk by 1SD increase in the VWF/ADAMTS-13 ratio was in agreement with the quartile-based analysis, with the strongest association also observed for unprovoked VTE (Supplementary Table S3). Because the VWF/ADAMTS-13 ratio was consistently associated with the VTE risk, we explored whether this association could be driven by either high VWF or low ADAMTS-13 plasma levels. As depicted in Supplementary Table S4, the risk estimates from model 2 remained essentially the same after additional adjustment for plasma VWF:Ag (model 3) or ADAMTS-13:Ag (model 4), respectively. Sensitivity analyses restricted to participants without self-reported CVD at baseline provided similar risk estimates as the main analyses and are reported in Supplementary Table S5.

To assess the possibility of underestimating the true association due to regression dilution bias, we estimated ORs for overall VTE as a function of time between blood sampling and the events (Figure 2). The ORs for VTE were somewhat higher with shortened time between blood sampling and VTE events for both ADAMTS-13:Ag (OR for the lowest vs highest quartiles) and VWF/ADAMTS-13 ratio (OR for the highest vs lowest quartiles), indicating a certain degree of regression dilution.

4 | DISCUSSION

In this population-based nested case-control study, we found that decreased plasma ADAMTS-13:Ag levels were associated with an increased risk of future VTE in age- and sex-adjusted analysis. In addition, an increasing VWF/ADAMTS-13 ratio displayed a linear association with the VTE risk, where subjects in the highest quartile of VWF/ADAMTS-13 ratio had a 70% higher OR of VTE than those in the lowest quartile, and the association was strongest for unprovoked VTE. The risk estimates were only slightly attenuated with further adjustment for BMI and inflammation, assessed by CRP. In addition, the risk estimates for VTE by ADAMTS-13 levels and VWF/ADAMTS-13 ratios were only moderately attenuated by increasing the time between blood sampling and the VTE events (regression dilution bias). Our findings support that ADAMTS-13 and in particular the VWF/ADAMTS-13 ratio may serve as biomarkers for the risk of future VTE and are involved in the pathogenesis of VTE.

Existing data on the association between ADAMTS-13 and VTE has been restricted to case-control studies involving highly selected study populations [24–27]. In accordance with most [24,25], but not all [27], previous case-control studies, Pagliari et al. [26] recently found that decreased ADAMTS-13 activity was associated with an increased risk of unprovoked DVT in a study comprising 365 patients and 292

TABLE 3 Odds ratios with 95% CIs for overall venous thromboembolism and subtypes across quartiles of plasma levels of ADAMTS-13

Quartiles of ADAMTS-13:Ag	Controls	Cases	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Overall VTE				
Q1 (<77%)	194	113	1.40 (0.99-1.99)	1.39 (0.98-1.99)
Q2 (77%-94%)	195	88	1.09 (0.76-1.57)	1.13 (0.78-1.63)
Q3 (94%-115%)	194	101	1.26 (0.88-1.79)	1.23 (0.86-1.76)
Q4 (≥115%)	197	81	1 (reference)	1 (reference)
P for trend			.12	.11
Provoked VTE				
Q1 (<77%)	194	61	1.34 (0.86-2.08)	1.31 (0.84-2.04)
Q2 (77%-94%)	195	61	1.35 (0.87-2.08)	1.37 (0.88-2.13)
Q3 (94%-115%)	194	59	1.32 (0.85-2.04)	1.27 (0.82-1.98)
Q4 (≥115%)	197	45	1 (reference)	1 (reference)
P for trend			.2	.2
Unprovoked VTE				
Q1 (<77%)	194	52	1.48 (0.92-2.38)	1.49 (0.92-2.42)
Q2 (77%-94%)	195	27	0.76 (0.44-1.31)	0.79 (0.46-1.36)
Q3 (94%-115%)	194	42	1.18 (0.72-1.93)	1.14 (0.70-1.88)
Q4 (≥115%)	197	36	1 (reference)	1 (reference)
P for trend			.2	.2
Deep vein thrombosis				
Q1 (<77%)	194	68	1.29 (0.85-1.95)	1.29 (0.85-1.96)
Q2 (77%-94%)	195	60	1.13 (0.74-1.72)	1.15 (0.75-1.76)
Q3 (94%-115%)	194	61	1.15 (0.76-1.75)	1.12 (0.74-1.71)
Q4 (≥115%)	197	54	1 (reference)	1 (reference)
P for trend			.3	.2
Pulmonary embolism				
Q1 (<77%)	194	45	1.63 (0.97-2.75)	1.59 (0.94-2.70)
Q2 (77%-94%)	195	28	1.02 (0.58-1.80)	1.06 (0.59-1.88)
Q3 (94%-115%)	194	40	1.48 (0.87-2.51)	1.41 (0.83-2.41)
Q4 (≥115%)	197	27	1 (reference)	1 (reference)
P for trend			.2	.2

Model 1: Adjusted for age and sex.

Model 2: model 1 + body mass index and C-reactive protein.

ADAMTS-13, a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13; OR, odds ratio; Q, quartiles; VTE, venous thromboembolism.

controls. Similarly, cohort studies exploring the relationship between ADAMTS-13 and VTE in patients with cancer [21–23] showed that decreased ADAMTS-13 levels were associated with the VTE risk in most [21,23], but not all [22], studies.

To the best of our knowledge, this is the first study to assess the association between plasma ADAMTS-13 levels and VTE with a prospective time sequence between the exposure and outcome (ie, blood samples for assessment of ADAMTS-13 were drawn before the VTEs occurred). We found that low ADAMTS-13 levels were associated with a 1.4-fold increased risk of VTE. The observed relationship between ADAMTS-13 levels and VTE risk pointed toward a threshold effect, as an increase in risk estimates became particularly apparent when comparing the lowest vs highest quartiles of ADAMTS-13. This finding is in agreement with previous studies on the association of ADAMTS-13 with arterial CVD and VTE, in which a threshold effect rather than a dose-response relationship was also suggested [19,26].

TABLE 4 Odds ratios with 95% CIs for overall venous thromboembolism and subtypes across quartiles of the plasma VWF/ADAMTS-13 ratio

VWF:Ag/ADAMTS-13:Ag ratio	Controls	Cases	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Overall VTE				
Q1 (<0.42)	194	74	1 (reference)	1 (reference)
Q2 (0.42-0.74)	195	77	1.04 (0.71-1.51)	1.00 (0.68-1.47)
Q3 (0.74-1.40)	194	106	1.45 (1.01-2.08)	1.41 (0.97-2.03)
Q4 (≥1.40)	197	126	1.70 (1.19-2.43)	1.61 (1.12-2.31)
P for trend			.001	.002
Provoked VTE				
Q1 (<0.42)	194	51	1 (reference)	1 (reference)
Q2 (0.42-0.74)	195	48	0.92 (0.59-1.44)	0.90 (0.58-1.41)
Q3 (0.74-1.40)	194	61	1.16 (0.76-1.79)	1.13 (0.74-1.75)
Q4 (≥1.40)	197	66	1.23 (0.80-1.89)	1.18 (0.76-1.81)
P for trend			.2	.3
Unprovoked VTE				
Q1 (<0.42)	194	23	1 (reference)	1 (reference)
Q2 (0.42-0.74)	195	29	1.29 (0.72-2.32)	1.28 (0.71-2.31)
Q3 (0.74-1.40)	194	45	2.09 (1.21-3.63)	2.09 (1.20-3.64)
Q4 (≥1.40)	197	60	2.81 (1.65-4.81)	2.68 (1.56-4.61)
P for trend			<.001	<.001
Deep vein thrombosis				
Q1 (<0.42)	194	50	1 (reference)	1 (reference)
Q2 (0.42-0.74)	195	43	0.87 (0.55-1.37)	0.86 (0.54-1.36)
Q3 (0.74-1.40)	194	64	1.32 (0.86-2.03)	1.31 (0.85-2.00)
Q4 (≥1.40)	197	86	1.77 (1.17-2.67)	1.67 (1.10-2.53)
P for trend			.001	.003
Pulmonary embolism				
Q1 (<0.42)	194	24	1 (reference)	1 (reference)
Q2 (0.42-0.74)	195	34	1.38 (0.78-2.41)	1.33 (0.75-2.35)
Q3 (0.74-1.40)	194	42	1.69 (0.97-2.94)	1.64 (0.94-2.87)
Q4 (≥1.40)	197	40	1.58 (0.90-2.76)	1.54 (0.87-2.70)
P for trend			.09	.11

Model 1: Adjusted for age and sex.
Model 2: Model 1 + body mass index and C-reactive protein.
ADAMTS-13, a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13; OR, odds ratio; Q, quartile; VTE, venous thromboembolism; VWF, von Willebrand factor.

The association between ADAMTS-13 levels and VTE risk was especially noticeable when modeling the metalloprotease as a continuous variable, with the assessment of the thrombosis risk by 1SD decrease in ADAMTS-13 levels. Importantly, in the present study, the effect of ADAMTS-13 levels on VTE risk remained virtually the same after adjustment for high-sensitivity CRP, implying that confounding by inflammation would be an unlikely explanation for the potential link between ADAMTS-13 and VTE. It is worth noting that rare genetic variants in *ADAMTS-13* were associated with slightly reduced activity of ADAMTS-13 and increased DVT risk, implying that ADAMTS-13 might be causally related to VTE [39,40]. However, a recent Mendelian randomization study did not find an association between genetically predicted ADAMTS-13 activity levels and VTE risk [41], suggesting that further research from a causal perspective is warranted.

Growing evidence advocates for a pivotal role of the VWF-ADAMTS-13 axis not only in hemostasis but also in thrombosis. In the present study, an imbalance between the plasma levels of VWF

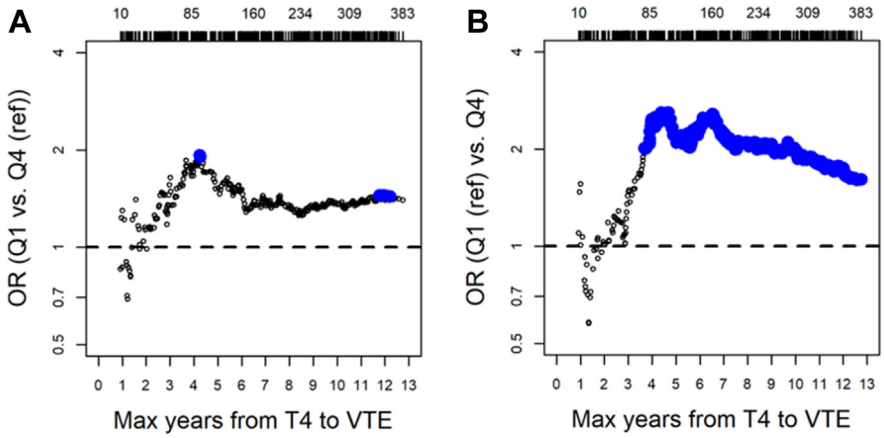

FIGURE 2 Plots of estimated ORs for overall VTE as a function of time between blood sampling in Tromsø 4 (T4, 1994–95) and VTE events. (A) Participants with plasma levels of ADAMTS-13:Ag in the lowest quartile (Q1) were compared with those with ADAMTS-13:Ag in the highest quartile (Q4, reference). (B) Participants with plasma VWF:Ag/ADAMTS-13:Ag ratio in the highest quartile (Q4) were compared with those with VWF:Ag/ADAMTS-13:Ag ratio in the lowest quartile (Q1, reference). ORs were adjusted for age, sex, body mass index, and high-sensitivity C-reactive protein. The large blue circles indicate ORs with *P* values of <.05. The number of VTE events is shown above the plots. ADAMTS-13, a disintegrin and metalloprotease with thrombospondin type 1 motif, member 13; OR, odds ratio; VTE, venous thromboembolism; VWF, von Willebrand factor.

and ADAMTS-13, as reflected by an increased VWF/ADAMTS-13 ratio, was dose-dependently associated with an increased risk of future VTE events. In patients with coronary artery disease and ischemic stroke, a high VWF/ADAMTS-13 ratio was consistently associated with cardiovascular complications, including recurrent thrombosis and death [28–31]. Others assessed the combined effect of high VWF levels and low ADAMTS-13 levels on thrombosis risk and found that the two risk factors displayed a synergistic effect on the risk of both arterial and venous thrombosis [26,42]. Thus, our findings expand the existing knowledge on the association of an imbalance between plasma VWF and ADAMTS-13 levels with arterial thromboembolic conditions to also embrace VTE. Moreover, although the ORs for VTE by increased VWF/ADAMTS-13 ratio were somewhat higher with shortened time between blood sampling and VTE events (Figure 2), the component of regression dilution due to intraindividual fluctuation of the VWF/ADAMTS-13 ratio over time was moderate at most. The stability of VWF/ADAMTS-13 ratio underscores its potential to serve as a reliable short- and long-term biomarker of VTE risk.

The consistent finding of increased VTE risk with the increase in VWF/ADAMTS-13 ratios suggests that the imbalance between VWF and ADAMTS-13 plays an important role in the pathogenesis of VTE. It is reasonable to speculate that in the presence of increased VWF levels due to genetic or acquired factors [43–45], the proteolytic activity of ADAMTS-13 toward VWF would be insufficient. The imbalance between the two proteins could also be a consequence of slightly to moderately decreased ADAMTS-13 levels due to acquired conditions (eg, advancing age, pregnancy, and comorbidities, such as cancer, liver disease, and inflammatory disease) [46–48] or genetic factors [39,40,49,50]. Interestingly, common genetic variants were reported to account for up to 20% of the variability in ADAMTS-13 antigen levels [50]. Of note, our results suggest that the association between the VWF/ADAMTS-13 ratio and VTE was not explained by either high levels of VWF or low levels of ADAMTS-13, as adjustment for these variables had a minor impact on the association. This implies that an imbalance between circulating levels of the two proteins contributes to the VTE risk. Irrespective of the primary cause for the imbalance in VWF and ADAMTS-13 levels, the regulation of the size of VWF multimers by ADAMTS-13 would be disrupted and could result in an excess of ULVWF multimers, which are more prone to bind platelets [16]. The interaction between VWF and platelets is well-known in the pathophysiology of arterial thrombosis [45], but growing evidence from mouse models suggests that this interaction is also implicated in venous thrombus formation [6,7]. The interplay between ADAMTS-13 and VWF is a complex and dynamic process in which shear stress has a key role [16]. Therefore, studies are needed to unravel how the VWF-ADAMTS-13 axis contributes to venous thrombosis formation, especially because ADAMTS-13 has the potential to be a target for therapeutic intervention. Indeed, previous experimental studies in mouse models showed that infusion of recombinant human ADAMTS-13 resulted in a reduction of both arterial [51–53] and venous [54] thrombi.

The strengths of the present study include the nested case-control design, which allows for prospective assessment and insight into the temporal sequence of the associations. The VTE cases and age- and sex-matched controls were recruited from the same unselected source population, minimizing the likelihood of selection bias. Still, the study has some limitations that require attention. In the quartile-based analysis of the association of ADAMTS-13 and VWF/ADAMTS-13 ratio with thrombosis risk, the number of VTE events was low in some subgroups, which resulted in limited statistical power and consequently the need for a cautious interpretation of the

findings, particularly for ADAMTS-13. Nonetheless, when both ADAMST13 and VWF/ADAMTS-13 ratios were entered into the regression models as continuous variables, the precision of the risk estimates was improved, as reflected by their 95% CIs. Although the number of plasma samples not available or of inadequate quality for the assessment of ADAMTS-13 antigen level was somewhat high, missing data on ADAMTS-13 was not related to the VTE status, occurring in 17% of the VTE cases and 16% of the controls. Additionally, baseline characteristics of the study participants with and without measurement of ADAMTS-13 were similar (data not shown). Thus, the missing data on ADAMTS-13 was presumably completely at random. Plasma samples were stored for more than 20 years between baseline sampling and measurement of ADAMTS-13 and VWF, thus introducing a possibility for discrepancy between true and measured levels. In the present study, ADAMTS-13 mean (242 ng/mL) and median (228 ng/mL) antigen levels in controls corresponded to about one-third of the levels generally reported for this metalloprotease in a healthy population [24,25,42,55–57]. However, because blood samples were stored in the same way and for the same duration for cases and controls, any potential misclassification would be nondifferential with regard to the VTE status, which could have led to an underestimation of the true associations [38]. Furthermore, previous studies have predominantly targeted ADAMTS-13 activity, as it expresses both quantitative and qualitative alterations of the metalloprotease, whereas in the present study, only ADAMTS-13 antigen levels were assessed as we only had stored EDTA plasma samples available for this cohort. In some physiological and pathologic conditions (eg, neonatal period, pregnancies of later maternal age, or cardiac surgery), plasma levels of ADAMTS-13 activity and antigen have been shown to not always be well correlated [47]. Nevertheless, our study population comprised adult individuals, where the majority had no major comorbidities at the time of baseline blood sampling, with the existing data indicating a substantial correlation between ADAMTS-13 antigen and activity levels in both healthy individuals and those with thrombotic disease [28,47]. Finally, VWF:Ag assay allows for quantitative assessment of VWF but does not evaluate its functional activity. VWF activity assays, which also reflect the protein multimeric size, such as VWF ristocetin-cofactor (VWF:RCo) activity, might provide more accurate and biologically meaningful results on the role of the imbalance between VWF and ADAMTS-13 in the risk of VTE. However, as already pointed out, because only stored EDTA plasma samples were available for this study, unfortunately, the VWF activity could not be assessed.

In conclusion, decreased plasma ADAMTS-13 levels and an imbalance between VWF and ADAMTS-13 levels, reflected by an increased VWF/ADAMTS-13 ratio, were associated with an increased risk of future VTE. Our findings suggest that the VWF-ADAMTS-13 axis might be involved in VTE pathogenesis and serve as a biomarker for the risk of future VTE. Further research is needed to unravel the mechanisms by which the VWF-ADAMTS-13 axis may contribute to venous thrombus formation and to explore its potential as a therapeutic target for VTE.

ACKNOWLEDGMENTS

The Thrombosis Research Center has received an independent grant from Stiftelsen Kristian Gerhard Jebsen (2014-2020).

AUTHOR CONTRIBUTIONS

M.S. Edvardsen analyzed data, interpreted the results, and drafted the manuscript. E.-S. Hansen interpreted the results and revised the manuscript. P. Aukrust and T. Ueland performed laboratory analysis, interpreted the results, and revised the manuscript. S.K. Brækkan designed the study, organized data collection, interpreted the results, and revised the manuscript. V.M. Morelli designed the study, interpreted the results, contributed to drafting of the manuscript, and also revised the manuscript. J.-B. Hansen designed the study, organized data collection, interpreted the results, contributed to drafting of the manuscript draft, and also revised the manuscript. All authors read and approved the final version of the article.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2023.01.024

Paper III

blood®
Regular Article

THROMBOSIS AND HEMOSTASIS

Combined effects of plasma von Willebrand factor and platelet measures on the risk of incident venous thromboembolism

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KEY POINTS

- **Platelet reactivity and platelet count interact biologically with high VWF plasma levels, resulting in an increased risk for VTE.**

Plasma von Willebrand factor (VWF) and platelet reactivity are risk factors for venous thromboembolism (VTE), and VWF can promote hemostasis by interaction with platelets. In this study, we explored the combined effects of plasma VWF and platelet measures on the risk of incident VTE. A population-based nested case-control study with 403 cases and 816 controls was derived from the Tromsø Study. VWF, platelet count and mean platelet volume (MPV) were measured in blood samples drawn at baseline. Odds ratios (ORs) with 95% confidence intervals (CIs) for VTE were estimated across VWF tertiles, within predefined MPV (<8.5, 8.5-9.5, and ≥9.5 fL) and platelet count (<230, 230-299, and ≥300 $\times 10^9$/L) strata. Here, participants with VWF levels in the highest tertile and with MPV ≥9.5 fL had an OR of 1.98 (95% CI, 1.17-3.36) for VTE compared with those in the lowest VWF tertile and with MPV <8.5 fL in the age- and sex-adjusted model. In the joint exposure group, 48% (95% CI, 15-96) of VTEs were attributable to the biological interaction between VWF and MPV. Similarly, individuals with VWF in the highest tertile and platelet count ≥300 × 10^9/L had an OR of 2.91 (95% CI, 1.49-5.67) compared with those with VWF in the lowest tertile and platelet count <230 × 10^9/L, and 39% (95% CI, −2 to 97) of VTEs in the joint exposure group were explained by the interaction. Our results suggest that platelet reactivity and platelet count interact biologically with high plasma VWF, resulting in an increased risk for incident VTE.

Introduction

Venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease associated with severe complications.[1,2] The incidence of VTE is increasing, contributing to a substantial disease burden globally.[3,4] Although the pathophysiology of VTE has gradually been unraveled over the last decades, up to half of all events arise in the absence of any known attributable factors.[5,6] Therefore, it is essential to identify risk markers and expand our insight into the mechanisms involved in the development of disease.

von Willebrand Factor (VWF) is a multimeric glycoprotein that is involved in hemostasis. It is synthesized by endothelial cells and megakaryocytes, and stored and released from Weibel-Palade bodies and platelet α-granules upon stimulation.[7-9] VWF has 2 distinct roles in hemostasis: to serve as the carrier and protector of coagulation factor VIII (FVIII) and to promote adhesion and aggregation of platelets through interaction with their glycoproteins.[10] We and other investigators have demonstrated that elevated plasma levels of VWF are associated with the risk of VTE.[11-13] Moreover, a recent Mendelian randomization analysis indicated a causal role for VWF in the development of VTE, but this causality could not be dissected from FVIII because of the tight correlation.[14] It is a common notion that the increased VTE risk by elevated VWF levels is mediated primarily through the parallel increase in FVIII levels.[12,14,15] However, studies in mice have suggested that VWF-mediated platelet adhesion has a critical role in VTE formation independent of FVIII.[16,17]

Platelets constitute a crucial part of hemostasis, and have been connected with thrombogenesis through several mechanisms.[18,19] A high platelet count is a marker of VTE risk in cancer patients, but not in cancer-free subjects.[20,21] Mean platelet volume (MPV), an indirect marker of platelet reactivity,[22,23] is associated with the future risk of VTE and unprovoked events, in particular.[24] However, studies using other measures of platelet function have provided conflicting results,[25] and the association between platelet function and VTE remains insufficiently understood.

Expanded knowledge on the role of the VWF-platelet interaction in the pathogenesis of VTE may facilitate development of novel therapeutic targets and improve strategies for prevention. To focus on the FVIII-independent role of VWF, our aim was to

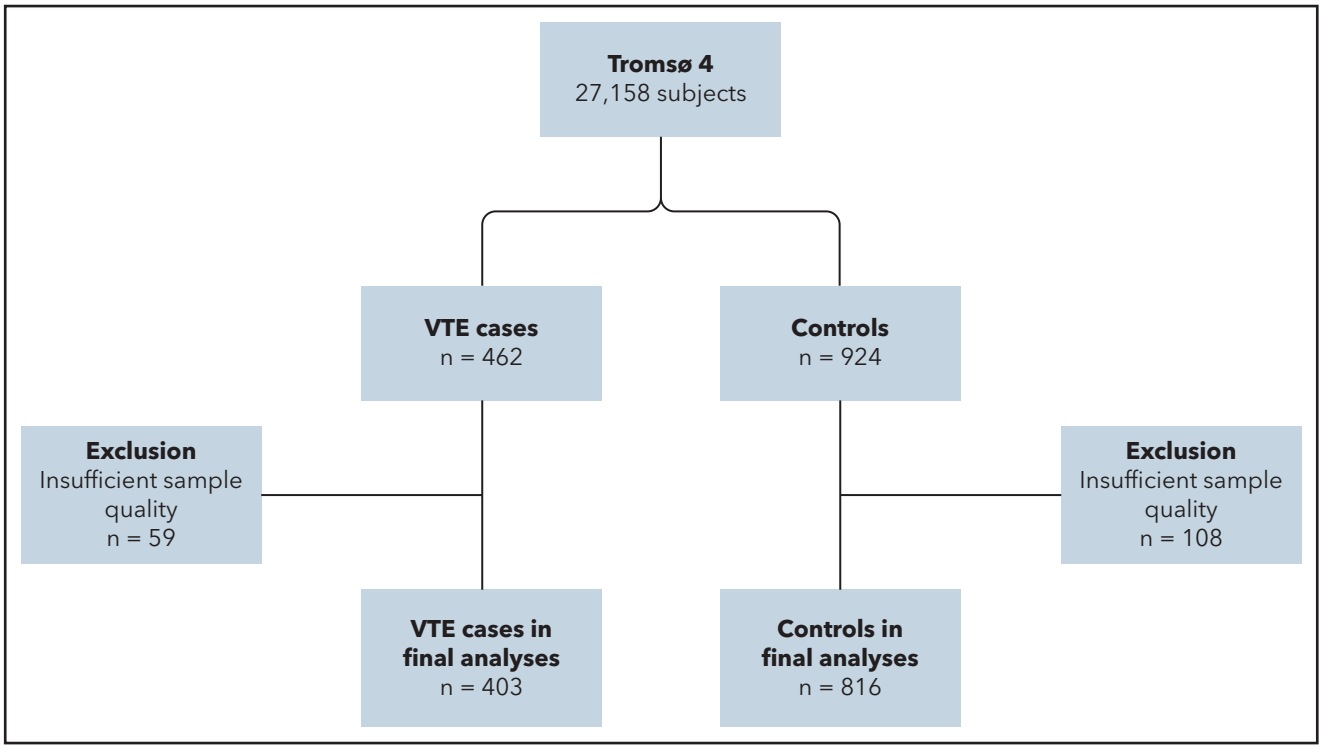

Figure 1. Flowchart of the study population. The chart illustrates the nested case-control design. Subjects (aged ≥25 years) were recruited from the general population. Cases and controls were matched on age and sex.

explore the combined effects of categories of VWF levels and platelet measures on the risk of VTE. To address this question, we carried out a nested case-control study derived from a population-based cohort, with the hypothesis that VWF levels and platelet measures would have more than an additive effect on VTE risk.

Methods

Study design

The Tromsø Study is a single-center, population-based cohort with repeated health surveys of the inhabitants of Tromsø municipality in Norway.[26] The fourth survey (Tromsø 4) was conducted in 1994 to 1995. All inhabitants aged ≥25 years were invited, and 27 158 individuals (77% of the eligible) took part in the survey. All participants were followed from the date of inclusion until an incident VTE, death, migration, or the end of follow-up (1 September 2007).

During follow-up, 462 participants developed an incident VTE event. For each case, 2 age- and sex-matched controls, who were alive at the index date of the case, were randomly sampled from the source cohort (Figure 1). The age matching was based on the same year of birth. Because of the insufficient quality of plasma samples, 59 VTE cases and 108 controls were excluded, leaving 403 cases and 816 controls in the final sample. Written informed consent was provided by all participants, and study approval was obtained from the regional committee for medical and health research ethics.

Validation of events

To identify all first lifetime VTE events, we searched the hospital discharge diagnosis registry, the radiology procedure registry, and the autopsy registry from the University Hospital of North Norway, which is the only hospital in the study region. The medical record of every patient with a potential VTE was reviewed by trained personnel, and the event was only recorded when clinical signs and symptoms were followed by objective radiological confirmation, resulting in a VTE diagnosis requiring treatment (unless contraindications were specified).

All events were classified as a DVT or PE, with simultaneous evidence of both conditions classified as a PE. Further classification into provoked and unprovoked events was also performed. If the patient had ≥1 provoking factor closely preceding the event, it was categorized as provoked. Provoking factors included trauma or surgery within 8 weeks prior to the event, an acute medical condition (myocardial infarction, ischemic stroke, or infectious disease), active cancer, and immobilization (>3 days' bed rest, wheelchair confinement, or long-distance travel ≥4 hours within the last 14 days). If the treating physician specified another factor to have provoked the event (eg, venous catheters), this was also recognized.

Baseline measurements and blood sampling

Baseline information was collected by physical examinations, self-administered questionnaires, and blood samples. Height (to the nearest centimeter) and weight (to the nearest 0.5 kg) were measured with participants in light clothing and no shoes; body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Trained personnel performed 3 consecutive blood pressure measurements on all participants, using an automatic device (Dinamap Vital Signs Monitor). Participants rested in a sitting position for 2 minutes before and between the measurements; the last 2 measurements were used to

calculate mean systolic and diastolic blood pressure. Self-administered questionnaires were used to obtain information regarding smoking habits and estrogen use, as well as history of cancer and cardiovascular disease (myocardial infarction, stroke, or angina pectoris).

Nonfasting blood samples were collected from an antecubital vein into 5-mL Vacutainers (Becton Dickinson, Le Pont-de-Claix, France) with EDTA (K_3-EDTA 40 μL, 0.37 mol/L per tube) as anticoagulant. Platelet-poor plasma was prepared by centrifugation at 3000*g* at room temperature for 10 minutes. The supernatant was transferred into cryovials (Greiner Labortechnik, Nürtingen, Germany) in 1-mL aliquots and stored at −80°C until further analysis.

Measurement of platelet count, MPV, and VWF

Platelet count and MPV were analyzed within 12 hours of blood sampling, using an automated blood cell counter (Coulter counter; Coulter Electronics, Luton, United Kingdom).

Measurement of VWF was performed at the Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet. Stored plasma samples were thawed in a water bath at 37°C for 5 minutes and then centrifuged at 13 500*g* for 2 minutes to obtain platelet-free plasma. Plasma VWF levels were measured by enzyme immunoassays with antibodies (A0082, P02256) obtained from Dako (Glostrup, Denmark), using a polyclonal antibody for coat (A0082) and a horseradish peroxidase–conjugated polyclonal antibody for detection (P02256). Parallel diluted pooled human plasma from 20 healthy individuals was used as standard, and the measurements were expressed as a percentage of the control population mean (100%). The intra- and interassay coefficients of variation were 2.6% and 10.8%, respectively.

Statistical analyses

STATA version 16.0 (Stata Corporation, College Station, TX) and R version 3.6.3 (The R Foundation for Statistical Computing, Vienna, Austria) were used to carry out the statistical analyses.

Categories of VWF (low, medium, high) were defined according to tertiles of plasma VWF levels in the control group. Categories of platelet variables were made with cutoffs at 8.5 and 9.5 fL for MPV and 230 and 300 × 10^9/L for platelet count, according to our previous study on platelets and VTE risk.[24] The 3-level variables of VWF and MPV were combined to yield a 9-level variable; subjects with concomitant low VWF levels and low MPV served as the reference group. A combined category of VWF and platelet count was created using the same approach.

Baseline characteristics across tertiles of VWF levels were expressed as mean (± standard deviation) or median (25th to 75th percentile) for continuous variables and as percentages (quantity) for categorical variables. Unconditional logistic regression was used to estimate odds ratios (ORs) with 95% confidence intervals (CIs) for VTE according to combined categories of VWF and platelet measures. The ORs were adjusted for age and sex in model 1 to take into account the matching factors in the analyses,[27] with further adjustment for BMI, C-reactive protein (CRP), smoking, hypertension, estrogen use, self-reported history of cancer at baseline, and platelet count/MPV in model 2.

The relative excess risk due to interaction (RERI)[28] was used to evaluate biological interaction: RERI = (OR_{AB} − 1) − (OR_A + OR_B − 2), where the exposures $_A$ and $_B$ represent high plasma VWF and high platelet measures, respectively. RERI >0 indicates that the combined effects of the 2 exposures exceed the sum of the individual effects, thereby suggesting biological interaction. The attributable proportion (AP; ie, the proportion of events in the joint exposure group that could be attributed to the biological interaction) was estimated as RERI/OR_{AB}.[28] RERI and AP were presented with 95% CIs.[28]

Results

The baseline characteristics of study participants across tertiles of plasma VWF levels are shown in Table 1. The mean age and proportion with hypertension increased, whereas platelet count decreased slightly, across tertiles of VWF. In the lowest tertile, history of cardiovascular disease was less frequent, whereas smoking was more common. Mean MPV, mean BMI, median CRP, proportion of men/women, estrogen use, and history of cancer did not differ across tertiles of VWF. Median time from baseline to VTE event was 7.5 years. The characteristics of the 403 VTE events are presented in Table 2. The mean age of the patients at the time of their first VTE was 67 years, and 48% were male. DVT (62%) was more common than PE (38%), and 58% of all events were classified as provoked.

The ORs for VTE by VWF levels within each MPV stratum and by the combined categories of VWF and MPV are displayed in Figure 2A and Table 3. In participants with low MPV (<8.5 fL), those with high VWF levels had an age- and sex-adjusted OR of 1.31 (95% CI, 0.80-2.17) for VTE compared with those with a low VWF level. In participants with high MPV (≥9.5 fL), high VWF levels yielded an OR of 2.18 (95% CI, 1.15-4.16) for VTE compared with those with low VWF. Compared with those with low MPV and low VWF levels, participants with high MPV and high VWF levels had an OR of 1.98 (95% CI, 1.17-3.36) in analyses adjusted for age and sex. The risk estimates were only modestly affected by multivariable adjustment (Figure 2A; Table 3). Interaction analyses yielded a RERI of 1.00 (95% CI, −0.04 to 2.03); by calculating the AP, we found that 48% (95% CI, 15-96) of the cases in the joint exposure group could be attributed to the biological interaction between VWF and MPV.

Subgroup analyses showed that the combined effect of VWF and MPV was particularly strong for unprovoked VTE events; participants with high VWF levels and MPV had an age- and sex-adjusted OR of 6.63 (95% CI, 2.89-15.17) compared with those with low MPV and low VWF levels. Multivariable adjusted analyses yielded similar risk estimates (Figure 2B). The RERI was 5.18 (95% CI, −2.29 to 9.93), and the AP implied that 59% (95% CI, 6-96) of the unprovoked VTEs in the joint exposure group were attributable to this interaction. Stratified analyses further pointed toward a stronger association for DVT (Figure 2C) than for PE; joint exposure of high VWF levels and high MPV yielded age- and sex-adjusted ORs of 2.23 (95% CI, 1.21-4.11) for DVT and 1.61 (95% CI, 0.75-3.46) for PE. RERI was 0.62 (95% CI, −0.50 to 1.83), and the AP suggested that 33% (95% CI, −16 to 99) of the DVT cases in the joint exposure group could be attributed to the interaction between VWF and MPV.

Table 1. Distribution of baseline characteristics according to tertiles of plasma levels of VWF

	Plasma VWF, %		
	Tertile 1 (< 90.4)	Tertile 2 (90.4-100.9)	Tertile 3 (≥100.9)
N	387	410	422
Platelet count, mean ± SD, $\times 10^9$/L	251 ± 54	245 ± 53	238 ± 55
MPV, mean ± SD, fL	8.8 ± 0.9	8.8 ± 0.9	8.9 ± 1.1
Age, mean ± SD, y	57.0 ± 14.3	60.0 ± 13.7	63.4 ± 12.9
Males	47.8 (185)	47.3 (194)	45.7 (193)
BMI, mean ± SD, kg/m^2	25.8 ± 3.9	26.6 ± 4.3	26.8 ± 4.6
CRP, median (IQR), mg/L	1.18 (0.65-2.23)	1.14 (0.63-1.89)	1.31 (0.65-2.33)
Hypertension*	49.2 (190)	56.5 (231)	60.7 (256)
Cancer†	5.0 (16)	6.6 (21)	5.4 (17)
CVD†	12.9 (50)	17.3 (71)	18.0 (76)
Estrogen use‡	4.7 (18)	5.1 (21)	4.3 (18)
Smoking‡	41.1 (159)	25.1 (103)	26.1 (110)

Unless otherwise noted, data are % (n).

CVD, cardiovascular disease; IQR, interquartile range; SD, standard deviation.

*Defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg.

†Self-reported history of cancer or myocardial infarction, angina, or stroke at baseline.

‡Self-reported daily use of oral contraceptives or hormonal replacement therapy (estrogen use) or smoking of cigarettes or cigars (smoking).

The ORs for VTE across combined categories of plasma VWF level and platelet count are shown in Figure 3A and Table 4. Table 4 also depicts the ORs for VTE according to VWF levels within each stratum of platelet count. In the low platelet count stratum, a high VWF level yielded an age- and sex-adjusted OR of 1.52 (95% CI, 0.95-2.44) compared with those with a low VWF. The corresponding OR in the high platelet count stratum was 2.77 (95% CI, 1.25-6.14). The combination of high platelet count and plasma VWF level in the highest tertile yielded an OR of 2.91 (95% CI, 1.49-5.67) for VTE compared with those with low platelet count and VWF level in the age- and sex-adjusted model. The ORs were minimally affected by further adjustment for BMI, CRP, smoking, hypertension, estrogen use, cancer at baseline, and MPV (Figure 3A; Table 4). Interaction analyses provided a RERI of 0.94 (95% CI, −0.59 to 2.52), indicating that the combined effects of high VWF and high platelet count on VTE risk were more than additive; the AP implied that 39% (95% CI, −2 to 97) of the events in the joint exposure group could be attributed to the interaction.

Table 2. Characteristics of VTE events (n = 403)

Characteristics	Data
Age at VTE, mean ± SD, y	67.4 ± 13.7
Sex, male	48.1 (194)
Deep vein thrombosis	62.3 (251)
Pulmonary embolism	37.7 (152)
Unprovoked VTE	41.9 (169)
Provoked VTE	58.1 (234)
Surgery/trauma	22.3 (90)
Acute medical condition	15.6 (63)
Active cancer	21.8 (88)
Immobilization	18.1 (73)
Other*	4.0 (16)

Unless otherwise noted, data are % (n).

SD, standard deviation

*Other factor specified by treating physician to have provoked the event (eg, intravascular catheter).

Subgroup analyses of the interaction between VWF and platelet count provided similar results as for MPV, with the strongest associations for unprovoked events and DVT. The combination of a high platelet count and high VWF yielded age-and sex-adjusted ORs of 6.54 (95% CI, 2.47-17.32) for unprovoked VTE and 3.01 (95% CI, 1.45-6.23) for DVT compared with those with low levels in both exposures. Multivariable adjustment did not affect the estimates substantially (Figure 3B-C). RERI for unprovoked VTE was 2.73 (95% CI, −0.93 to 4.36), and the AP indicated that 40% (95% CI, 10-100) of unprovoked VTEs in those with combined high platelet count and high VWF could be attributed to the interaction. For DVT, the RERI was 1.36 (95% CI, −0.68 to 3.54), and the AP implied that 56% (95% CI, 8-100) of all DVTs in the joint exposure group were attributable to the interaction.

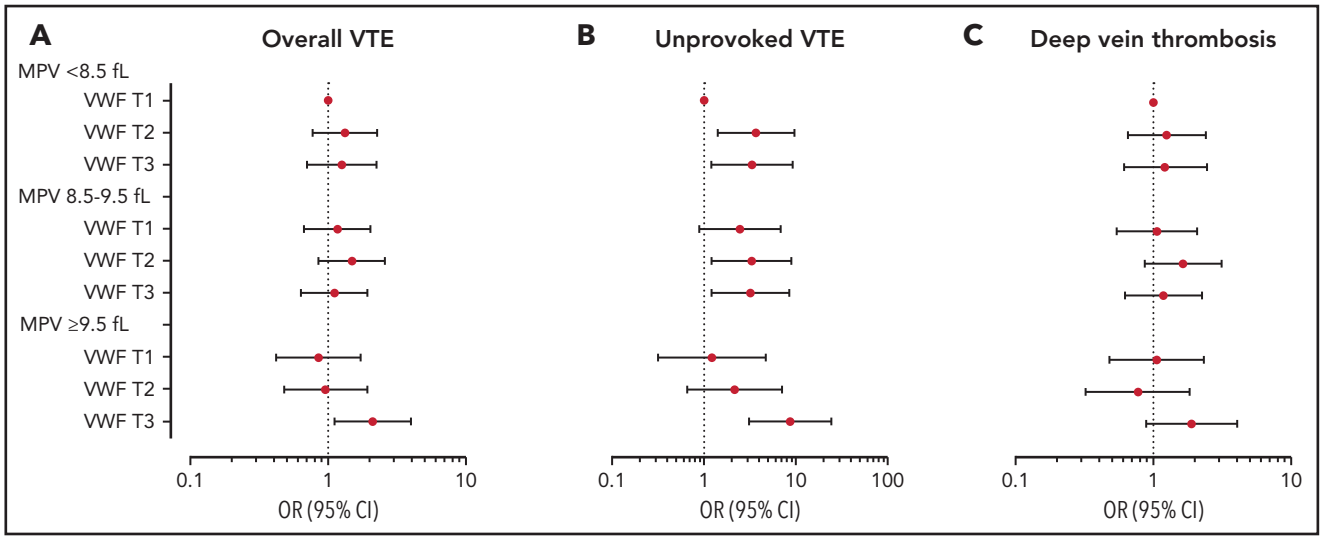

Figure 2. Forest plot of VTE risk by MPV and VWF levels. ORs and 95% CIs in multivariable analyses for overall VTE (A), unprovoked VTE (B), and DVT (C) by categories of VWF and MPV. The group with VWF level in the lowest tertile (T) and low MPV (<8.5 fL) is set as reference. ORs were adjusted for age, sex, BMI, CRP, smoking, hypertension, estrogen use, self-reported history of cancer at baseline, and platelet count.

Discussion

In the present population-based nested case-control study, we found that the combination of elevated plasma VWF levels and high MPV or a high platelet count had a more than additive effect on the VTE risk. In those with high MPV and high VWF, 48% of the VTE events could be attributed to the biological interaction. Similarly, 39% of the VTE events in those with high platelet count and high VWF could be attributed to the interaction. The combinations of elevated VWF levels and high platelet measures were most strongly associated with unprovoked VTE events. Our findings suggest that the presence of high VWF levels and a high platelet count or reactivity is required to yield an increased risk for VTE, implying a codependency between VWF and platelet measures in the biology of the VTE risk.

MPV is a marker of platelet reactivity and functional capacity,[29] because large platelets are observed to have a higher turnover of thromboxane A2 and release larger amounts of signaling

Table 3. ORs with 95% CIs for VTE across tertiles of plasma VWF and categories of MPV

MPV, fL	VWF, %	Controls (n = 816)	Cases (n = 403)	Within stratum, OR (95% CI)		Combined effects, OR (95% CI)	
				Model 1*	Model 2†	Model 1*	Model 2†
<8.5		314	148				
	T1	112	46	Ref	Ref	Ref	Ref
	T2	106	54	1.27 (0.79-2.05)	1.42 (0.81-2.48)	1.25 (0.78-2.01)	1.32 (0.77-2.27)
	T3	96	48	1.31 (0.80-2.17)	1.40 (0.77-2.54)	1.24 (0.76-2.03)	1.25 (0.70-2.24)
	P for trend			.3	.3		
8.5-9.5		324	163				
	T1	99	47	Ref	Ref	1.16 (0.71-1.90)	1.16 (0.67-2.02)
	T2	107	57	1.12 (0.70-1.81)	1.33 (0.77-2.27)	1.31 (0.82-2.11)	1.48 (0.85-2.58)
	T3	118	59	1.05 (0.65-1.69)	0.97 (0.57-1.65)	1.24 (0.77-1.98)	1.11 (0.64-1.91)
	P for trend			.9	.9		
≥9.5		178	92				
	T1	62	21	Ref	Ref	0.84 (0.46-1.53)	0.85 (0.42-1.72)
	T2	60	26	1.24 (0.63-2.45)	1.08 (0.49-2.41)	1.07 (0.60-1.90)	0.95 (0.48-1.90)
	T3	56	45	2.18 (1.15-4.16)	2.16 (1.01-4.60)	1.98 (1.17-3.36)	2.10 (1.11-3.98)
	P for trend			.014	.041		

Ref, reference; T, tertile.

*Adjusted for age and sex.

†Adjusted for age, sex, BMI, CRP, smoking, hypertension, estrogen use, self-reported history of cancer at baseline, and platelet count.

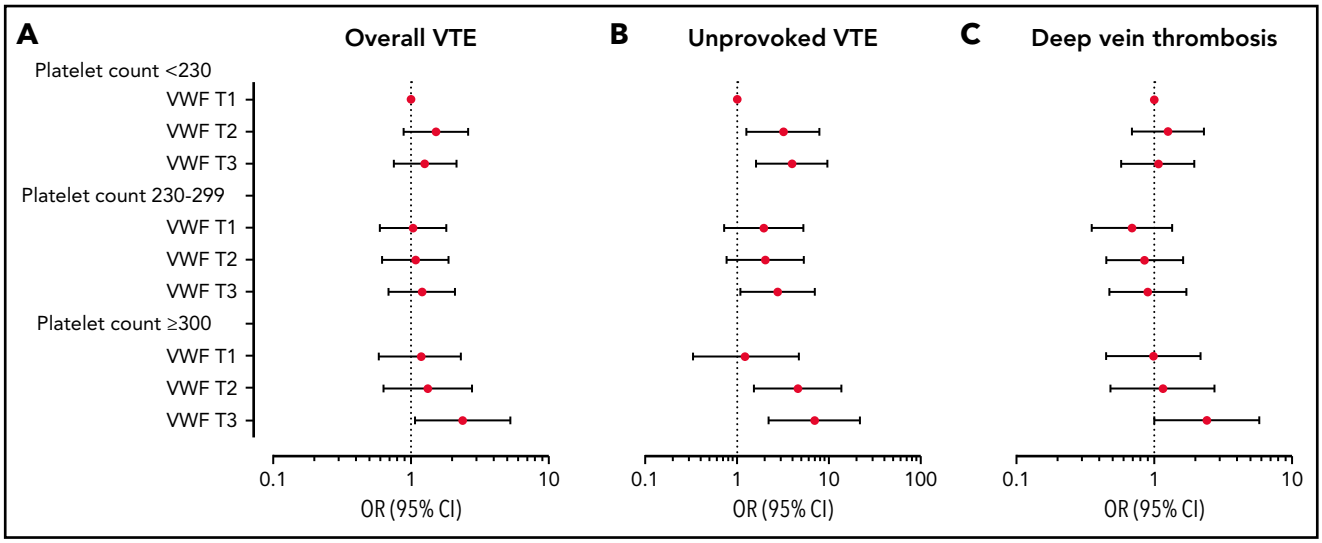

Figure 3. Forest plot of VTE risk by platelet count and VWF levels. ORs and 95% CIs in multivariable analyses for overall VTE (A), unprovoked VTE (B), and DVT (C) by categories of VWF and platelet count. The group with VWF level in the lowest tertile and low platelet count (<230 × 10^9/L) is set as reference. ORs were adjusted for age, sex, BMI, CRP, smoking, hypertension, estrogen use, self-reported history of cancer at baseline, and MPV. T, tertile.

substances upon activation.[22,30] Platelet size is primarily determined by the state of the parent megakaryocyte at the time of discharge.[31,32] In situations with enhanced platelet consumption, such as hypoxia, smoking, high BMI, and immune thrombocytopenic purpura, the megakaryocytes have higher cytoplasmic volumes and release larger platelets.[22,33,34] Large and more reactive (and hemostatically active) platelets are a risk factor for arterial[35,36] and venous[24] thrombosis.

Plasma VWF levels are shown to be associated with future risk of VTE.[11,13] VWF is acknowledged as an important protagonist of arterial thrombosis under high-flow conditions,[37] whereas the role of VWF in the causal pathway of VTE is still not fully understood. Since the discovery of the association between VWF and DVT in 1995, it has been perceived that it is primarily explained by concurrent high levels of FVIII.[12] However, in the Longitudinal Investigation of Thromboembolism Etiology study, VWF and FVIII

Table 4. ORs with 95% CIs for VTE across tertiles of plasma VWF and categories of platelet count

Platelet count, ×10^9/L	VWF, %	Controls (n = 816)	Cases (n = 403)	Within stratum, OR (95% CI)		Combined effects, OR (95% CI)	
				Model 1*	Model 2†	Model 1*	Model 2†
<230		349	176				
	T1	112	41	Ref	Ref	Ref	Ref
	T2	112	64	1.56 (0.97-2.51)	1.44 (0.84-2.45)	1.57 (0.98-2.52)	1.52 (0.90-2.59)
	T3	125	71	1.52 (0.95-2.44)	1.13 (0.66-1.95)	1.56 (0.98-2.49)	1.28 (0.76-2.17)
	P for trend			.1	.7		
230-299		349	157				
	T1	111	49	Ref	Ref	1.22 (0.74-1.99)	1.04 (0.60-1.82)
	T2	117	52	1.01 (0.63-1.61)	1.06 (0.62-1.83)	1.22 (0.75-1.99)	1.09 (0.63-1.90)
	T3	121	56	1.03 (0.64-1.64)	1.17 (0.69-2.00)	1.29 (0.80-2.08)	1.23 (0.71-2.10)
	P for trend			.9	.6		
≥300		118	70				
	T1	50	24	Ref	Ref	1.30 (0.71-2.39)	1.18 (0.59-2.33)
	T2	44	21	1.09 (0.53-2.27)	1.30 (0.54-3.13)	1.32 (0.70-2.48)	1.34 (0.64-2.83)
	T3	24	25	2.77 (1.25-6.14)	2.52 (0.95-6.69)	2.91 (1.49-5.67)	2.40 (1.08-5.34)
	P for trend			.017	.073		

T, tertile.

*Adjusted for age and sex.

†Adjusted for age, sex, BMI, CRP, smoking, hypertension, estrogen use, self-reported history of cancer at baseline, and MPV.

were reported to be independently associated with the risk of VTE.[11] A recent Mendelian randomization analysis was not able to address the independent role of VWF, because no locus was found to regulate VWF independently of FVIII.[14] In addition to serving as the carrier of FVIII, VWF promotes hemostasis by interacting with platelet glycoprotein Ib-IX-V and αIIbβ3 integrin, resulting in adhesion of platelets to the endothelium and to other platelets, respectively.[38] Therefore, it is reasonable to assume that VWF is also involved in FVIII-independent mechanisms in the pathogenesis of VTE.

To our knowledge, this is the first study to explore the combined effects of VWF with platelet count and platelet reactivity on VTE risk. The observation that VWF and platelet measures displayed a synergistic effect on the risk of VTE suggests that a biological interaction may contribute to the pathophysiology of VTE. Our results add knowledge about the presence and impact of this interaction in the general population. Notably, for overall VTE, VWF and platelet measures only yielded increased VTE risk when both components were elevated. Specifically, the 2 variables did not merely interact to add excess risk[39] but appeared to be dependent on each other to mediate increased risk. Thus, codependency, rather than synergism, may be a more fitting term for the interaction.

Similar to previous studies investigating the separate association of VWF and MPV in relation to VTE risk,[24] we found that the combined effect was strongest for unprovoked events. This implies a contribution by the VWF-platelet interaction on unprovoked events, in particular.[40,41] Furthermore, the combined exposure of high VWF levels and high MPV or high platelet count resulted in a particularly strong increase in the risk for DVT compared with PE. This finding is analogous with our previous study on VWF and the risk for VTE[13] and suggests that thrombi formed in the presence of high plasma levels of VWF and either many or highly reactive platelets may be more tightly anchored to the endothelium and, thus, be less likely to embolize.

Evidence from mouse studies suggests an essential role for VWF-platelet interaction in the pathogenesis of VTE. First, Chauhan and colleagues found that VWF-deficient mice had impaired thrombus growth in ferric chloride–injured veins.[16] Second, Brill and colleagues reported that the VWF-platelet interaction was necessary for the development of venous thrombosis in a flow-restricted model.[17] Traditionally, an increased thrombin potential because of concurrent elevated FVIII levels has been thought to explain the observed VWF-associated VTE risk.[12,14] However, infusion of recombinant FVIII in VWF-deficient mice only resulted in increased thrombus stability and did not increase thrombus formation.[16,17] Recently, Michels and colleagues found that VWF is critical in obesity-associated DVT in mice and demonstrated that targeting the VWF-platelet interaction with antibodies or nanobodies protected against thrombogenicity.[42] Furthermore, observational studies have shown that VWF and FVIII are independent risk factors for VTE.[11,43] Taken together, growing evidence suggests that enhanced platelet reactivity contributes to the increased VTE risk in those with elevated plasma VWF levels. The VWF-platelet interaction may therefore represent a promising target for VTE prevention and treatment, as for arterial thrombosis.[42,44] Several studies have been carried out on possible therapeutic approaches for VWF inhibition, but the focus has been on arterial thrombosis.[45]

Platelet count is inversely correlated with MPV and bleeding time[22] but is not regarded as a useful marker of a prothrombotic state because no association is established with arterial or venous thrombotic events,[24,46,47] and both low and high platelet count are associated with increased mortality and the risk of bleeding events in the general population.[48-52] However, the risk of VTE was substantially increased in subjects with high platelet count and concomitant high VWF, suggesting that the VWF-platelet interaction is also important for thrombogenesis in the presence of a high platelet count.

The strengths of this study include the population-based nested case-control design with a large population of cases and controls sampled from the same parent cohort with close follow-up. Because of the prospective design, we were able to gain insight into the temporal sequences of the associations. The study also has some limitations that require attention. Guidelines recommend collecting blood into tubes containing citrate as anticoagulant to measure VWF levels,[53] but in the present study, only plasma obtained from samples collected into EDTA was available. Of note, previous studies of healthy volunteers suggested a strong positive correlation between VWF antigen levels measured in EDTA and citrate plasma.[54,55] Although participants from this population-based nested case-control study were not necessarily healthy at baseline, it is unlikely that they would have clinically relevant coagulation disorders. Still, because blood samples were collected into EDTA in cases and controls, any discrepancy between true and measured levels of VWF would be nondifferential in relation to VTE status. In addition, blood samples were stored for >20 years between baseline sampling and measurement of VWF and were subjected to 1 additional freeze-thaw cycle before assessment of VWF, which could have introduced a discrepancy between true and measured levels. However, the samples were stored in the same way, for the same duration, and were subjected to the same number of freeze-thaw cycles in cases and controls; potential alterations would be nondifferential with regard to VTE status, thereby introducing a possibility for regression dilution bias and a weakening of the results compared with the true associations. Interaction analyses have inherent statistical limitations because they require dividing the study population into smaller groups.[56] Thus, our results on measures of biological interaction and their 95% CIs should be interpreted with caution, especially for the VTE subgroups. The majority of participants in this study were white; therefore, we encourage caution when extrapolating these findings to individuals of other ethnicities.

In summary, we found a synergistic effect of elevated plasma VWF levels and high MPV on the risk for VTE, which was most prominent for unprovoked events. High platelet count also yielded an increased risk for VTE when VWF levels were high. Our findings suggest that the presence of high VWF levels and a high platelet count or reactivity are required to yield an increased risk for VTE. Further research is necessary to disentangle the mechanisms of interaction between VWF and platelet number and reactivity and explore potential targets for VTE prevention and treatment.

Authorship

Contribution: M.S.E. performed statistical analyses, interpreted data, and drafted the manuscript; K.H., S.K.B., and L.H.E. performed statistical analyses, interpreted data, and revised the manuscript; E.-S.H. and V.M.M. interpreted data and revised the manuscript; T.U. and P.A. performed laboratory analyses, interpreted data, and revised the manuscript; and J.-B.H. conceived and designed the study, interpreted data and revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Footnotes

Submitted 5 March 2021; accepted 15 June 2021; prepublished online on *Blood* First Edition 23 June 2021. DOI 10.1182/blood.2021011494.

Data sharing requests should be sent to Magnus S. Edvardsen (magnus.s.edvardsen@uit.no).

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Paper IV

Received: 3 April 2023 | Revised: 10 June 2023 | Accepted: 22 June 2023

https://doi.org/10.1016/j.jtha.2023.06.022

ORIGINAL ARTICLE

Combined effect of high factor VIII levels and high mean platelet volume on the risk of future incident venous thromboembolism

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Funding information
The Thrombosis Research Center has received an independent grant from Stiftelsen Kristian Gerhard Jebsen (2014-2020).

Abstract

Background: High factor VIII (FVIII) levels and large platelets, as reflected by a high mean platelet volume (MPV), are separately associated with increased risk of venous thromboembolism (VTE). Whether the combination of high FVIII levels and large platelets has a supra-additive effect on VTE risk is unknown.

Objectives: We aimed to investigate the joint effect of high FVIII levels and large platelets, as reflected by high MPV, on the risk of future incident VTE.

Methods: A population-based nested case-control study with 365 incident VTE cases and 710 controls was derived from the Tromsø study. FVIII antigen levels and MPV were measured in blood samples drawn at baseline. Odds ratios with 95% CIs were estimated across FVIII tertiles (<85%, 85%-108%, and ≥108%) and within predefined MPV strata (<8.5, 8.5-9.5, and ≥9.5 fL).

Results: VTE risk increased linearly across FVIII tertiles (P_{trend} < .001) in models adjusted for age, sex, body mass index, and C-reactive protein. In the combined analysis, participants with FVIII levels in the highest tertile and an MPV of ≥9.5 fL (ie, joint exposure) had an odds ratio for VTE of 2.71 (95% CI, 1.44-5.11) compared with those with FVIII levels in the lowest tertile and an MPV of <8.5 fL (reference). In the joint exposure group, 52% (95% CI, 17%-88%) of VTEs were attributable to the biological interaction between FVIII and MPV.

Conclusion: Our results suggest that large platelets, as reflected by high MPV, might play a role in the mechanism by which high FVIII level increases the risk of incident VTE.

KEYWORDS
deep vein thrombosis, factor VIII, interaction, mean platelet volume, venous thromboembolism

Manuscript handled by: Alan Mast

Final decision: Alan Mast, 22 June 2023

1 | INTRODUCTION

Venous thromboembolism (VTE), a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease associated with serious short- and long-term complications, including postthrombotic syndrome, post-PE syndrome, recurrence, and death [1–3]. Effective prevention and subsequent reduction of VTE burden in the general population are dependent on expanded clarification of risk factors and molecular disease mechanisms. However, up to 50% of incident VTE events occur in the absence of any recognized predisposing factor [1,4], implying that there are still major knowledge gaps in data on the pathophysiological mechanisms of the disease.

During the past decades, a substantial body of evidence has implicated that coagulation factor VIII (FVIII) is a key component in the pathogenesis of VTE [5–7]. FVIII is a plasma sialoglycoprotein that plays an essential role in hemostasis. Upon cleavage by thrombin, activated FVIII dissociates from its carrier protein von Willebrand factor (VWF) and acts as a cofactor for activated factor IX (FIX) on the surface of negatively charged phospholipid membranes provided mainly by platelets, leading to accelerated thrombin generation [8]. The association between FVIII and VTE in epidemiologic studies was first recognized by Koster et al. [9] in 1995, who reported that high FVIII levels were associated with increased VTE risk in a dose-response manner. In the following years, an increasing number of studies, mostly with a case-control design, have supported an association between high FVIII levels and increased risk of a first-lifetime VTE [10–14]. Still, only a few studies have prospectively evaluated this relationship in the general population [15–17]. The role of FVIII in venous thrombosis is further reinforced by animal models, which have shown that elevated FVIII levels enhance thrombus formation and stabilization [5–7].

Although the association between FVIII levels and VTE is well established, the mechanisms underlying this association are not fully understood, especially regarding to what extent other components of the hemostatic system modify the VTE risk in the presence of high FVIII levels. One of the possible components is platelets. Large platelets, as reflected by a high mean platelet volume (MPV), are associated with increased risk of incident VTE [18]. *In vitro*, large platelets display increased reactivity and are more prone to expose phosphatidylserine on their membrane [19–21]. As the exposure of phosphatidylserine upon platelet activation is a fundamental step for activated FVIII and FIX to form the tenase complex that facilitates thrombin generation [8], we hypothesized that the combination of high FVIII levels and large platelets could have a supra-additive effect on VTE risk due to biological interaction. To examine this hypothesis, we first assessed the association between FVIII plasma levels and risk of future incident VTE in a nested case-control study derived from the general population and secondly investigated the combined effect of high FVIII levels and large platelets, as reflected by a high MPV, on the risk of VTE.

Essentials

- How high factor VIII (FVIII) levels combined with large platelets affect venous thromboembolism (VTE) risk is unknown.
- This combined effect was investigated in a population-based nested case-control study.
- High FVIII plasma levels were robustly associated with increased risk of future VTE.
- High FVIII levels combined with large platelets resulted in a supra-additive effect on VTE risk.

2 | METHODS

2.1 | Study population and study design

The Tromsø study is a population-based cohort, with repeated health surveys of inhabitants in Tromsø, Norway [22]. All inhabitants aged ≥25 years living in the Tromsø municipality were invited to participate in the fourth survey (Tromsø 4, 1994-1995). A total of 27 158 individuals participated (77% of those invited) and were followed from the date of inclusion until an incident VTE, migration, death, or end of follow-up (September 1, 2007), whichever came first. All first-lifetime VTE events occurring during follow-up were identified using the hospital discharge diagnosis, autopsy, and radiology procedure registries from the University Hospital of North Norway, which is the only hospital in the Tromsø region. Trained personnel confirmed and recorded each VTE event via extensive review of medical records. A VTE was confirmed if there were signs and symptoms of PE or DVT combined with objective confirmation by radiological procedures, which resulted in treatment initiation [23]. A VTE event was further classified as provoked or unprovoked based on provoking factors closely preceding the VTE diagnosis. A provoked VTE was defined as an event occurring in the presence of ≥1 of the following provoking factors: trauma, surgery, or acute medical conditions (acute ischemic stroke, acute myocardial infarction, or acute infection) within 8 weeks before the event; active cancer at the time of VTE diagnosis; immobilization (bed rest for >3 days, confinement to a wheelchair within the past 8 weeks, or long distance travel of ≥4 hours within the past 14 days); or other factors specifically described as provoking by a physician (eg, intravascular catheter).

We created a nested case-control study derived from the Tromsø 4 cohort for the assessment of biological variables from stored blood samples that were obtained at cohort inclusion, as previously described [24,25]. Briefly, during the follow-up period (1994-2007), 462 individuals experienced an incident VTE. For each case, 2 age- and sex-matched controls were randomly sampled from the parent cohort, who were alive at the index date of the VTE event (n = 924). From this

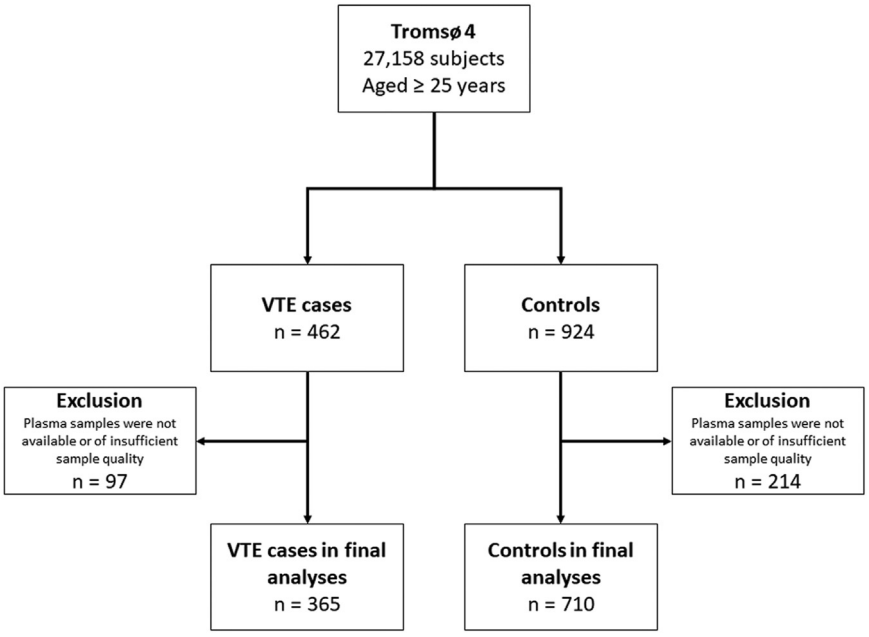

FIGURE 1 Flowchart of the study population. The flowchart illustrates the nested case-control study derived from the fourth survey of the Tromsø study (1994-1995). VTE, venous thromboembolism.

population, 97 cases and 214 controls were excluded because plasma samples were not available or were of insufficient quality for the analyses. Therefore, 365 VTE cases and 710 controls were included in the final analysis (Figure 1). The regional committee for medical and health research ethics approved the study, and all participants provided written informed consent.

2.2 | Baseline measurements and blood sampling

Height (to the nearest centimeter) and weight (to the nearest 0.5 kg) were measured in participants wearing light clothing and no shoes [23]. Body mass index (BMI) was calculated as weight divided by the square of height in meters (kilograms per square meter). A self-administered questionnaire was used to collect a detailed history of previous cancer and arterial cardiovascular disease (CVD) events (ie, stroke, angina pectoris, transient ischemic attack, and myocardial infarction).

Procedures for blood collection and storage of blood products have been previously described elsewhere [24,25]. In brief, at baseline inclusion in 1994 to 1995 (Tromsø 4), nonfasting blood was collected from an antecubital vein into 5-mL vacutainers (Becton Dickinson) containing EDTA (K_3-EDTA 40 μL, 0.37 mol/L per tube) as an anticoagulant. Platelet-poor plasma was prepared by centrifugation at 3000*g* for 10 minutes at room temperature, after which the supernatant was transferred into cryovials (Greiner Bio-One) in 1-mL aliquots and stored at −80 °C until further analysis.

2.3 | Laboratory analyses

Platelet parameters (platelet count and MPV) were analyzed within 12 hours of blood sampling, using an automated blood cell counter (Coulter Counter, Coulter Electronics) [18]. Measurements of FVIII and C-reactive protein (CRP) were performed at the Research Institute of Internal Medicine at Oslo University Hospital, Rikshospitalet. Platelet-poor plasma samples were thawed at 37 °C in a water bath for 5 minutes and prepared for analyses by centrifugation at 13 500*g* for 2 minutes to obtain platelet-free plasma [25].

Plasma levels of FVIII antigen were measured by enzyme immunoassay (EIA) with matched antibodies from Affinity Biologicals. EIA was performed in a 384-format using a combination of a pipetting robot and a BioTek dispenser/washer. Absorption was read at 450 nm with wavelength correction set to 540 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Rad). FVIII intra-assay and interassay coefficients of variation were <10%. The mean value of FVIII in the control population was set to 100%, and all other values were adjusted accordingly and expressed in percentages. High-sensitivity CRP was measured by EIA, as previously described [26].

2.4 | Statistical analyses

2.4.1 | Association between plasma FVIII levels and risk of future VTE

Statistical analyses were performed using Stata version 16 (StataCorp LLC) and R version 4.0.5 (The R Foundation for Statistical Computing). Participants were categorized according to tertiles of plasma FVIII antigen levels, with cutoffs determined in the control population (<85%, 85%-108%, and ≥108%). Baseline characteristics across FVIII tertiles were expressed as proportions for categorical variables, and as mean (±SD) or median (25th to 75th percentiles) for continuous variables using descriptive statistics.

Unconditional logistic regression was used to calculate odds ratios (ORs) for VTE with 95% CIs according to tertiles of FVIII, and the lowest tertile served as the reference. We performed analyses for overall VTE and for subgroups according to VTE location (DVT or PE) and presence of provoking factors (provoked or unprovoked). The *P* value for linear trend of VTE risk was estimated across increasing tertiles of FVIII levels. The association between FVIII levels and VTE was adjusted for age and sex in a first model to take into account the matching variables in the analyses [27]. BMI and inflammation, as reflected by high-sensitivity CRP, can influence both FVIII levels [28,29] and VTE risk [30,31], thereby acting as potential confounders in the association between FVIII and VTE. We therefore added BMI and high-sensitivity CRP to a second model. We chose not to adjust plasma FVIII for VWF because the levels of these factors are known to be closely related as they circulate in a tight noncovalent complex and can be considered as one entity [32]. Further, in this scenario, potential errors in the measured levels could have a substantial impact on the adjustment, limiting the ability to draw reliable conclusions [33]. Because cancer and arterial CVD have been reported to be associated with both FVIII levels [34,35] and VTE risk [36–38], we performed sensitivity analyses for overall VTE after excluding participants with a self-reported history of cancer or arterial CVD at cohort baseline. In all analyses, plasma FVIII was also entered into the logistic regression models as a continuous variable. For this purpose, we calculated the mean and SD of FVIII levels based on the distribution of the control population. The ORs for VTE were assessed by 1 SD increase in FVIII levels.

Results based only on baseline measurement of FVIII could be affected by regression dilution bias due to the long follow-up time in the parent cohort [39]. To address this, we took into account the time elapsed between blood sampling at baseline (ie, when samples to measure FVIII were drawn) and the occurrence of VTE events. We performed analyses that restricted the maximum follow-up time for the VTE cases, while keeping all controls in the analyses. The logistic regression analyses on time restrictions were set to require at least 10 VTE events, and ORs adjusted for age, sex, BMI, and CRP (model 2) were generated at every time point a new VTE occurred and plotted as a function of this maximum time.

2.4.2 | Combined effect between plasma FVIII levels and MPV on the risk of future VTE

To investigate the combined effect between plasma FVIII antigen levels and MPV, categories of MPV were conceived based on cutoffs at 8.5 and 9.5 fL, according to our previous study on platelets and VTE risk [18]. The 3-level variables of FVIII (<85%, 85%-108%, and ≥108%) and MPV (<8.5, 8.5-9.5, and ≥9.5 fL) were combined to yield a 9-level variable; subjects with both low FVIII levels (<85%) and low MPV (<8.5 fL) served as the reference group. Unconditional logistic regression was used to calculate ORs for overall VTE according to combined categories of FVIII and MPV using the aforementioned adjustment models but with the addition of platelet count to the second model. The presence of interaction on an additive scale between FVIII and MPV was assessed by calculating the following measures of biological interaction: the relative excess risk due to interaction (RERI) and the attributable proportion (AP) due to interaction with corresponding 95% CIs [40]. The RERI can be explained as the part of the total effect on the outcome that is attributable to the interaction, and the AP as the proportion of cases in the joint exposure group that is due to the interaction between the 2 exposures. An RERI and AP of >0 indicate positive interaction or more than additivity, meaning that the effect of the joint exposure to the 2 risk factors is greater than the sum of the separate effects [41].

TABLE 1 Distribution of baseline characteristics of the study population across tertiles of plasma factor VIII antigen levels.

Characteristic	FVIII levels		
	Tertile 1 <85%	Tertile 2 85%-108%	Tertile 3 ≥108%
No. of participants, *n*	320	358	397
Age, (y)	54.9 ± 13.5	61.6 ± 13.3	64.1 ± 13.0
Sex, men	50.9 (163)	46.9 (168)	44.8 (178)
BMI (kg/m^2)	25.7 ± 3.9	26.2 ± 4.1	27.1 ± 4.6
hsCRP (mg/L)	1.08 (0.63-2.20)	1.33 (0.74-2.54)	1.76 (0.90-3.51)
MPV (fL)	8.8 ± 0.99	8.9 ± 0.99	8.8 ± 0.96
Platelet, (×10^9 L^{-1})	252 ± 53	245 ± 55	240 ± 54
Cancer[a]	2.2 (7)	4.8 (17)	5.8 (23)
CVD[a]	13.4 (43)	17.6 (63)	17.4 (69)

Continuous variables are shown as mean ± SD or median (25th percentile to 75th percentile). Categorical variables are shown as percentages with numbers in parentheses.

BMI, body mass index; CVD, cardiovascular disease; FVIII factor VIII; hsCRP, high-sensitivity C-reactive protein; MPV, mean platelet volume.

[a] Self-reported history of cancer or arterial cardiovascular disease (myocardial infarction, angina, stroke) at baseline.

3 | RESULTS

The distribution of baseline characteristics across tertiles of plasma FVIII antigen levels in the study population is shown in Table 1. The mean age and BMI and the median levels of CRP increased across FVIII tertiles, while the mean MPV did not substantially differ according to FVIII tertiles. Characteristics of the patients with VTE are shown in Table 2. The mean age at the time of VTE was 67 years, 48.5% were men, 63.3% of the events were DVTs, and 59.5% were provoked VTEs.

3.1 | Association between plasma FVIII levels and risk of future VTE

The ORs for overall VTE and subgroups (ie, DVT, PE, and provoked and unprovoked VTE) according to tertiles of plasma FVIII antigen levels are shown in Table 3. The ORs for VTE increased linearly across FVIII tertiles in the age- and sex-adjusted model (P_{trend} < .001).

TABLE 2 Characteristics of the venous thromboembolism events (n = 365).

Characteristics	Value
Age at VTE (y)	67.4 ± 13.8
Sex (males)	48.5 (177)
Deep vein thrombosis	63.3 (231)
Pulmonary embolism	36.7 (134)
Unprovoked	40.5 (148)
Provoked VTE	59.5 (217)
Surgery/trauma	21.9 (80)
Cancer	23.6 (86)
Immobilization	19.2 (70)
Acute medical condition	15.9 (58)
Other factors	4.4 (16)

Age is shown as mean ± SD, and categorical variables are shown as percentages with numbers in parentheses.

VTE, venous thromboembolism.

Participants with FVIII levels in the highest tertile had a 2.1-fold higher OR for VTE (OR, 2.13; 95% CI, 1.53-2.98) than that of those with FVIII levels in the lowest tertile. A dose-response relationship between FVIII levels and thrombosis risk was also observed for the VTE subgroups, except for PE. Of note, FVIII levels were more strongly associated with the risk of DVT (OR for highest vs lowest tertile, 2.63; 95% CI, 1.77-3.92) than with the risk of PE (OR for highest vs lowest tertile, 1.46; 95% CI, 0.89-2.39). For overall and subgroup analyses, further adjustment for BMI and CRP had a minor impact on risk estimates. The thrombosis risk by 1 SD increase in FVIII levels was in line with the tertile-based analysis, with the strongest association being for DVT (Table 3). In the sensitivity analyses, exclusion of participants with a self-reported history of arterial CVD (Supplementary Table S1) or cancer (Supplementary Table S2) at baseline yielded results similar to those obtained in the main analysis.

To investigate the possibility of underestimating the true association due to regression dilution bias, we considered the time elapsed between the blood sampling at baseline and the occurrence of VTE events, estimating ORs for VTE among participants with high (highest tertile) vs low (lowest tertile) FVIII levels. As shown in Figure 2, the ORs for overall VTE by high levels of FVIII were higher with shortened time between blood sampling and VTE events, with risk estimates being especially high during the first 4 years after blood sampling. However, along the entire study period (≥12 years for many individuals), FVIII levels remained significantly associated with VTE.

TABLE 3 Odds ratios with 95% CIs for overall venous thromboembolism and subgroups according to plasma factor VIII antigen levels.

Tertiles of factor VIII	Controls	Cases	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Overall VTE				
T1 (<85%)	238	82	1 (reference)	1 (reference)
T2 (85%-108%)	236	122	1.58 (1.13-2.22)	1.57 (1.11-2.21)
T3 (≥108%)	236	161	2.13 (1.53-2.98)	1.97 (1.40-2.76)
P for trend			<.001	<.001
Per 1 SD increase			1.32 (1.16-1.50)	1.28 (1.12-1.45)
DVT				
T1 (<85%)	238	48	1 (reference)	1 (reference)
T2 (85%-108%)	236	72	1.64 (1.09-2.49)	1.62 (1.07-2.46)
T3 (≥108%)	236	111	2.63 (1.77-3.92)	2.45 (1.63-3.67)
P for trend			<.001	<.001
Per 1 SD increase			1.41 (1.22-1.63)	1.37 (1.19-1.59)
PE				
T1 (<85%)	238	34	1 (reference)	1 (reference)
T2 (85%-108%)	236	50	1.46 (0.90-2.37)	1.44 (0.88-2.35)
T3 (≥108%)	236	50	1.46 (0.89-2.39)	1.31 (0.79-2.16)
P for trend			.150	.334
Per 1 SD increase			1.11 (0.92-1.33)	1.05 (0.87-1.27)
Provoked				
T1 (<85%)	238	50	1 (reference)	1 (reference)
T2 (85%-108%)	236	69	1.42 (0.94-2.15)	1.41 (0.93-2.13)
T3 (≥108%)	236	98	2.04 (1.37-3.05)	1.90 (1.27-2.86)
P for trend			<.001	.002
Per 1 SD increase			1.25 (1.07-1.45)	1.21 (1.04-1.41)
Unprovoked				
T1 (<85%)	238	32	1 (reference)	1 (reference)
T2 (85%-108%)	236	53	1.85 (1.14-3.01)	1.82 (1.12-2.97)
T3 (≥108%)	236	63	2.29 (1.42-3.72)	2.03 (1.24-3.31)
P for trend			.001	.006
Per 1 SD increase			1.37 (1.17-1.62)	1.32 (1.12-1.55)

Model 1 was adjusted for age and sex. Model 2 was adjusted for age, sex, body mass index, and high-sensitivity C-reactive protein. The mean and SD (32%) of plasma factor VIII antigen levels were determined in the control population.

DVT, deep vein thrombosis; OR, odds ratio; PE, pulmonary embolism; T, tertile; VTE, venous thromboembolism.

3.2 | Combined effect between plasma FVIII levels and MPV on the risk of future VTE

The ORs for VTE according to plasma levels of FVIII within each stratum of MPV are described in Table 4. In each MPV stratum, there was a dose-response relationship between FVIII levels and VTE risk. It is worth noting that the highest estimates were observed in the highest MPV stratum (≥9.5 fL), where subjects with FVIII levels in the highest tertile had an OR for VTE of 3.42 (95% CI, 1.60-7.30) compared with those with FVIII in the lowest tertile in age- and sex- adjusted analysis, with only a

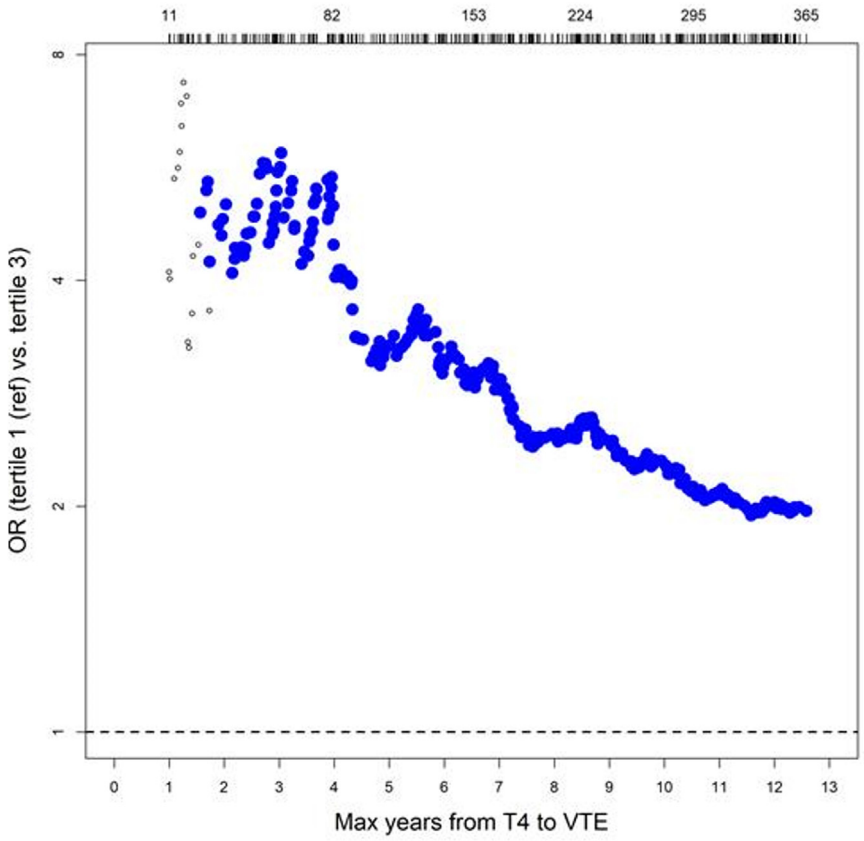

FIGURE 2 Plots of estimated odds ratios (ORs) for overall venous thromboembolism (VTE) as a function of time from blood sampling in T4 (Tromsø 4; 1994-1995) to VTE events. Participants with plasma factor VIII levels in the highest tertile (T3) were compared with those with factor VIII levels in the lowest tertile (T1, reference [ref] category). Analyses were adjusted for age, sex, body mass index, and high-sensitivity C-reactive protein. Blue solid circles indicate that risk estimates were statistically significant at a *P* value of <.05. The number of VTE events is depicted above the plot. Max, maximum.

slight change in the OR after further adjustment for BMI, CRP, and platelet count (OR, 3.74; 95% CI, 1.69-8.29).

As shown in Table 4, participants in the combined high-high category (ie, FVIII in the highest tertile and an MPV of ≥9.5 fL) had an OR for VTE of 2.77 (95% CI, 1.52-5.03) compared with those in the combined low-low category (ie, FVIII in the lowest tertile and an MPV of <8.5 fL) in analysis adjusted for age and sex. Additional adjustment for BMI, CRP, and platelet count had a minor impact on the risk estimate (OR, 2.71; 95% CI, 1.44-5.11). The combined exposure to high FVIII and high MPV had a supra-additive effect on VTE risk, with a RERI of 1.42 (95% CI, 0.01-2.85) in the fully adjusted analyses. The estimation of AP revealed that 52% (95% CI, 17%-88%) of the VTE events in the joint exposure group could be attributed to the biological interaction between FVIII and MPV. The combined effect of high FVIII levels and high MPV on VTE risk did not seem to be driven by platelet count as adjustment for platelet count did not affect the ORs. Moreover, the combination of high FVIII level and high platelet count (≥300 × 10^9/L) had no supra-additive effect on VTE risk, with RERI and AP estimates being approximately 0 (Supplementary Table S3).

4 | DISCUSSION

In this population-based nested case-control study, FVIII levels were linearly associated with the risk of future incident VTE in analyses adjusted for age and sex, and the association was particularly strong for DVT. The risk estimates were only slightly attenuated after further adjustment for BMI and CRP. Although the ORs for VTE were higher with shortened time between blood sampling and the thrombotic events, the association between high FVIII levels and VTE remained significant even several years after blood sampling. Furthermore, high FVIII levels combined with high MPV displayed a supra-additive effect on VTE risk. In the joint exposure group, 52% of the VTE events could be attributed to the biological interaction between high FVIII levels and high MPV. Our results indicate that plasma FVIII levels are robustly associated with the risk of future VTE in the general population and suggest that large platelets, as reflected by a high MPV, might play a role in the mechanism by which a high FVIII level increases thrombosis risk.

Although there are several case-control studies addressing the association between FVIII and VTE [9–14,42], only a few reports have prospectively evaluated this relationship in the general population [15–17]. In the Longitudinal Investigation of Thromboembolism Etiology study, with a cohort of 19 237 participants, of whom 159 experienced a VTE event during a median follow-up time of 7.8 years, the authors observed a linear relationship between FVIII levels and VTE risk in models adjusted for age, sex, race, BMI, diabetes, factor VII, and VWF [15]. Results from a case-cohort study derived from the Reasons for Geographic and Racial Differences in Stroke study [16] and from a cohort (Multi-Ethnic Study of Atherosclerosis) [17] also showed that high FVIII plasma levels were associated with increased risk of incident VTE in analyses adjusted for several demographics and comorbidities.

TABLE 4 Odds ratios with 95% CIs for overall venous thromboembolism across tertiles of plasma factor VIII antigen levels and strata of mean platelet volume.

MPV (fL)	FVIII (%)	Controls (*n* = 710)	Cases (*n* = 365)	Within stratum OR (95% CI)		Combined effects OR (95% CI)	
				Model 1	Model 2	Model 1	Model 2
<8.5	T1	92	33	1 (reference)	1 (reference)	1 (reference)	1 (reference)
	T2	81	44	1.73 (0.99 to 3.01)	1.68 (0.96 to 2.95)	1.61 (0.93 to 2.79)	1.57 (0.90 to 2.72)
	T3	98	55	1.85 (1.08 to 3.17)	1.70 (0.98 to 2.96)	1.69 (1.00 to 2.87)	1.56 (0.91 to 2.65)
P for trend				.032	.071		
8.5 to 9.5	T1	90	35	1 (reference)	1 (reference)	1.11 (0.63 to 1.94)	1.10 (0.62 to 1.94)
	T2	104	47	1.20 (0.70 to 2.03)	1.25 (0.73 to 2.14)	1.35 (0.79 to 2.30)	1.40 (0.81 to 2.41)
	T3	93	66	1.91 (1.13 to 3.21)	1.79 (1.05 to 3.05)	2.18 (1.29 to 3.66)	2.06 (1.21 to 3.50)
P for trend				.012	.028		
≥9.5	T1	56	14	1 (reference)	1 (reference)	0.71 (0.35 to 1.44)	0.73 (0.35 to 1.50)
	T2	51	31	2.32 (1.09 to 4.94)	2.41 (1.10 to 5.26)	1.84 (1.00 to 3.36)	1.79 (0.95 to 3.37)
	T3	45	40	3.42 (1.60 to 7.30)	3.74 (1.69 to 8.29)	2.77 (1.52 to 5.03)	2.71 (1.44 to 5.11)
P for trend				.002	.001		
RERI (95% CI)						1.37 (−0.03 to 2.76)	1.42 (0.01 to 2.85)
AP (95% CI)						0.49 (0.13 to 0.85)	0.52 (0.17 to 0.88)

T1 corresponds to FVIII <85%, T2 corresponds to FVIII 85% to 108%, and T3 corresponds to FVIII ≥108%. Model 1 was adjusted for age and sex. Model 2 was adjusted for age, sex, body mass index, high-sensitivity C-reactive protein, and platelet count.

AP, attributable proportion; FVIII, factor VIII; MPV, mean platelet volume; OR, odds ratio; RERI, relative excess risk due to interaction; T, tertile.

In the present study, we confirmed the previous findings on the prospective association between high FVIII levels and the risk of incident VTE in the general population [15–17]. Moreover, our results are consistent with the linear relationship between FVIII levels and VTE risk observed in several reports [9,13,17]. Because FVIII levels were dose-dependently associated with VTE risk in the present study, it was not surprising that FVIII levels were associated with increased risk of VTE even within the normal range, eg, at percentiles 33.3th (85%) and 66.6th (108%) of the distribution of the controls. In the Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis (MEGA) study, a large population-based case-control study, participants with FVIII antigen levels in the 25th to 50th percentile of the control distribution (vs those with FVIII antigen levels in the <25th percentile) were already at a significantly increased risk of VTE [13]. In the MEGA study, the VTE risk increased linearly up to the extreme levels of FVIII (ie, >99th percentile). Unfortunately, due to limited statistical power, the assessment of the association between extreme levels of FVIII and VTE risk was not feasible in our study. As FVIII is an acute phase protein [43], it is crucial to take inflammation into account when studying the association between FVIII and VTE. Here, we demonstrated that the association was not explained by inflammation as adjustment for high-sensitivity CRP had minor impact on risk estimates. Another relevant feature of our study is the fact that although the ORs for VTE by increased FVIII levels were higher with shortened time between blood sampling and VTE events (Figure 2), the strength of the association between plasma FVIII and VTE risk remained substantial even several years after blood sampling. Importantly, results from a Mendelian randomization study advocated a causal relationship between FVIII levels and VTE [44]. Taken together, these findings highlight that FVIII not only behaves as a robust short- and long-term biomarker of VTE risk in the general population but may also be causally related to VTE.

Interestingly, the association between plasma FVIII levels and VTE was mostly driven by the relationship with DVT, which is in agreement with results from a case-control study [13], but was not reported in previous prospective studies [15–17]. In a mouse model of venous thrombosis [5], authors observed increased embolization in VWF-deficient mice compared with that in wild-type mice. This was due to reduced FVIII levels since infusion of recombinant FVIII in VWF-deficient mice resulted in thrombus stabilization, with significantly less embolization. The critical role that activated FVIII plays for efficient propagation of the coagulation system and thrombin generation [8] is a plausible mechanism by which FVIII promotes thrombus stabilization, thus predominantly affecting DVT risk. It is worth noting that factor V Leiden (FVL) is also associated with a higher risk of DVT than of PE, a phenomenon known as the FVL paradox [45]. According to experimental data on mice, FVL carriers develop larger and more stable thrombi that are less likely to embolize compared with wild-type mice [46]. Because FVIII increases activated protein C resistance [47], the same phenotype associated with FVL, FVIII may also contribute to thrombus stabilization through activated protein C resistance.

To our knowledge, we are the first to investigate the combined effect of FVIII and MPV on VTE risk. Exposure to both high FVIII levels and high MPV resulted in a supra-additive effect on VTE risk,

with 52% of VTEs in the combined exposure group being attributed to the biological interaction between the 2 exposures. Our findings suggest that the risk of VTE conferred by high FVIII levels synergistically increases in the presence of large platelets, as determined by a high MPV, which have been shown to be more hemostatically active than small platelets [19–21]. Indeed, upon stimulation, large platelets are more prone to expose negatively charged phospholipids (phosphatidylserine in particular) on their membranes compared with small platelets [20]. Hence, large platelets may provide a more efficient surface for the assembly of activated FVIII and FIX, accelerating factor X activation and subsequent thrombin generation [8,48].

As FVIII circulates in a tight noncovalent complex with VWF [32], it might be argued that the interaction between high FVIII levels and high MPV could have been mainly driven by plasma VWF. We previously reported that high plasma VWF levels had a synergistic effect on VTE risk not only in the presence of high MPV but also in the presence of a high platelet count [49]. If the synergistic effect between FVIII and MPV on VTE risk were due to VWF, the combination of FVIII and platelet count would be expected to have a similar impact on thrombosis risk. However, there was no biological interaction between high FVIII and high platelet count (RERI and AP estimates were close to 0), making VWF an unlikely explanation for the synergism that we found between FVIII and MPV in relation to VTE risk. In light of these findings, one might speculate that the interaction between high FVIII levels and high MPV could be due to an increased exposure of negatively charged phospholipids on the surface of large platelets that would accelerate thrombin generation in the presence of high FVIII levels, ultimately resulting in a prothrombotic state and increased risk of VTE. However, it is important to address that because plasma levels of FVIII and VWF are closely related [32], any epidemiologic approach would be insufficient to reliably disentangle the effect of FVIII from VWF (and vice versa) on thrombosis risk. Experimental models of venous thrombosis conceived to fully disentangle the effect of these coagulation factors would be warranted to reveal to what extent high FVIII levels in combination with large platelets would affect thrombogenesis independent of VWF. Finally, platelet size, as reflected by MPV, is reported to have a strong genetic component [50,51] and to be relatively stable within an individual over time [52,53]. Whether the identification of subjects with high FVIII levels and high MPV could be useful for risk stratification and targeted VTE prevention, particularly in high risk situations for developing a VTE, is a further open question worth pursuing.

The main strengths of our study include the nested case-control study design, where the VTE cases and controls were selected from the same source population (ie, the Tromsø study cohort), thus mitigating the likelihood of selection bias. Because of the prospective design, where samples used to measure plasma FVIII levels and platelet parameters were collected at cohort baseline, we could make assumptions on the temporal sequence between exposure and outcome. Some limitations of this study merit attention. Although the number of plasma samples not available or of inadequate quality for the assessment of FVIII antigen levels was somewhat high, missing data on FVIII did not seem to be related to the VTE status, occurring in 21% of the VTE cases and 23% of the controls. Additionally, relevant baseline characteristics were similar for study participants with and without measurement of FVIII (data not shown). Thus, the missing data on FVIII was presumably completely at random. Blood samples were drawn in 1994 to 1995 and stored for >20 years before analyses, and this could potentially have affected FVIII levels. However, because blood samples were stored in the same way and for the same duration in cases and controls, any potential misclassification would be nondifferential with regards to VTE status, thereby introducing a possibility for underestimation of the true associations. Finally, prior studies have predominantly assessed FVIII activity, while in the present study, FVIII antigen levels were investigated since we only had stored EDTA plasma samples available in the parent cohort. It is important to note that FVIII activity and FVIII antigen levels have shown similar impact on VTE risk in previous studies [13,54].

In conclusion, increasing levels of plasma FVIII were linearly associated with increased risk of future incident VTE, and the association was particularly strong for DVT. We found a supra-additive effect of high FVIII levels and high MPV on the risk of VTE. Our findings suggest that large platelets, as reflected by a high MPV, interact biologically with high FVIII levels and play a role in the pathophysiological mechanism by which FVIII increases VTE risk. Future studies are needed to confirm our findings and unravel the mechanisms that underlie the interaction between large platelets and FVIII.

ACKNOWLEDGMENTS

The Thrombosis Research Center has received an independent grant from Stiftelsen Kristian Gerhard Jebsen (2014-2020).

AUTHOR CONTRIBUTIONS

E.-S.H. analyzed the data, interpreted the results, and drafted the manuscript. M.S.E. interpreted the results and revised the manuscript. P.A. and T.U. performed the laboratory analysis, interpreted the results, and revised the manuscript. J.-B.H. and S.K.B. designed the study, organized data collection, interpreted the results, and revised the manuscript. V.M.M. designed the study, interpreted the results, contributed to the manuscript draft, and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2023.06.022