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Role of Mannose-Binding Lectin and Complement Activation in Venous Thromboembolism

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List of papers

The thesis is based on the following papers:

I. Complement Activation Assessed by the Plasma Terminal Complement Complex and Future Risk of Venous Thromboembolism.
Høiland II, Liang RA, Brækkan SK, Pettersen K, Ludviksen JK, Latysheva N, Snir O, Ueland T, Hindberg K, Mollnes TE, Hansen JB
J Thromb Haemost. 2019;17:934-943.

II. Plasma levels of Mannose-Binding Lectin and Future Risk of Venous Thromboembolism.
Liang RA, Høiland II, Ueland T, Aukrust P, Snir O, Hindberg K, Brækkan SK, Garred P, Mollnes TE, Hansen JB
J Thromb Haemost. *2019;00:1-9.*

III. ABO Status Affects Plasma Mannose-Binding Lectin Levels and the Association Between MBL Levels and Risk of Venous Thromboembolism.
Liang RA, Hindberg K, Ueland T, Aukrust P, Snir O, Brækkan SK, Garred P, Mollnes TE, Hansen JB
Manuscript

Summary

The complement and coagulation systems are two interrelated plasma protein cascades. Evidence from observational and animal studies has proposed a role for the complement system in the development of venous thromboembolism (VTE), but the exact mechanisms remain obscure.

The aim of this thesis was to investigate whether activation of the complement system impacts the risk of VTE and to investigate which pathways may be involved. The procoagulant properties of the lectin pathway, in particular, demanded closer inspection. Genetic variations associated with plasma levels of complement protein or activation products were also explored to discover novel genetic regulators that could potentially contribute to thrombosis risk.

The fourth survey of the Tromsø Study was used as the parent cohort for all of the nested case-control studies discussed in this thesis. There were 462 individuals who experienced a VTE in the follow-up period (1994/95-2007). For each VTE case, two age- and sex-matched controls who were alive at the index date of the VTE event were randomly sampled from the source cohort.

The findings in this thesis support a role for the complement system in the development of VTE. Higher baseline levels of complement activation as measured by the soluble terminal C5b-9 complement complex (TCC) were associated with future risk of VTE, as were high levels of plasma mannose-binding lectin (MBL). The rs8176719 SNP of the *ABO* gene, which determines O and non-O blood types and is itself a known risk factor for VTE, was found to be significantly associated with high plasma MBL levels. Individuals with high plasma MBL levels and type O blood were found to have a similar risk for VTE as individuals with non-O blood type, regardless of plasma MBL levels.

These studies implicate that the lectin pathway is involved in the mechanisms leading to venous thrombosis. Functional studies are warranted. Additionally, the discovery of a relationship between blood type and plasma MBL levels requires further investigation, both alone and as a risk factor for venous thromboembolism.

Abbreviations

aHUS	atypical hemolytic uremic syndrome
APS	antiphospholipid syndrome
APC	activated protein C
BMI	body mass index
C	complement factor
CI	confidence interval
CRP	C-reactive protein
CTEPH	chronic thromboembolic pulmonary hypertension
CVD	cardiovascular disease
DVT	deep vein thrombosis
EDTA	ethylenediaminetetraacetic acid
EV	extracellular vesicle
F	factor
FVL	Factor V Leiden
GWAS	genome-wide association study
HMWK	high molecular weight kininogen
hs	high-sensitivity
MAC	membrane attack complex
MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MI	myocardial infarction
NET	neutrophil extracellular traps
OR	odds ratio
PAR	protease-activated receptor
PE	pulmonary embolism
PNH	paroxysmal nocturnal hemoglobinuria
PTS	post-thrombotic syndrome
PRM	pattern recognition molecule

pQTL	protein quantitative trait loci
PS	phosphatidylserine
Q	quartile
QoL	quality of life
rh	recombinant human
SLE	systemic lupus erythematosus
SD	standard deviation
SNP	single nucleotide polymorphisms
TAFI	thrombin-activatable fibrinolysis inhibitor
TCC	terminal complement complex
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TIA	transient ischemic attack
VTE	venous thromboembolism
vWF	von Willebrand Factor

1. Introduction

Venous thromboembolism (VTE) is a term that encompasses two serious conditions that are part of the same disease entity: deep vein thrombosis (DVT) and pulmonary embolism (PE). Deep vein thrombosis is a condition that occurs when a blood clot forms in the deep veins, most commonly in the lower extremities. Signs and symptoms of DVT include pain, redness, and swelling in the affected limb due to obstructed blood flow. Pulmonary embolism is a potentially life-threatening condition that occurs when a piece of a growing venous blood clot breaks off, travels through the bloodstream, and lodges in the narrow vasculature of the lungs. Though often assumed to be a sequela of DVT, up to 50% of PE have an untraceable origin [1]. It has been proposed that intracardiac thrombus formation as a result of atrial fibrillation is a source of up to 20% of PE [2], and other pathological mechanisms of PE may include local thrombus formation in the lung vasculature [1]. Symptoms of this serious condition include chest pain, tachypnea, dyspnea, and coughing. Severe cases can result in circulatory collapse and death.

VTE is a common disease with an annual incidence of 1-2 per 1000 persons in Western Europe [3, 4], but the mechanisms in the pathogenesis of VTE remain elusive. Immunothrombosis is thrombus formation as a result of the activation and interplay of several intravascular protein cascades: the coagulation, fibrinolytic, complement, and kinin-kallikrein systems [5].

Hemostasis is the physiological mechanism by which the body is able to stop bleeding at a site of injury. The process of hemostasis is divided into primary hemostasis, or the formation of a platelet plug, and secondary hemostasis, or the formation of a fibrin mesh that strengthens and stabilizes the plug and forms the resultant clot. Secondary hemostasis is a process controlled by the coagulation cascade, an ancient intravascular proteolytic cascade that ultimately results in the formation of a fibrin mesh. As the ability to stop bleeding is vital to survival, hemostasis must be tightly regulated to ensure effective clotting and limit bleeding, and just as importantly, to avoid inappropriate clotting. Thrombosis, or the pathological formation of clots in blood vessels in the absence of bleeding, can result from dysregulation and excessive activation of the coagulation system. Thrombosis is essentially “hemostasis in the wrong place and at the wrong time [6].”

At a site of open injury it is critical that the body protects against the introduction of foreign pathogens in addition to stopping bleeding. The complement system is an ancient protein cascade closely connected to the coagulation cascade. This mainly intravascular proteolytic system has a wide range of functions, including: integration of the innate and adaptive immune systems by clearing and lysing foreign pathogens, promotion of inflammation, contribution to normal development of tissues and organs, and promotion of tissue repair [7]. The activation of complement or other components of the intravascular innate immune system interact with and amplify each other to drive the thromboinflammatory process [8].

This thesis explores the role of the complement system in the development of venous thrombosis, and aims to unravel the potential role of mannose binding lectin (MBL) and the lectin pathway in the pathogenesis of VTE.

1.1. Epidemiology of venous thromboembolism

VTE is the third most common cardiovascular disease (CVD) following myocardial infarction and stroke [9], and is a major cause of morbidity and mortality around the world. The incidence of VTE is 1-2 per 1000 person-years [3, 4], and has remained stable [10] or even increased in the last 20 years [4, 11], in alarming contrast to the 25-40% decrease in incidence of arterial CVD over the same time period [12]. The incidence of VTE (both DVT and PE) increases exponentially with age for both sexes [13, 14]. While women have a twice higher incidence in childbearing years [13, 15], the incidence rate is higher in men of all ages when reproductive risk factors are taken into account [16]. People of different ethnic background have differing incidence rates of VTE, with African-American populations having the highest risk, especially for PE, and decreasing risk in those of Caucasian, Hispanic, and Asian/Pacific Islander descent, respectively [17].

VTE presents as deep vein thrombosis in approximately two-thirds of patients, while about one-third of VTE events present as pulmonary embolism [18]. However, this ratio is changing, with recent studies showing that the rate of PE has increased approximately 250% over the last two decades [4, 11], likely due to more frequent diagnosis by better imaging

techniques (e.g. computed tomographic pulmonary angiography) [11]. These studies also found that rates of DVT have decreased over the same time period.

Despite the improvements in diagnostic methods as well as in treatment of VTE, the rate of adverse events remains high. The 30-day case fatality rate for overall VTE was found to be between 6.4-10.6%, with 30-day case-fatality rates being roughly twice as high in PE patients than in DVT patients. This difference disappeared within one year [13, 19]. Several studies have found one-year case fatality rates to be between 21-24% [13, 19, 20], with rates significantly higher in cancer-related VTE (as high as 88%)[21].

VTE is a common and chronic disease that recurs in approximately 30% of patients within 10 years [14]. The highest risk of recurrence is between 6-12 months after the first event [22]. Patients with an initial DVT have a 1.4-fold higher risk of recurrence than those with a first PE [20]. Several studies have found that the initial disease usually recurs at the same site [20, 23], i.e. DVT tends to recur as DVT and PE recurs most commonly as PE. Unprovoked VTE events are more than twice as likely to recur than events provoked by transient risk factors [24].

As VTE is a chronic disease, patients can suffer from several long-term complications that constitute a major health burden for society. The sequelae of VTE include post-thrombotic syndrome (PTS) and chronic thromboembolic pulmonary hypertension (CTEPH). PTS presents with edema, swelling, chronic pain, and skin changes in the limb affected by DVT – severe cases can lead to leg ulceration [25]. Between 20-50% of DVT patients develop PTS [15, 25], with 5-10% developing severe PTS [26, 27], due to damage to the venous valves and subsequent venous hypertension and impaired venous return. Female sex, obesity, proximal DVT, recurrent DVT, and varicose veins are risk factors for PTS [26]. The Villalta score, a clinical severity scoring system for PTS, correlates well with both clinical measurements and patient-perceived quality of life (QoL) [28]. In addition to increasing healthcare costs due treatment and hospitalization, PTS also negatively affects QoL [29, 30]. Patients suffering from PTS reported a worse QoL compared to other chronic diseases, including angina, chronic lung disease, and osteoarthritis [31]. In a recent cohort of over 60,000 individuals of working age in the general population, VTE was associated with considerable work-related disability, with the risk of work-related disability in subjects with VTE being 62% higher when compared to individuals without VTE in a model adjusted for age

and sex [32]. Those with unprovoked VTE had a 52-67% higher risk of work-related disability, while it is notable that no association was found between provoked VTE and subsequent risk of disability [32]. DVT was associated with disability, while PE was not. This is likely attributable to the frequency of PTS and the rarity of CTEPH.

CTEPH is a rare complication of acute PE, affecting between 2-4% of PE patients [33]. Risk factors for CTEPH include young age, unprovoked PE, and recurrent VTE [33, 34]. Symptoms of CTEPH include fatigue, dyspnea, and chest discomfort, and in severe cases hemoptysis due increased pulmonary hypertension from vascular remodeling after thrombus organization [35]. CTEPH is a disease with progressive pulmonary hypertension and ultimately right heart failure, if untreated [35]. CTEPH is the most severe form of the post-pulmonary embolism syndrome, an increasingly recognized long-term complication of PE [36]. Recent studies suggest that post-PE syndrome affects almost half of PE patients, and these patients suffer an impaired QoL [37]. Post-PE syndrome manifests as dyspnea and exercise intolerance, along with diminished right ventricular function and perfusion defects. Currently, however, there are no clear diagnostic criteria for this condition.

1.2. Pathophysiology of venous thromboembolism

Already in the 1856, Rudolph Virchow proposed that there were three main factors involved in thrombus formation, which later became known as Virchow's Triad (Figure 1)[38]. These factors are changes in blood flow (stasis), changes in blood composition (hypercoagulability), and vessel wall injury. For over one and a half centuries, these three key elements have proven relevant and critical in the understanding of the pathogenesis of venous thrombosis. Established risk factors for venous thromboembolism can be classified under at least one element in Virchow's triad [39].

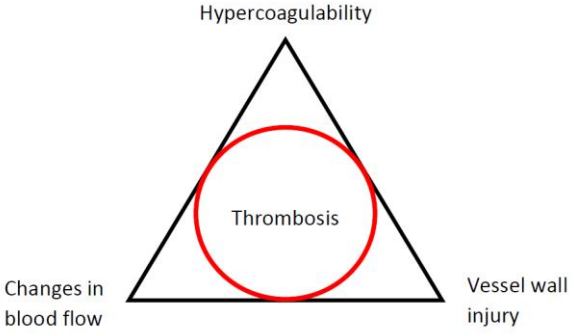

Figure 1. Virchow's Triad

Changes in blood flow (stasis), the composition of blood (hypercoagulablity), and vessel wall injury (endothelial dysfunction) were proposed by Rudolph Virchow to be important factors in thrombus formation.

As blood flows in the veins, it follows a low-pressure system back to the right heart. To counteract the effects of gravity, skeletal muscle contraction helps compress venous vessels, and backward flow is prevented by venous valves. From autopsy and phlebography studies, it has been established that the deepest recesses of the venous valves are the site of initiation of thrombus formation [40].

There are several changes that occur to blood flow in the areas around these valves. First, some blood begins vortical flow as it crosses the leaflets of the venous valve. Blood and its cellular components become trapped in the deepest recesses of the valvular sinuses (Figure 2). In this pocket of stasis, the cells are cut off from a fresh blood supply and a hypoxic environment begins to form. Hamer et al. showed that stasis resulted in severe hypoxia and low oxygen tension in the deepest recesses of the venous valves in immobilized dogs [41]. Indeed, this is the same location that had previously been described as the origin of venous thrombi found in human autopsy studies [40, 42]. Stasis in the venous valves is also evident from venography studies that observed contrast material lingers for up to an hour after the procedure in the elderly [43]. With age, veins lose their compliance and venous valves stiffen from fibrosis, causing disrupted blood flow. These physical changes in the veins likely contribute to the increased incidence of VTE with increased age [40].

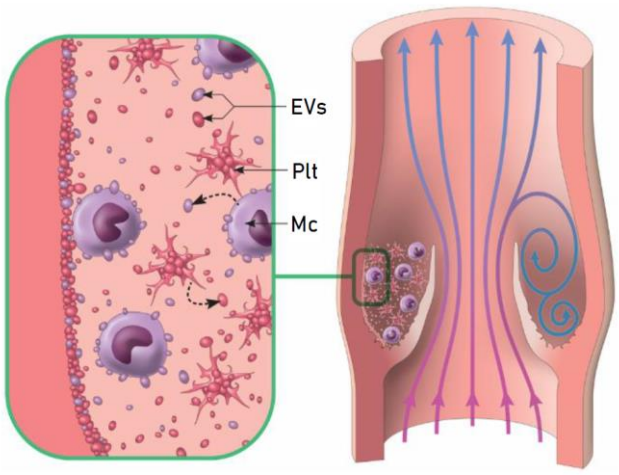

Figure 2. Venous valvular sinuses

Deep vein thrombosis has been shown to originate in the valvular pockets of the deep veins. The changes in blood flow due to the valves result in vortical flow and stasis. The lack of flow creates a hypoxic environment which activates the endothelium and the trapped cellular components, including leukocytes. Tissue factor (TF)-bearing monocytes (Mc) and extracellular vesicles (EVs) as well as activated platelets (Plt) are key factors in the initiation of thrombus formation.

The hypoxic milieu induces several stress responses within the trapped cells and affected endothelium. Hypoxia induces the formation of reactive oxygen species (ROS) and

activates oxygen dependent transcription factors hypoxia-inducible factor-1 (HIF-1) and early growth response-1 (Egr-1)[40]. These transcription factors upregulate a multitude of genes, including those responsible for glycolysis, vasomotor control, and angiogenesis [44]. Of note, Egr-1 has been found to upregulate tissue factor (TF) (a key initiator of the coagulation system) on monocytes following hypoxia [44].

Hypoxia also activates endothelial cells to express a proinflammatory and procoagulant phenotype (Figure 3). Activated endothelium releases Weibel-Palade bodies, which contains von Willebrand Factor (vWF) and several adhesion molecules. vWF binds platelets and thereby promotes platelet aggregation at the endothelial surface. P-selectin from Weibel-Palade bodies is exposed on the surface of the activated endothelium, and binds P-selectin glycoprotein ligand-1 (PSGL-1) expressed on circulating leukocytes and extracellular vesicles (EVs). The activated endothelium produces plasminogen activator inhibitor-1 (PAI-1) to inhibit fibrinolysis. It also produces ROS, which recruits and activates monocytes. No gross vessel injury has been found in autopsy studies on non-traumatic DVT [42], but activated endothelium represents stress and dysfunction in the lining of vessel walls.

The proteins and adhesion molecules expressed by activated endothelial cells in response to hypoxia (e.g. P-selectin, E-selectin, vWF) capture leukocytes, platelets, and EVs. Histological studies that venous thrombi form in alternating layers of red, fibrin-rich layers with multiple trapped erythrocytes and white, platelet-rich layers known as the lines of Zahn. The layer found immediately on the endothelial surface is fibrin-rich and red, and is indicative of TF-induced fibrin formation [40]. TF is expressed mainly on circulating monocytes and EVs [45, 46] and activates the extrinsic pathway of coagulation. Indeed, a recent study in mice elegantly showed the recruitment of TF-bearing leukocytes to the intact but stenotic vessel wall using in vivo microscopy in an inferior vena cava model [47]. The procoagulant environment of the venous valvular sinuses thus also represents the hypercoagulable element of Virchow's triad.

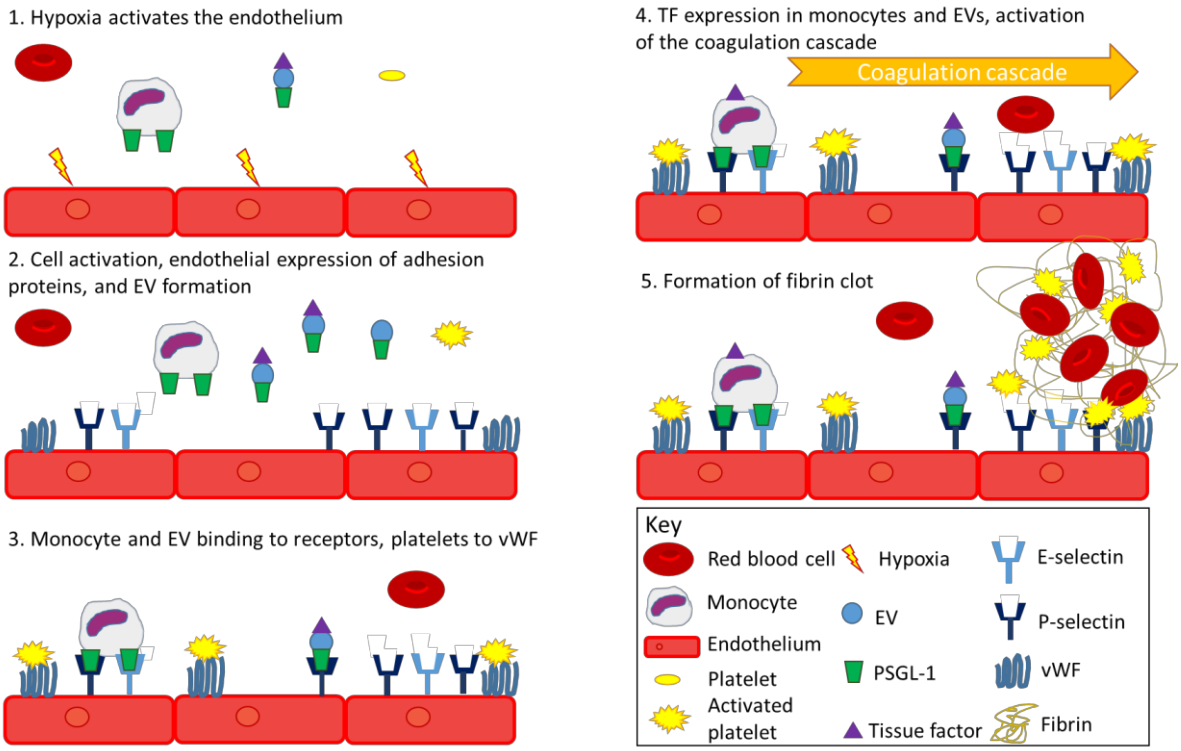

Figure 3. Pathophysiology of venous thromboembolism

Hypoxia in the venous valvular sinuses activates endothelial cells, which release von Willebrand factor (vWF) and adhesion molecules from Weibel-Palade bodies. The Weibel-Palade bodies then bring P-selectin and vWF to the endothelial surface, where they bind P-selectin glycoprotein ligand-1 (PSGL-1)-bearing leukocytes and extracellular vesicles (EVs) and platelets, respectively. Monocytes and EVs also carry tissue factor (TF) to the site, which initiates the clotting cascade and begins the formation of a fibrin-rich clot.

(Adapted from Mackman N. *New Insights into the mechanisms of venous thrombosis*. Journal of Clinical Investigation, 2012.)

1.3. Risk factors for venous thromboembolism

Venous thrombosis is a complex, multicausal disease with many risk factors [48]. These risk factors are classifiable by the elements of Virchow's triad [39, 48], but are usually classified as genetic or acquired risk factors. The presence of several risk factors in one individual is common, and often required before venous thrombosis manifests. These risk factors interact dynamically, and the additive and supra-additive effects of several risk factors is represented in the thrombosis potential model (Figure 4)[48]. In this model, VTE manifests when an individual's thrombosis potential exceeds the thrombosis threshold.

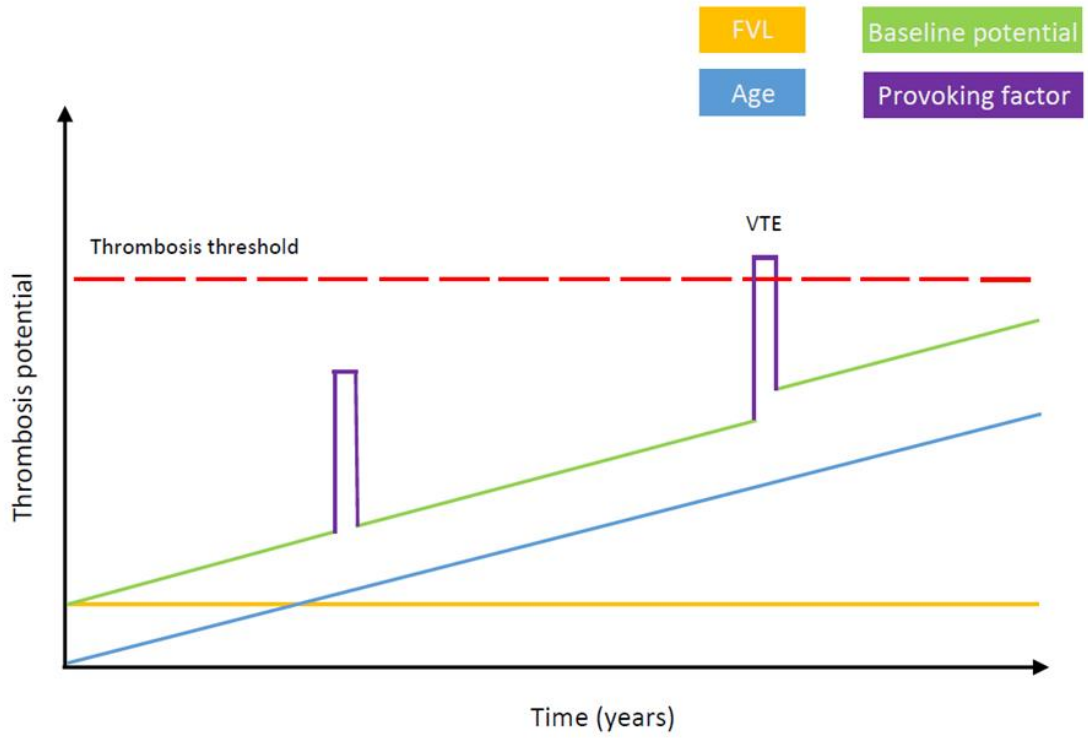

Figure 4. The thrombosis potential model

Individual thrombosis potential is influenced by both genetic and acquired risk factors. These risk factors interact dynamically and have an additive or even supra-additive effect. If several factors are present, the thrombosis potential may approach or exceed the thrombosis threshold, which leads to development of venous thromboembolism (VTE). In this example, the baseline thrombosis potential of the individual is elevated due to the presence of the Factor V Leiden mutation (FVL), and increases with age. Provoking factors at various time points additionally push the thrombosis potential toward the thrombosis threshold, until finally exceeding it, leading to a VTE. A provoking factor early in life may not cross the thrombosis threshold, but with increased age the same factor may cross the thrombosis threshold and provoke VTE.

(Adapted from Rosendaal FR. *Venous thrombosis: a multicausal disease.* The Lancet, 1999.)

1.3.1. Heritable risk factors

Heritable, or genetic risk factors, are major contributors to the risk of VTE. From family and twin studies the heritability of the disease has been estimated to be around 50-60% [49, 50]. Thrombophilia is a term indicating an increased genetic predisposition to VTE as a result of hypercoagulability [51].

Genetic defects in inhibitors of the coagulation pathway, including antithrombin, Protein C, and Protein S were among the first thrombophilic disorders identified. In 1965, Egeberg discovered that clustering of VTE events in a Norwegian family occurred due to a

partial antithrombin deficiency. Though very rare (<1% for all), deficiencies in these natural anticoagulants are strong risk factors for VTE, increasing risk 5- to 50-fold [50, 52, 53].

There are also several moderate genetic risk factors for VTE. Factor V Leiden (FVL) is a mutation in coagulation factor V. The prevalence of this mutation among Europeans is about 5%, and it gives a 2-4-fold increased risk of DVT [54, 55]. This mutation affects the activated Protein C (APC) cleavage site on Factor V, thus causing resistance to APC anticoagulation [55]. Interestingly, FVL seems to have a differential effect on DVT and PE, known as the "FVL paradox", giving an OR of 4.5 for DVT and OR 1.7 for PE [56, 57].

Prothrombin G20210A is the second most common inherited thrombophilia and occurs in about 2% of Caucasians [58]. It is another moderate risk factor for DVT, increasing the risk roughly 3-fold [59]. The prothrombin G20210A mutation is a gain-of-function mutation, giving 30% increased levels of prothrombin. The mutation also inhibits fibrinolysis through a thrombin-activatable fibrinolysis inhibitor (TAFI)-mediated mechanism, further contributing to thrombosis risk [60].

Although the risk of VTE conferred by non-O blood types is less than that of FVL or prothrombin G20210A, due to its high frequency in the population (60-70%), non-O blood type is the commonest genetic risk factor [61] with a population attributable risk of 20% [62]. Compared to type O blood group, B and A1 blood groups have a relative risk of about 2 for venous thrombosis [54, 63, 64]. ABO blood group determines plasma levels of vWF and FVIII. Plasma vWF levels are 25% higher in non-O individuals compared to those with type O [65]. Increased levels of vWF and FVIII are also known risk factors for VTE, even after adjusting for ABO blood group [66]. However, increased vWF/FVIII levels only account for part of the increased thrombotic risk, and the risk remains elevated for non-O blood groups even after accounting for vWF and FVIII [67, 68].

The fibrinogen gamma gene (FGG) encodes the fibrinogen γ chain. The γA chain is the main variant, but an alternatively spliced γ′ version also occurs. Patients with VTE were found to have reduced γA/γ′ fibrinogen levels and reduced γ′ fibrinogen/total fibrinogen ratios [54, 69]. Decreased γ′ fibrinogen levels in the FGG-H2 haplotype give an approximately 1.45-fold increased risk of VTE [69]. Studies suggest that the γ′ fibrinogen chains contain binding sites for FXIII and thrombin that ultimately inhibit thrombin [69]. Fibrin clots formed

from γA/γ′ fibrinogen contain finer chains and more extensive cross-linking than γA/γA fibrinogen [70]. These changes along with the observed resistance to fibrinolysis likely contribute to the increased risk of thrombus formation in individuals with low γ′ fibrinogen levels [69].

Many genetic factors for VTE have been discovered since the 1990s. Genome wide association studies (GWAS) have identified several single nucleotide polymorphisms (SNPs) associated with VTE [50, 71]. As of 2015, 17 VTE-associated genes have been discovered [50]. Some of these new SNPs are novel and surprising, as they do not belong to the known coagulation pathways nor have they been previously associated with other CVD [72, 73]. These SNPs provide new areas to investigate in the pathophysiology of VTE. De Haan et al. proposed a genetic risk score using SNPs in the criteria for a risk prediction model for VTE [74]. Risk scores incorporating risk alleles of 31 SNPs performed similarly to a model incorporating five SNPs most strongly associated with VTE. The model was further improved by combining genetic and non-genetic risk factors [74]. Discoveries based on whole exome and whole genome sequencing studies hold promise in determining novel risk prediction models in the future [50].

1.3.2. Acquired risk factors

There are several acquired risk factors for VTE. Some of these environmental factors are considered provoking factors, and can be transient or persistent risk factors [75].

Age is one of the strongest risk factors for VTE. The incidence of VTE increases exponentially with age [13, 14], with the risk of VTE being 50- to 80-fold higher in the elderly [76]. Studies have suggested that between 78% [76] and 90% [77] of the population attributable risk of VTE is due to age. Blood becomes more hypercoagulable with age, due to an increase in procoagulant factors without a concomitant increase in natural anticoagulants [78, 79]. Additionally, body mass index (BMI) increases with age [80], while activity decreases and immobilization increases due to illness, infection, and co-morbidity [81]. Fibrotic changes that stiffen the venous valves predispose to stasis, while inactivity and loss of muscle mass also contribute to reduced venous return and stasis in the lower extremities.

Obesity, or a body mass index above 30 kg/m^2, is an important modifiable risk factor for venous thrombosis. The risk of VTE increases linearly with BMI [82], and obese individuals have a 2 to 3-fold higher risk of VTE compared to persons of normal BMI [3, 83]. Obesity was found to account for 33% of incident unprovoked VTE [10]. Weight gain has also been described as increasing the risk for VTE [84]. Waist circumference has been proposed as a better anthropomorphic measure of obesity as it is associated with the highest risk of VTE, and identifies the most people at risk [85]. Visceral adipose tissue found in abdominal obesity is metabolically active and releases a plethora of proinflammatory and procoagulant substances [86, 87]. Though the exact mechanisms are unknown, it is likely that raised intraabdominal pressure and reduced venous return may play a role in thrombogenesis in the obese. Recent Mendelian randomization studies have found that there is a causal relationship between BMI and VTE [88-90].

Cancer is a major provoking risk factor for VTE, increasing the risk between 4- and 7-fold [91]. It is estimated that 20-30% of VTEs are associated with cancer [91], and in many cases VTE is often the first manifestation of cancer. Indeed, up to 10% of patients are diagnosed with cancer within a year after an unprovoked thrombotic event [92]. The risk of VTE varies according to the type of cancer, with hematological, lung, brain, gastrointestinal tract, and pancreatic cancers having the highest incidence of VTE [93]. Malignancy often produces a hypercoagulable state, due to the expression of TF by cancer cells and release of procoagulant TF-bearing EVs into the bloodstream [94]. Inflammation and leukocytosis, and possibly the presence of neutrophil extracellular traps (NETs) also likely contribute to the procoagulant environment bestowed by cancer. Apart from the cancer itself, hospitalization and treatment regimens including surgery, chemotherapy, and the use of central venous catheters also increase the risk of VTE in cancer patients [95, 96]. Prothrombotic genotypes also influence the risk of VTE in cancer, and individuals with FVL, prothrombin G20210A and non-O blood types have increased risks of cancer-related VTE [97]. These risks were often supra-additive. To illustrate, it was found FVL mutation gave a 3-fold higher risk of VTE, cancer gave a 5-fold risk, and FVL and cancer together gave a 12-fold increased risk [97, 98].

Major general surgery is also an established provoking factor for VTE, giving up to 22-fold increased risk [95, 99]. The highest risk surgeries were major orthopedic surgery, neurosurgery, and major vascular surgery [100]. The increased risk for VTE is a result of the

inflammatory response and hypercoagulable state that develops during the postoperative period [101-103]. Trauma is also an important risk factor, giving an over 12-fold increased risk [95]. It is estimated that without thromboprophylaxis up to about 58% of trauma patients may suffer from DVT [104].

Acute medical conditions are also independent risk factors for VTE. Arterial **cardiovascular disease** including myocardial infarction (MI) [105, 106] and ischemic stroke [106, 107] are associated with increased risk of VTE in the general population, especially during the first few months following an acute cardiovascular event. The risk of VTE was almost five-fold higher in subjects having suffered an MI compared to subjects without MI during the first 6 months after diagnosis of MI [105]. Moreover, it was found that over 72% VTE events were attributable to an MI, while almost 79% of PE were attributable to MI [105]. Ischemic stroke was associated with a 3-fold higher risk of VTE compared to individuals without stroke, with the greatest risk being 20-fold higher in the first month after stroke [107]. Several diseases that have a longer course are also associated with an increased risk of VTE. Congestive heart failure [108] reflects a condition in which all components of Virchow's triad are fulfilled, thereby conferring an increased risk for both DVT and PE [109]. Lung disease including chronic obstructive pulmonary disease (COPD) [110] or respiratory failure [93] and rheumatologic disorders [111] such as systemic lupus erythematosus are also risk factors for VTE.

Immobilization is another recognized risk factor for VTE, conferring a 2- to 5-fold increased risk of VTE [112, 113]. There is a bidirectional relationship between immobilization and infection, with infection causing bed-rest and immobilization, and immobilization predisposing to infections such as pneumonia [114]. A recent case-crossover study in the general population found that immobilization led to an OR of 72.5 for VTE, and this risk almost doubled when infection was also present [115]. **Infection** is also a known risk factor for both DVT and PE [116].

Pregnancy and **oral contraceptives** are also risk factors for VTE. Among the many changes with pregnancy is a transient hypercoagulable state due to increased coagulation factors FVII, FVIII, FX, fibrinogen, vWF and PAI-1 [117, 118]. Compared to women who are not pregnant, pregnant women have a 4- to 5-fold increased risk for VTE, while the risk during the postpartum period is 20-fold increased [117, 119]. For women taking oral

contraceptives, the risk for VTE is transiently increased (in the first year) about 2- to 4-fold [120]. In individuals with FVL mutation who were taking hormonal contraceptives the risk increased to 36-fold [120], showing the strong synergistic effect of multiple risk factors on overall thrombotic risk.

1.4. The coagulation system

The coagulation system is a proteolytic cascade that culminates in the formation of fibrin and the activation of platelets, which form the blood clot. The coagulation system is comprised of two pathways: the extrinsic/tissue factor pathway and the intrinsic pathway. These two pathways merge into one common pathway to form thrombin, which cleaves fibrinogen to give fibrin (Figure 5). While the extrinsic pathway is involved in normal hemostasis, it is believed that both pathways contribute to pathological thrombosis [121].

The **extrinsic pathway** is also known as the tissue factor (TF) pathway. TF is an integral membrane protein expressed by extravascular cells, including pericytes, vascular smooth muscle cells, and adventitial fibroblasts within the walls of blood vessels [45]. It is also expressed by monocytes under pathological conditions. TF serves a critical role in hemostasis, underscored by the fact that a deficiency in humans has never been discovered, and murine studies have shown that TF-deficiency is incompatible with life [45]. Extravascular TF is exposed upon vascular injury and binds circulating coagulation factor (F)VII and the miniscule amount (~1%) of circulating activated FVII(a) with high affinity [122]. Upon complexing with TF, the inactive zymogen FVII is rapidly converted to FVIIa. Negatively charged phospholipids such as phosphatidylserine (PS) are a prerequisite for FIX and FX binding to cell surfaces as well as the decryption of TF, which expresses binding sites of the TF:FVIIa complex for its substrates [121, 123]. The TF:FVIIa complex then activates its substrates FIX and FX to FIXa and FXa, respectively [124]. Small amounts of thrombin are formed as a result, which then activates cofactors FV and FVIII. The clotting cascade then continues to propagate when FIXa and FXa assemble on appropriate membrane surfaces together with their respective cofactors (FVIIIa and FVa, respectively). Binding of these coagulation factors to PS on activated platelets or damaged cells immensely enhances the reaction rate of proteolytic conversion of zymogens to active serine proteases, thereby

amplifying the reaction [125]. Indeed, in the absence of appropriate lipid membranes, most coagulation enzymes show little activity with their substrate in a biologically relevant time frame. The prothrombinase complex, FXa and cofactor FVa, activates prothrombin to thrombin. Thrombin is another key component of the coagulation cascade, and has key roles in amplification of the clotting cascade and platelet activation. With formation of large amounts of thrombin, fibrinogen is cleaved to fibrin monomers. These fibrin monomers polymerize spontaneously and FXIIIa, also activated by thrombin, cross-links fibrin chains to stabilize the clot [126].

The **intrinsic pathway** of the coagulation cascade is activated in the absence of TF. It is triggered upon contact of plasma factor XII with a number of negatively charged artificial surfaces. There are also several proposed activators *in vivo*, such as platelet-derived polyphosphates [127] and extracellular nucleic acids [128, 129]. Contact with an activator results in a conformational change in FXII that produces small amounts of FXIIa. A positive feedback loop starts when FXIIa activates prekallikrein (PK) to kallikrein, which activates FXII in a reciprocal manner. FXIIa further activates FXI to FXIa. FXIa converts FIX to FIXa, which forms the tenase complex with its cofactor FVIIIa [130]. This activates FX to FXa in the final common pathway of coagulation to produce thrombin and finally, fibrin. Interestingly, the intrinsic pathway does not seem to contribute significantly to hemostasis *in vivo.* Unlike TF, FXII is not “essential” to life, and neither mice nor humans who are deficient for FXII have defects in hemostasis [131]. Several studies have implicated a role for FXII and the intrinsic pathway in thrombosis [132, 133], and have proposed both FXII and FXI as potential therapeutic targets [134].

The coagulation cascade should be considered in context of the cell-based model of hemostasis. In this model, there are three key phases of hemostasis: initiation of coagulation by TF-bearing cells, amplification on platelet surfaces, and propagation on activated platelets [135]. Fibroblasts, pericytes, activated endothelial cells, and monocytes are the main cells that express TF. Extracellular vesicles (EVs), a term encompassing exosomes and microvesicles/microparticles, are membranous structures 30-1000 nm in diameter derived from cells. Exosomes are vesicles secreted from the cell, while microvesicles (formerly called microparticles) bud off from the membrane of cells. EVs are found in biological fluids and have been linked to important physiological and pathological processes. These include the

elimination of waste products as well as intercellular communication and signaling through exchange of nucleic acids, lipids, and proteins expressed on or carried in EVs [136]. The surface markers, proteins, and nucleic acids expressed by EVs are derived from the parent cells, and thus EVs are heterogeneous and have varied biological activity [137]. EVs are an important source of TF as well as negatively charged phospholipids such as PS, and are thus highly procoagulant [128].

Upon vessel wall injury, TF-expressing extravascular cells bind FVII in plasma, forming the TF:FVIIa complex and initiating the extrinsic pathway of the coagulation cascade. The small amount of thrombin produced activates platelets, which adhere to the site of injury and set the stage for the amplification of coagulation by binding cofactors FVa and FVIIIa to their surfaces. The tenase (FVIIIa/IXa) and prothrombinase (FXa/FVa) complexes then assemble on platelet surfaces and produce large amounts of thrombin in the propagation phase. Thrombin converts fibrinogen to fibrin, forming a fibrin clot.

Coagulation must be kept tightly regulated, as excessive clotting is harmful to the host. Tissue factor pathway inhibitor (TFPI) directly inhibits FXa and thus inhibits the prothrombinase (FXa/FVa) complex. It also inhibits the TF:FVIIa complex mostly through feedback inhibition [138]. The importance of TFPI is underscored by the finding that mice deficient in TFPI die *in utero* and humans deficient for TFPI have never been reported, strongly suggesting that TFPI deficiency is incompatible with life [139]. Another important natural anticoagulant is antithrombin, which primarily inhibits FXa and thrombin of the common pathway, as well as the TF:FVIIa complex in the presence of heparin [140]. Protein C is another natural anticoagulant. Activated protein C (APC) inactivates FVa and FVIIIa when bound to its cofactor protein S [141]. As discussed previously, antithrombin, protein C, and protein S deficiencies are genetic risk factors for development of VTE [50, 52].

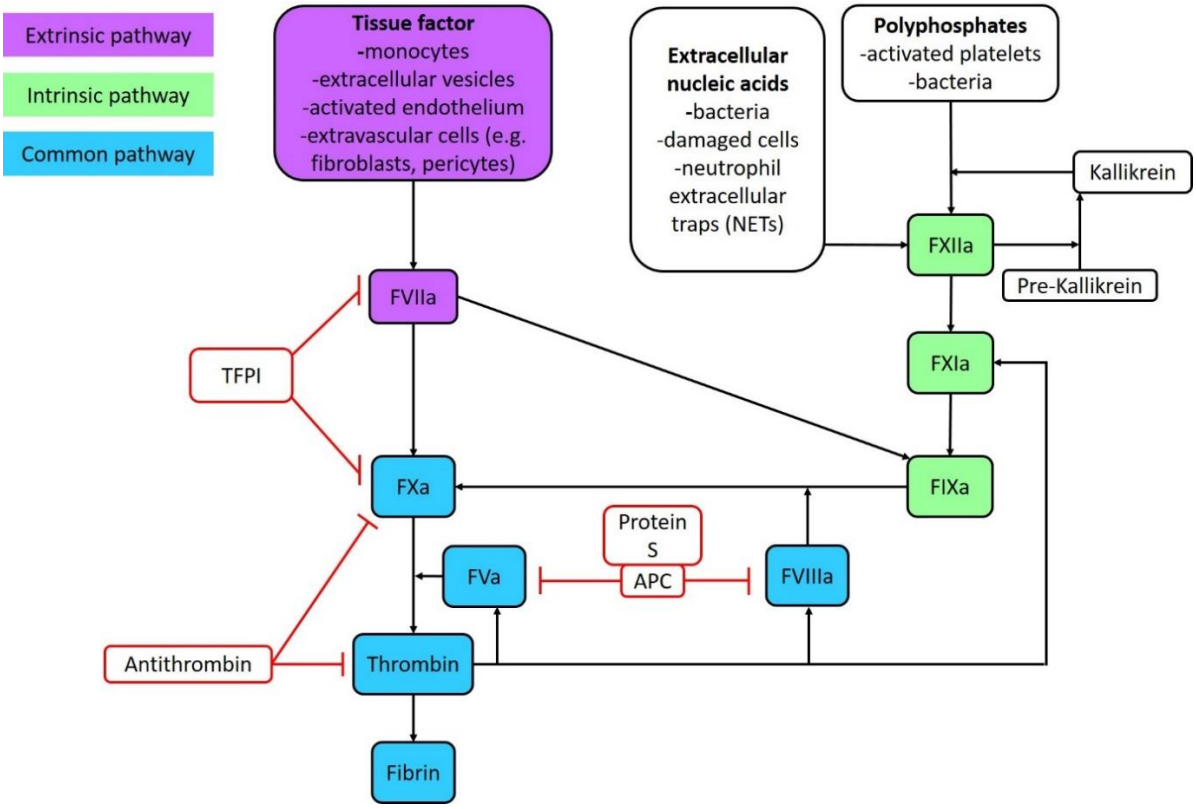

Figure 5. A simplified overview of the coagulation system

The extrinsic pathway is activated by tissue-factor (TF)-bearing monocytes, extracellular vesicles (EVs), and possibly activated endothelial cells. The intrinsic pathway is activated *in vivo* by polyphosphates released from platelets or invading bacteria as well as extracellular nucleic acids. These pathways converge at the common pathway to form thrombin, and ultimately, fibrin.

(Adapted from Mackman N. *New Insights into the mechanisms of venous thrombosis*. Journal of Clinical Investigation, 2012.)

1.5. The complement system

The complement system is an ancient network of over 50 proteins with pleiotropic functions in innate immunity, a first line defense against pathogens. It also plays an important role in development and organogenesis, the clearance of tissue debris, apoptotic cells and immune complexes, and the promotion of tissue repair following injury, and is thus critically important to the preservation of homeostasis [7]. Traditionally, it has been viewed as a blood-based system, but recent research points to there being an intracellular system as well [142]. There are three pathways with distinct triggers that converge at complement component 3 (C3): the classical, lectin, and alternative pathways (Figure 6). Complement also has key roles in communication between innate and adaptive immunity by substantially

potentiating the antibody [7, 143]. It is not surprising, therefore, that dysfunction of the complement system can transform it into a pathological effector rather than one of homeostasis. Indeed, complement has been found to be involved in a number of immune, inflammatory, and other diseases [144].

The classical pathway is initiated by activation of the pattern recognition molecule (PRM) C1q. C1q recognizes antibodies (the Fc portion of IgM and IgG clusters) as well as pentraxins (such as C-reactive protein) and distinct structures on microbial and apoptotic cells [145, 146]. C1r and C1s are subsequently activated upon C1q binding to a recognized pattern [146]. C1s thereby cleaves C4 into C4a and C4b. C4a has been found to act as an untethered ligand for protease-activated receptors (PAR)1 and PAR4, and thereby activates the endothelium [7]. C4b binds covalently to surfaces near the activation sites in a process called opsonization. C2 binds to C4b and is cleaved by C1s to C2a and C2b [147]. Thus, C1s is involved in the generation of the C3 convertase (C4b2b), which cleaves C3 and initiates the common complement pathway. Complement effector functions are produced from this point and downstream.

The lectin pathway is initiated in a similar way to the classical pathway. Mannose-binding lectin (MBL)[148], ficolins, and collectins are PRMs that bind to specific carbohydrate patterns found on microbes and damaged host cells. Upon binding to an appropriate surface pattern, the PRM activates MBL-associated serine proteases (MASPs) to which the PRMs are in complex with [149]. MASP-2 cleaves both C4 and C2, and is thus capable of generating C3 convertase [8] and initiating the common complement pathway.

The alternative pathway of complement is activated by spontaneous hydrolysis of a small fraction of C3 molecules in a ‘tick-over’ mechanism for constant surveillance [150]. This hydrolysis exposes new binding sites on $C3H_2O$, to which factor B (FB) can bind. Factor D (FD) then cleaves FB to Bb and Ba, generating the C3 convertase of the alternative pathway $C3(H_2O)Bb$. C3b produced by the C3 convertases of the other pathways can also bind FB, forming C3 convertase C3bBb [146]. Like the C3 convertases of the classical and lectin pathways, these C3 convertases cleave C3 to C3a and C3b. The C3b molecule contains a temporary thioester moiety which tags foreign cells by reacting with carbohydrates on foreign microbes but not host cells. Properdin (factor P) can associate with and stabilize C3bBb convertase by forming the C3bBbP complex, which slows its deactivation [151]. The

alternative pathway has an important role in the amplification loop in complement activation. Amplification through the alternative pathway makes up to 80-90% of total complement activation and thereby produces the majority of C3b and C5b-9 regardless of the initiating pathway [152, 153].

Regardless of initiation pathway, generation of C3 convertases leads continuation of the common pathway as well as amplification of the alternative pathway [154]. The anaphylatoxins C3a and C5a are produced in the common pathway and have important effects in proinflammatory signaling and phagocytosis [155]. The anaphylatoxins are potent chemoattractants that lead neutrophils and monocytes to the site of complement activation and promote phagocytosis. Activation of the common pathway of complement culminates in the formation of the terminal complement complex (TCC) C5b-9. The TCC forms when C5b associates with C6, C7 and this lipophilic complex inserts into a cell membrane, after which it associates with C8 and then binds multiple C9 units. When TCC assembles on a membrane surface it is called the membrane attack complex (MAC). Sublytic C5b-9 does not lyse cells, but form small pores that induce proinflammatory responses via calcium influx [156]. When complement activation occurs in the fluid phase, TCC forms in a soluble form (sC5b-9). While sC5b-9 still has no known biological function [157], it is measurable in plasma and as such is an invaluable tool to monitor complement activation [158].

Like the coagulation system, the complement system must be tightly regulated to avoid pathological activation. Complement regulators exist in both soluble and cell-bound form. C1-esterase inhibitor (C1-INH) inhibits several proteases in the classical (C1r and C1s) and lectin pathways (MASP-1 and -2)[159], as well as the coagulation and fibrinolytic cascade [160], thus it is not a specific complement inhibitor. sMAP and MAP-1 are splice products of the *MASP2* and *MASP1* genes and are competitive inhibitors of MASPs as they bind to MBL and ficolins [8, 161]. Factor H (FH) is the main inhibitor of the C3 convertase of the alternative pathway [162]. Factor I cleaves C4b and C3b to give inactivated products (e.g. iC3b, C3c, and C3dg) that are unable to assemble into C3 convertases [163]. C4b-binding protein (C4BP) is a soluble regulator that binds to C4b and thereby inhibits the classical and lectin pathways [164]. Finally, regulators of the convertases accelerate their enzymatic decay and include CR1 (CD35), decay accelerating factor (DAF/CD55), and membrane cofactor

protein (MCP/CD46). CD59 is a cell-based regulator that prevents formation of sublytic and lytic TCC complexes on surface membranes [165].

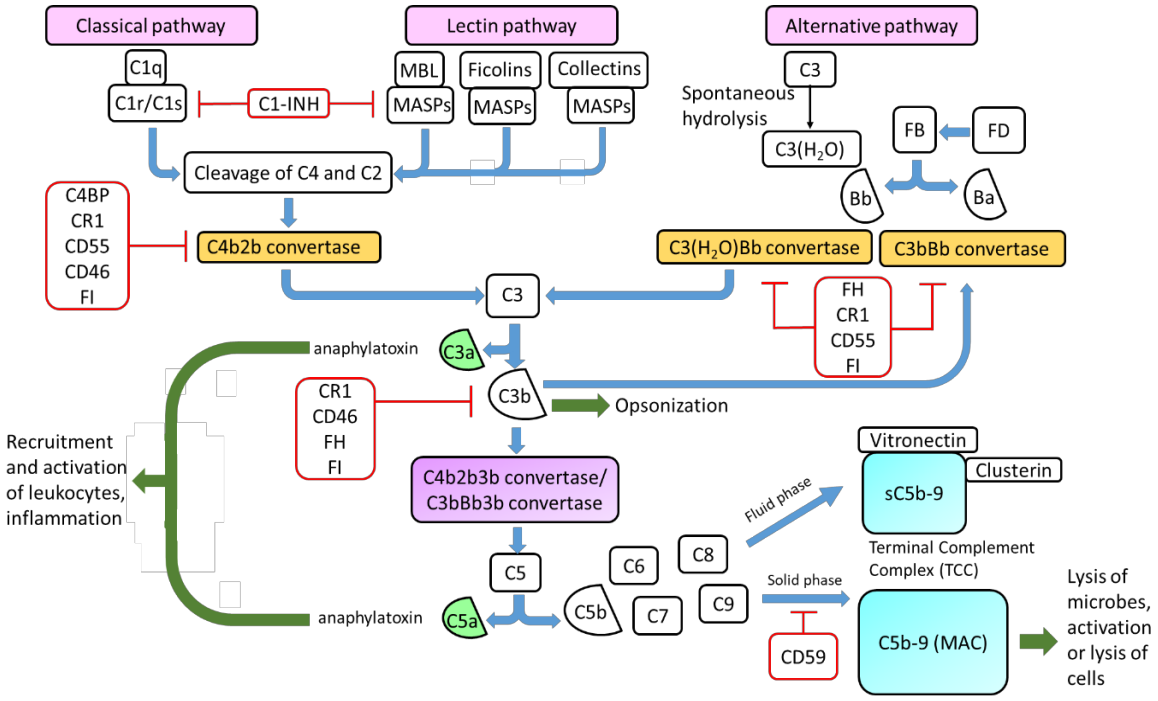

Figure 6. A simplified overview of the complement system

The complement system is comprised of three activation pathways: the classical, lectin, and alternative pathways. These pathways converge at complement factor C3. The final common pathway produces several important effector molecules, including anaphylatoxins, opsonins, and the terminal complement complex (TCC), which can be either soluble (s)C5b-9 or the membrane attack complex (MAC).

(Adapted from Poppelaars F. *The complement system in dialysis: a forgotten story?* Frontiers in Immunology, 2018.)

1.5.1. The lectin pathway of complement

The lectin pathway is the most recently discovered pathway of the three activation pathways of the complement system. PRMs of the lectin pathway (e.g. MBL, ficolin-1, ficolin-2, ficolin-3, and collectin-10, collectin-11, and collectin-12) bind to carbohydrate patterns on pathogenic microorganisms and apoptotic cells. These PRMs are specific to steric and spatial organization of carbohydrates, thus preventing the erroneous recognition of healthy self-cells [166]. MBL, ficolins, and collectins circulate in complex with MASPs, which mediate

complement activation through generation of the C3 convertase upon binding of the PRM on a carbohydrate surface.

MASP-1 autoactivates upon PRM binding, and in turn activates MASP-2 [167] to cleave C4 and C2 to form the C4b2a convertase [8]. MASP-1 is also able to cleave C2, but not C4. Complexes can cross-activate MASPs in nearby complexes [149]. Though the function of MASP-3 has remained obscure after its discovery, recent evidence suggests MASP-3 may be involved in alternative pathway activation as it can cleave Factor D [168]. There are also two non-catalytic, alternatively spliced products of the *MASP1* and *MASP2* genes, MAP-1 and sMAP, respectively [8]. Although most MASPs are associated with PRMs of the lectin pathway, some MASPs circulate unbound as well [169]. In addition to cleaving complement components, MASP-1 and -2 have been shown to have loose substrate-binding specificities that overlap with thrombin, therefore giving these proteins a role in coagulation. MASP-1 is an especially promiscuous protein, shown to cleave fibrinogen, FXIII, and prothrombin [170]. It also activates TAFI and inhibits fibrinolysis. In plasma systems, MASP-1 promoted the formation of a loose clot with thick fibrin fibers in a thrombin-dependent manner [171]. MASP-2 has also been shown to cleave prothrombin to thrombin [172]. The procoagulant activity of the lectin pathway is discussed in more detail later.

1.5.2. Mannose-binding lectin

Mannose-binding lectin (also called mannan-binding lectin) was the first pattern recognition molecule discovered in the lectin pathway. It is a calcium-dependent C-type lectin that recognizes carbohydrate moieties such as mannose and N-acetyl-D-glucosamine on membranes of pathogens and damaged or apoptotic host cells. MBL initiates the lectin pathway of complement.

The *MBL2* gene is found on chromosome 10q11.2-21. Plasma MBL levels vary greatly and are largely determined by polymorphisms of the *MBL2* gene. There are four exons within the protein-encoding region of *MBL2*, and base substitutions in exon 1 at codon 52, 54, or 57 cause changes to amino acids resulting in lowered plasma MBL levels and decreased function [169]. Individuals of the same genotype can have MBL levels that differ 10-fold [173]. Protein expression of MBL is also dependent on polymorphisms of the

promoter region of the gene, thus leading to a wide range of plasma levels of MBL in individuals even of the same haplotype [174]. An alternative promoter region 1kb upstream of exon 1 of *MBL2* has been suggested to affect MBL expression as well, albeit to a much smaller degree (up to 15%) [175]. Though these polymorphisms account for most of the interracial variation in MBL concentrations, there is still a significant and yet unexplained variation in circulating MBL levels [169]. Therefore, it is likely that there are additional epigenetic or hormonal factors that affect plasma MBL levels [169].

Although there is a great degree of inter-individual variation in plasma MBL levels, an individual's MBL levels in plasma are quite stable throughout life [176]. Age and diurnal variation do not appear to affect MBL levels, nor do moderate exercise [177] or weight loss [178].

It is estimated that between 5 and 20% of the population is "MBL-deficient" with levels below 100 ng/mL, though the cut-off level is disputed due to high rates of haplotype variation [179-181]. MBL-deficiency has been associated with several diseases, including a number of infectious diseases, autoimmune disorders and CVD [176, 182, 183]. Infants and children with MBL-deficiency were found to have defects in opsonization and suffer from frequent infections [176]. Low MBL levels have been shown to be associated with rheumatoid arthritis, systemic lupus erythematosus (SLE), and celiac disease, and in general predict a poor prognosis [176].

1.6. Crosstalk between the coagulation and complement systems

Dysregulation of complement can lead to several clinical disorders, including atypical hemolytic-uremic syndrome (aHUS) and paroxysmal nocturnal hemoglobinuria (PNH). aHUS is most commonly caused by either genetic mutations in or autoantibodies produced against Factor H, a key regulator of the C3 convertase of the alternative pathway. This results in chronic and excessive complement activation. Patients with aHUS suffer from hemolytic anemia, thrombocytopenia, and acute renal failure [184]. PNH is caused by a mutation affecting glycosylphosphatidylinositol anchors on complement regulatory proteins CD55 and CD59, leading to decreased expression of these proteins on cell membranes. This results in excessive complement activation. Intravascular hemolytic anemia and red urine are the main

signs of PNH. Both disorders are also characterized by thrombosis, manifesting as thrombotic microangiopathy in aHUS and as thromboembolism in PNH, highlighting the interaction between complement and coagulation [184].

Indeed, the recently described concepts of immunothrombosis [5] and thromboinflammation [8] reflect the idea that thrombus formation is the result of the activation and crosstalk between coagulation and components of the immune system. The coagulation and complement systems are linked at several points.

The coagulation system has been found to interact with the complement system. Thrombin was found to cleave C5 to biologically active C5a and C5b independently of C3 and the C3 convertase [185, 186]. (This has recently been challenged by our group (unpublished data) since the experiments were done in purified, non-physiological conditions.) Additionally FXa, plasmin, thrombin, FIXa, and FXIa were able to cleave both C3 and C5 to produce functional anaphylatoxins in a descending order of enzymatic activity for C3 cleavage [187]. A fragment of FXIIa can activate the classical pathway [188].

In parallel, the complement system has several procoagulant effects. C5a induces TF expression on endothelial cells [189]. The anaphylatoxins induce platelet activation and aggregation [190], through which C3b can bind to P-selectin and assemble the C3 convertase [191]. Sublytic concentrations of C5b-9 also activate platelets and expose procoagulant lipids such as PS on the surface of platelets and endothelial cells that are necessary for the assembly of the prothrombinase complex [192, 193]. Sublytic C5b-9 complexes also induce the expression of adhesion molecules on endothelial cells and platelets, and induce the secretion of vWF and proinflammatory cytokines. Platelet-derived EVs expressing CD55, CD59, clusterin, P-selectin and MAC are also released from activated platelets [194].

Another interesting area of coagulation-complement crosstalk is through vWF. Ultra large multimeric strings of vWF released from Weibel-Palade bodies in activated endothelial cells function as a binding surface for C3b, thereby promoting assembly of the C3 and C5 convertases of the alternative pathway [195]. Plasma vWF also binds C3b, but acts as a cofactor for C3b cleavage by factor I, thus degrading C3b to inactive iC3b and inhibiting complement activation [196].

The MASPs represent another important bridge between complement and coagulation (Figure 7). MASP-1 is a rather promiscuous protease, activating prothrombin [171], TAFI, fibrinogen, and FXIII [185]. MASP-1 not only has thrombin-like activity in cleaving FXIII and fibrinogen, it can also activate endothelial cells through PAR4 [197]. MASP-2 is also able to cleave prothrombin [172]. Both MASP-1 and -2 are efficiently inhibited by antithrombin in the presence of heparin [198] and α2-macroglobulin [199], though the physiological role of α2-macroglobulin on MASP inhibition has been questioned [200]. Although MASPs have procoagulant effects, they are less efficient in cleaving prothrombin [172, 201], fibrinogen [201, 202], and FXIII [201] than the native coagulation factors (FXa and thrombin, respectively) and therefore their biological role in coagulation are likely supplementary rather than redundant [169]. This supporting role is confirmed by a study showing that MASP-1 is unable to initiate the clotting cascade [171], but plays a role in amplification processes. Another study showed both MASP-1 and -2 are activated by activated platelets and fibrin formation *in vitro* and *in vivo*, and thereby likely contribute to the amplification of thrombosis [203].

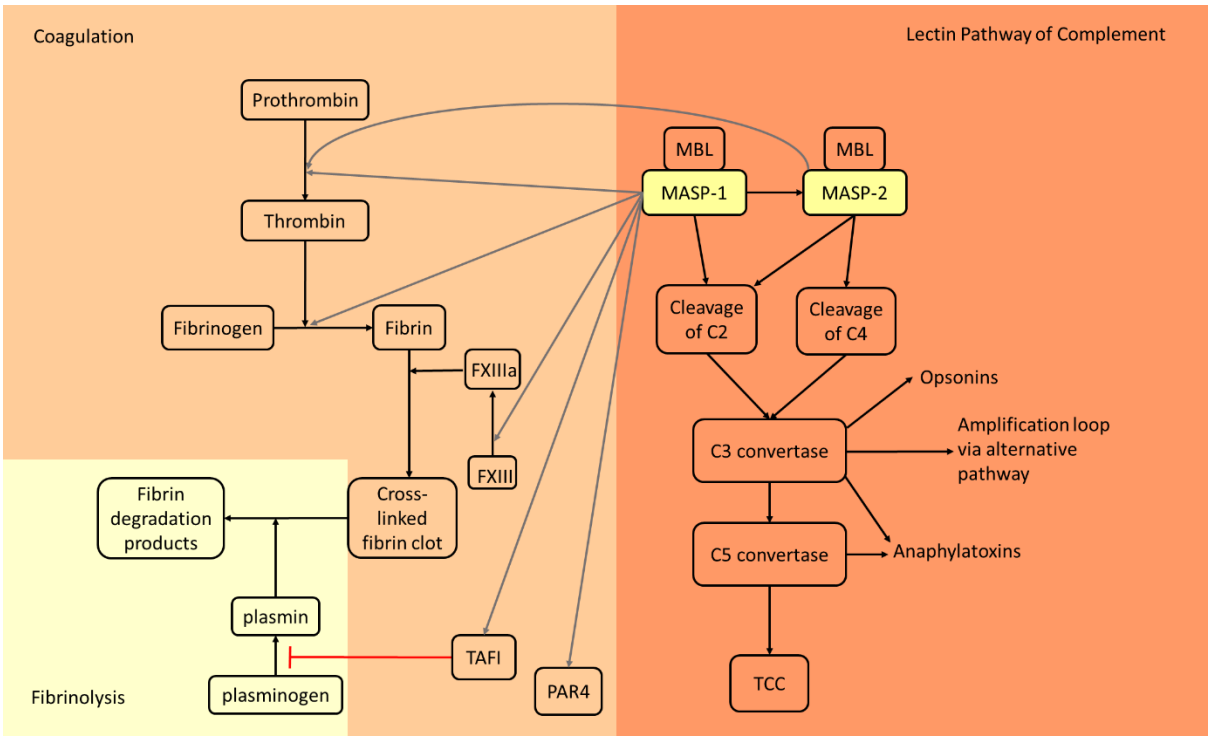

Figure 7. Crosstalk between MASPs and the coagulation system

MBL-associated serine protease (MASP)-1 and -2 cleave prothrombin. MASP-1 has thrombin-like activity and cleaves FXIII, TAFI, and fibrinogen. MASP-1 also activates PAR4 on endothelial cells.

(Adapted from Garred P. *A Journey through the lectin pathway of complement- MBL and beyond.* Immunological Reviews, 2016.)

1.7. Complement and the risk of venous thromboembolism

Few studies have investigated the role of complement in the development of venous thromboembolism. Given the extensive crosstalk between the complement and coagulation systems, as well as the role of complement in arterial CVD, it stands to reason that complement activation also plays a role in the development of VTE.

Complement factor deposition on platelets has been investigated as a potential player in the pathogenesis of VTE [204]. Systemic lupus erythematosus (SLE) patients with a history of VTE were found to have increased deposition of C1q, C3d, and C4d on platelets compared to SLE patients without VTE. The researchers concluded that classical pathway activation may be involved in the pathogenesis of VTE in SLE patients.

Previously only two studies have investigated the effects of MBL on risk of VTE. A longitudinal study on *MBL2* genotypes in SLE patients showed that although MBL alleles conferring deficient phenotypes had an increased risk for arterial thrombosis compared to those with normal alleles, there was no association between genotype and risk for VTE [205]. In a cross-sectional study on SLE patients, Font and colleagues found that individuals with *MBL2* genotypes giving low plasma MBL levels had an increased risk of VTE events than those with genotypes giving clinically normal MBL levels [206].

These studies have implicated the involvement of complement in thrombogenesis, but the mechanisms remain elusive. More research is needed to clarify the role of complement activation in the development of VTE, including determining which pathways are involved in clot formation. Additionally, the association between MBL and risk of VTE needs to be investigated in the general population. New insights into the pathological mechanisms of the disease are paramount in the improvement and development of novel preventative and therapeutic measures of VTE.

2. Aims of the thesis

The aims of the thesis were:

- To investigate whether complement activation, as measured by plasma levels of the terminal complement complex (TCC), is associated with future risk of VTE in a nested-case control study (Paper I).

- To investigate whether low plasma mannose-binding lectin (MBL) levels are associated with risk of venous thromboembolism (VTE) in a population–based nested case-control study (Paper II).

- To investigate whether high plasma levels of MBL were associated with genetic variants that could influence the association between plasma MBL levels and the risk of VTE (Paper III).

3. Methods

3.1. Study populations

3.1.1. The Tromsø Study

The Tromsø Study is a single center, population-based cohort with repeated health surveys of the inhabitants of the municipality of Tromsø in northern Norway. In total, seven surveys have been conducted, the first in 1974, and most recently, the seventh in 2015/16. The primary aim of the cohort was originally to investigate the causes of the unusually high cardiovascular mortality in the region and to find ways to prevent arterial CVD such as myocardial infarction (MI) and stroke [207]. The cohort has since been invaluable in investigating a broader range of chronic diseases and conditions.

The fourth survey of the Tromsø Study (Tromsø 4) was conducted in 1994/95. All inhabitants 25 years old or older living in the municipality of Tromsø were invited to participate in the survey. Of those, 27,158 (77%) participated in the study and were followed from date of inclusion (1994/95) until migration from the region, death, or end of follow-up (September 1, 2007). Baseline information was collected through self-administered questionnaires, blood sampling, and physical examinations in all surveys of the Tromsø Study. The questionnaires assessed the participant's health and lifestyle habits and provided information about dietary habits, smoking and alcohol consumption, physical activity, use of oral contraceptives or hormone replacement therapy, and diseases such as CVD and diabetes. Trained personnel conducted physical examinations on the participants. Height and weight measurements were taken in subjects wearing light clothing and no shoes. Body mass index (BMI) was calculated by weight in kilograms (kg) divided by height in meters (m) squared (kg/m^2). Blood samples were drawn from an antecubital vein in a non-fasting state and were analyzed in the Department of Clinical Chemistry at the University Hospital of North Norway (UNN). Blood cell counts were assessed from blood collected in 5mL vacutainer tubes (Becton Dickinson, Meylan Cedex, France) containing the anticoagulant EDTA (K_3-EDTA 40µL, 0.37mol/L per tube) and were analyzed by an automated blood cell counter (Coulter Counter®, Coulter Electronics, Luton, UK) within 12 hours. DNA samples were isolated from blood and were stored at the National CONOR Biobank.

3.1.2. Outcome measurements and validation

Participants in Tromsø 4 were followed from date of inclusion (1994/95) until VTE event, migration from the region, death, or the end of the study period (September 1, 2007). As UNN is the only hospital that serves the municipality of Tromsø, it provides all hospital-based and outpatient medical care in the region, including diagnosis and treatment of VTE. All VTE events during follow-up were identified using the following registries at UNN: the hospital discharge registry, the radiological procedure registry, and the autopsy registry. International Classification of Diseases, revision 9 (ICD-9) codes 325, 415.1, 452, 453, 671.3, 671.4, and 671.9 were used to identify VTE events that occurred between 1994 and 1998, while revision 10 (ICD-10) codes I26, I80, I82, I67.6, O22.3, O22.5, O87.1, O87.3 were used for the period 1998 until 2007. After identification of events, trained personnel who were blinded to the baseline variables of the patients reviewed medical records for every potential case of VTE for validation. VTE cases from the hospital discharge registry and radiological procedure registry were recorded if all of the following criteria were fulfilled:

1) Clinical signs or symptoms of a DVT or PE, or both, were present
2) the VTE event was objectively confirmed by an objective diagnostic procedure (i.e. compression ultrasound, venography, computed tomography (CT), perfusion-ventilation scan (VQ-scan), pulmonary angiography, or autopsy)
3) a physician noted the diagnosis of DVT or PE in the patient's medical record
4) the patient was given therapy for VTE (i.e. anticoagulant medication, thrombolysis, vascular surgery) unless specifically contraindicated

VTE cases from the autopsy registry were recorded if VTE was indicated as the cause of death or was noted as a significant condition contributing to death in the autopsy report.

VTE events were classified as either a DVT or PE. If both DVT and PE were present, the event was recorded as a PE. Each VTE event was further classified as provoked or unprovoked according to the presence or absence of factors provoking VTE at the time of diagnosis. An event was classified as provoked if at least one of the following were present: active cancer, trauma or surgery within the previous eight weeks, acute medical conditions (major infection, acute MI, or stroke), immobilization (bedrest $\geq$ three hours, wheelchair use,

or long-haul travel ≥ four hours in the last two weeks prior to the event), or any other factor specifically described in medical records to be provoking.

3.1.3. Study design

A nested case-control study conducted with participants from Tromsø 4 (1994/95-2007) was used in all papers in this thesis. During the follow-up period, 462 individuals experienced a VTE event. Two age- and sex-matched controls who were alive at the index date were randomly sampled from the source cohort for each VTE case (n=924). A number of individuals (47 cases and 76 controls) did not have plasma of sufficient quality for laboratory measurements of TCC and were therefore excluded. In Paper I, the final study included 415 VTE cases and 848 controls. The aim was to investigate the association between plasma levels of TCC and future risk of incident VTE.

In Paper II, we used the same nested case-control study from Tromsø 4. A total of 45 cases and 75 controls did not have plasma samples of sufficient quality for laboratory analysis of MBL levels. The study therefore included 417 VTE cases and 849 age- and sex-matched controls, all of whom were followed until the date of adjudicated VTE event, migration, death, or end of follow-up. The aim of the study was to investigate whether there was an association between plasma MBL levels and future risk of VTE.

In Paper III, we used the same nested case-control study as in papers I and II. Plasma samples were of insufficient quality in 45 cases and 75 controls, and were therefore excluded from this study. This study included 417 VTE cases and 849 controls. Additionally, whole exome sequencing was only available in a subgroup of this population (355 VTE patients and 354 controls). We conducted a protein quantitative trait loci (pQTL) analysis using whole exome sequencing data to find potential genetic variants that are associated with high MBL levels (highest quartile from our previous study in paper II).

3.2. Laboratory measurements

Non-fasting blood was drawn into 5mL vacutainer tubes (Becton Dickinson, Meylan Cedex, France) containing the anticoagulant EDTA (K_3-EDTA 40µL, 0.37mol/L per tube) in the

Tromsø 4 study. Platelet-poor plasma was then prepared by centrifugation at 3000x*g* for 10 minutes at room temperature, and the supernatant was then transferred into cryovials (Greiner Laboratechnik, Nürtringen, Germany) in 1mL aliquots and stored at -80°C until further analysis. In all analyses, platelet-poor plasma samples were thawed in a water bath at 37°C for 5 minutes and then centrifuged at 13000x*g* for 2 minutes to obtain platelet-free plasma.

In Paper I, plasma TCC levels were measured using an in-house enzyme-linked immunosorbent assay (ELISA) as described in detail previously [208]. ELISA plates (Nunc, Immunoplate II, Copenhagen, Denmark) were coated with monoclonal antibody aE11 specific for a neoantigen exposed in the poly-C9 unit after formation and incorporation into the TCC. Biotinylated anti-C6 monoclonal antibody (9C4) was used as the detection antibody and streptavidin horseradish peroxidase (GE Healthcare UK) was added in the final step. The optical density was measured at 405 nm (Dynatech MR580, Dynatech Laboratories Inc., Alexandria, VA, USA). Results are presented in Complement Arbitrary Units (CAU)/mL. The intra-assay coefficient of variation was negligible, and the inter-assay coefficient of variation was 10.3%. The limit of detection was 0.3 CAU/mL.

In Paper II and III, plasma levels of MBL were measured using an enzyme-linked immunosorbent assay (Bioporto Diagnostics A/S, Hellerup, Denmark) according to the manufacturer's instructions. Briefly, samples were brought to room temperature and 100 µL of diluted plasma were added to each microwell pre-coated with monoclonal antibody against MBL and allowed to incubate for 60 minutes on a shaking platform. After addition of 100 µL biotinylated MBL antibody and incubation under shaking for 60 minutes, the wells were washed. 100 µL HRP-conjugated streptavidin was added to each well and allowed to incubate for 60 minutes on the shaking platform before washing. Finally, 100 µL chromogenic substrate (TMB) was added to each well and allowed to develop for 15 minutes in the dark before the reaction was stopped chemically and the plates were read at 450 nm in a plate reader. The intra-assay coefficient of variation is between 3.8-5.5%. The limit of detection was 5 pg/mL.

In all papers in this thesis, plasma levels of CRP were measured using an in-house enzyme-linked immunosorbent assay (CRP antibody: DY1707, R&D). Briefly, samples were brought to room temperature and pipetted in duplicates using a SELMA pipetting robot

(Jena, Germany) and a BioTek dispenser/washer (EL406, Winooski, USA). Commercially available reagents were used for enzyme-immunoassay (R&D Systems, USA) in a 384 format. An EIA plate reader (Synergy H1 Hybrid, BioTek, USA) was used with absorption at 450 nm and wavelength corrected to 540 nm. The intra- and inter-individual coefficients of variation were 2.6% and 9.1%, respectively. The limit of detection was 0.1 mg/mL.

In Paper III, we used the same measurements for MBL and CRP as in paper I. In addition, genotyping of DNA for the non-O vs. O blood group SNP (rs8176719) was performed using the Sequenom platform. Molecular mass of the extended primers were measured by single-base extension followed by mass spectrometry. DNA (10-20 ng) isolated from whole blood was genotyped with the Sequenom iPlex Gold Assay according to the recommended protocol and analyzed by the MassARRAY Analyzer 4. Only genotypes with a high quality score (A. conservative or B. moderate) were used. Non-O blood type was assigned to individuals with 1 or 2 alleles for the rs8176719 SNP, and type O was assigned to individuals with no alleles. Whole exome sequencing at high-coverage (≈100x) was performed in a subgroup of the nested case-control study (355 VTE patients and 354 controls) using the Agilent SureSelect 50 Mb capture kit.

3.3. Statistical analyses

All statistical analyses were conducted using STATA version 15 (StataCorp LLC, Texas, USA) or R version 3.4.4 (The R Foundation for Statistical Computing c/o Institute for Statistics and Mathematics, Wien, Austria).

In Paper I, plasma TCC levels were categorized according to quartile cut-offs in the control population (≤0.80, 0.80-1.04, 1.04-1.40, >1.40 CAU/mL). Descriptive statistics were used to calculate means and proportions of baseline characteristics. Logistic regression models were used to estimate odds ratios (OR) of VTE with 95% confidence intervals (CI) according to quartiles of TCC levels. The lowest quartile of TCC was used as the reference group. The *p*-value for linear trend was calculated across increasing quartiles of TCC, as well as the OR per 1 standard deviation (SD) increase in TCC.

In Paper II, plasma MBL levels were categorized according to quartile cut-offs in the control population (<435, 435-1367, 1367-2422, ≥2423 ng/mL). Descriptive statistics were

used to calculate the means and proportions of baseline characteristics across quartiles of MBL levels. Logistic regression models were used to calculate ORs of VTE and subgroups with 95% CI across quartiles of MBL, using the highest quartile of MBL as the reference group. We also calculated the *p*-value for linear trend in VTE risk across decreasing quartiles of MBL.

In Paper III, plasma MBL levels were categorized into quartile cut-offs in the control population (<435, 435-1367, 1367-2422, ≥2423 ng/mL). The lower three quartiles (Q1-3) were then merged to produce two groups for analysis: participants with low (merged quartiles Q1-3) and high (Q4) MBL levels. Descriptive statistics were used for calculation of means and proportions of baseline characteristics in the low and high MBL groups.

The pQTL analysis of whole exome sequencing data including 1,034,910 genetic variants. The commonly used significance threshold of $5x10^{-8}$ was used to adjust for multiple testing. Plasma MBL levels were divided into low and high categories (outcomes), with the lower three quartiles defined as the low MBL category and the highest quartile making up the high MBL category. The pQTL analysis was performed using EPACTS (Efficient and Parallelizable Association Container Toolbox) software edition 3.3.0 (University of Michigan, 2016). Within EPACTS, we used the logistic regression Wald test adjusted for age, sex, BMI, and VTE status to test for associations between high and low MBL levels (outcome) and allele counts of different genetic variants modelled as a linear variable.

Logistic regression models were used to estimate ORs with 95% CI for VTE according to blood groups (type O blood group and non-O blood group). Finally, ORs with 95% CI for VTE and subgroups were estimated using logistic regression for combinations of MBL and blood groups (low/high MBL and O/non-O blood group) using the low MBL / O blood group as the reference group. Sensitivity testing was also conducted to eliminate the possibility that co-morbidities that may be triggers for VTE influenced our findings. MI, stroke, infection, or cancer that occurred within 3 months prior to VTE event were excluded from our analysis.

4. Main results

4.1. Paper I

Complement activation assessed by the plasma terminal complement complex and future risk of venous thromboembolism

Observational and animal studies have suggested that components of the complement system (e.g. C3 and C5) are involved in the pathogenesis of venous thromboembolism (VTE). However, it remains unknown whether there is an association between plasma levels of the terminal complement complex (TCC) and future risk of incident VTE.

We aimed to investigate whether plasma TCC levels were associated with risk of incident VTE in a nested-case control study and to explore whether there were any genetic variants associated with plasma TCC using protein quantitative trait loci (pQTL) analysis of exome sequencing data.

Plasma TCC levels from 415 subjects with VTE and 848 age- and sex-matched controls from the population-based cohort (the Tromsø Study) were measured using ELISA. Whole exome sequencing using the Agilent SureSelect 50Mb capture kit was performed on 709 individuals (355 VTE patients and 354 controls). Logistic regression models were used to estimate odds ratios (OR) with 95% confidence intervals (CI) across quartiles of plasma TCC levels.

The risk of VTE increased across quartiles of plasma TCC, especially for unprovoked VTE (*p* for trend 0.02). Individuals with plasma TCC levels in the highest quartile (>1.40 CAU/mL) had an OR 1.33 (95% CI: 0.96-1.86) for overall VTE and OR 1.72 (95% CI: 1.08-2.75) for unprovoked VTE compared to individuals with plasma TCC levels in the lowest quartile (<0.80 CAU/mL) in a logistic regression model which adjusted for age, sex, and body mass index. The OR for VTE increased with shortened time between blood sampling and VTE event. There was no significant association between genome-wide or complement-related gene variants and plasma TCC levels. Our findings imply that plasma TCC levels are independent of gene regulation and that high plasma TCC levels are associated with risk of VTE, particularly unprovoked VTE.

4.2. Paper II

Plasma Levels of Mannose-Binding Lectin and Future Risk of Venous Thromboembolism

Mannose-binding lectin (MBL) is a C-type lectin that binds to repetitive carbohydrate patterns such as mannose on damaged host cells. The binding of MBL to such repetitive patterns activates the lectin pathway of the complement system. Individuals who are clinically deficient in MBL (<100ng/mL) have a high prevalence of associated diseases, including various types of infectious diseases, autoimmune diseases, and arterial cardiovascular disease.

We aimed to investigate the association between plasma levels of MBL and risk of VTE. We conducted a nested case-control study derived from the general population (the Tromsø Study), with 417 VTE cases and 849 age- and sex-matched controls who were alive at the index date of the VTE event. Plasma MBL levels were measured using ELISA. Logistic regression models were used to estimate odds ratios (OR) and 95% confidence intervals (CI) for VTE across quartiles of plasma MBL levels.

Individuals with plasma MBL levels in the lowest quartile (<435 ng/mL) had a decreased OR for overall VTE (OR 0.79, 95% CI: 0.56-1.10) and deep vein thrombosis (DVT) (OR 0.70, 95% CI: 0.47-1.04) compared to individuals with plasma MBL levels in the highest quartile (>2423 ng/mL) after adjusting for age, sex, body mass index, and C-reactive protein in the regression model. The ORs decreased substantially with decreasing time between blood sampling and VTE event for VTE, DVT, and pulmonary embolism (PE).

Our findings suggest that low plasma levels of MBL are associated with a reduced risk of VTE, and especially DVT.

4.3. Paper III

ABO Status Affects Plasma Mannose-Binding Lectin Levels and the Association Between MBL Levels and Risk of Venous Thromboembolism

The lectin pathway of the complement system is a potentially procoagulant pathway. Our aims in this study were therefore to investigate whether high plasma MBL levels were associated with any genetic variations that could influence the association between plasma MBL levels and risk for VTE.

We performed a nested case-control study with 417 VTE patients and 849 age- and sex-matched controls from a population-based parent cohort (The Tromsø Study). Plasma MBL levels were measured using ELISA. Whole exome sequencing was performed using the Agilent SureSelect 50Mb capture kit on 709 subjects (355 VTE patients and 354 controls) and the data was used in a protein quantitative trait loci (pQTL) analysis. Logistic regression models were used to estimate odds ratios (OR) and 95% confidence intervals (CI) for VTE and subgroups across groups combining MBL levels and ABO blood groups.

The pQTL analysis found that the rs8176719 SNP of the *ABO* gene, which differentiates between type O and non-O blood groups, was significantly associated with high plasma MBL levels. Mean plasma MBL levels were 40% higher in individuals with non-O blood type than in individuals with type O blood (1842±1614 ng/mL vs 1318±977 ng/mL, respectively, $p=2.8\text{x}10^{-8}$). Individuals with type O blood and high plasma MBL levels had increased ORs for VTE, DVT, and PE compared to individuals with low MBL levels in the same blood group. High MBL levels did not have an additional impact VTE risk in individuals with non-O blood type.

Our findings suggest that ABO blood group is partly responsible for determining plasma MBL levels, and individuals with high plasma MBL levels and type O blood have increased risk for VTE, with ORs of similar magnitude to individuals with non-O blood types.

5. General discussion

5.1. Methodological considerations

5.1.1. Study design

All the papers in this thesis used data derived from the Tromsø Study, a population-based cohort study. A **cohort** study follows a defined population from inclusion until the outcome of interest [209]. In the studies in this work, participants in The Tromsø Study were followed until VTE or death, migration from the municipality, withdrawal from the study, or end of the study period. Exposure status was recorded at inclusion in the study. Blood samples were also taken at inclusion in the study. Relative risk for VTE, the probability of developing DVT or PE, when comparing different groups with different exposures can be estimated using the cohort study design. The prospective nature of these studies makes them useful in studies investigating risk factors or the natural history of a given disease [210]. Other strengths of cohort studies are large participant pools and long follow-up times. Additionally, the cohort design allows for determination of both absolute risk and relative risk. The clear temporal relationship between exposure and outcome is a strength of cohort studies, and the most important prerequisite for causality. Hill's guidelines suggest other criteria that should also be considered for causality, including experimental evidence, the strength of the association, dose-response, biologic plausibility, and consistency across studies [211]. Challenges with the cohort study design include selection bias, confounders, and bias due to differential loss to follow-up, which will be discussed in the next section [209].

Case-control studies have a retrospective design, where a group with the outcome of interest is compared with a group without the outcome. The exposure information is acquired for both groups, and an odds ratio is estimated for the proportion of cases with the exposure compared to the proportion of controls with the same exposure. This study design is cost-efficient because the known outcome requires a smaller sample size. However, case-control studies are subject to selection bias, recall bias, and confounding (discussed in following sections) [209]. Another potential problem with case-control studies is **reverse causality**. Reverse causality is a type of temporal bias that occurs when the observed association is the opposite of the hypothesized causal relationship (i.e. the outcome of interest causes changes in the exposure of interest) [211].

Papers I-III utilize a **nested case-control study** design. Nested case-control studies are case-control studies carried out within a cohort, and thereby share features and advantages of both cohort and case-control studies [211]. All participants in this type of study (cases and controls) are derived from the parent cohort. In our studies, two age- and sex- matched controls alive at the index date of VTE event were sampled from the parent cohort for each VTE case to reduce confounding and to ensure that there were no losses to follow-up. The nested case-control study design was chosen for our investigations primarily because it is efficient with regards to time and costs, and the prospective temporal sequence between the exposure and outcome of the parent cohort study is preserved. Additionally, the random sampling of two matched controls from the source population that the cases were derived from increases the likelihood that our controls are representative of the general population. Moreover, there is better control for confounding [209, 211]. In our studies, we used logistic regression models and adjusted for known confounders to estimate ORs for VTE and subgroups by plasma levels of TCC or MBL.

5.1.2. Bias

A systematic error in the design of a study resulting in incorrect estimates of a true association between exposure and outcome is termed **bias**. Bias is commonly classified as selection bias or information bias.

Selection bias is a result of systematic errors in the recruitment or retention of participants in a study [211]. Cohort studies are less likely to be affected by selection bias, as all participants (both cases and controls) are included in the study before the outcome of interest occurs. Despite this, cohort studies may be prone to a particular type of selection bias called **non-response bias** (also known as participation bias), where participants differ from non-participants [212]. Individuals who choose to participate in cohort studies may be healthier and more health conscious than non-attendees. Individuals who are institutionalized or immobilized due to illness or disease are unlikely to attend health surveys. In general, those who do not participate in an epidemiological study are more likely to be men, unmarried, at extremes of age (younger, older), have less education, and be of lower socioeconomic status than those who choose to participate [212]. Tromsø 4 recruited

77% of the eligible population (all individuals in the general population aged 25 and older) [207]. Indeed, it has been found that subjects who did not attend the Tromsø Study tended to be <35 years old or >80 years old, single, and have a higher proportion of men than attendees [207].

In all papers in this work, controls were randomly sampled and matched with cases on age and sex to minimize potential differences in the exposure groups. The average age of individuals suffering a VTE event in these three papers was 67 years old, which is similar to the average of VTE found in other cohort studies in Scandinavian populations [213]. There were an equal proportion of men and women experiencing VTE in all papers, as has been previously established by other studies [213] . Finally, the proportions of DVT and PE (62% and 38%, respectively) were comparable to previous studies [18]. The cases in our nested-case control studies are therefore representative of individuals with VTE in the general population, giving them a high external validity. Controls were randomly sampled from the same parent cohort (Tromsø 4), and matched to cases on age and sex, as well as time in the study.

The generalizability of a study is the extent to which the results can be applied to a defined population. This is also known as the external validity of a study [214]. Tromsø 4 had a 77% participation rate among those eligible [207]. The participants included a higher proportion of women and individuals between the ages of 35 and 80 years than in the non-participant group [207], and the generalizability is therefore decreased in individuals belonging to subgroups representing non-attendees (i.e. men, <35 and >80 years old). Inhabitants of Tromsø municipality are predominantly Caucasian, with a Sami minority. The homogeneity of the study population thus likely makes it comparable to other Caucasian populations. It is less clear whether findings in the Tromsø Study are generalizable to different ethnic populations, as the incidence of VTE [17] and the gene polymorphisms for the *MBL2* [215, 216] and *ABO* [217] genes differ between ethnicities.

The internal validity of a study is the extent to which the observed results reflects the true association in the population observed. Randomized control trials are the gold standard of study design to minimize effects of bias and confounding and maximizing internal validity. Despite this, the strict inclusion and exclusion criteria also limits the external validity of these

studies [209]. The high participation rate (77%) of our population-based cohort study increases its external validity.

In paper I, a similar proportion of cases and controls (47 (10%) and 76 (8%), respectively) did not have plasma samples of sufficient quality to analyze for TCC levels. Similarly, in papers II and III, 45 (10%) cases and 75 (8%) controls lacked plasma of sufficient quality for analysis of MBL. These individuals were excluded from the studies. The proportion of missing data was low in both groups and most likely non-differential (as sample quality does not depend on the outcome), thereby allowing for exclusion of these individuals without a major loss of statistical power. Any measurement error that may have occurred under analysis of plasma samples is likely to be a random error rather than a systematic error as it occurs by chance.

Another type of selection bias that may affect cohort studies is **loss to follow-up** [218]. This occurs when there is a difference in the probability of completing the study between the exposure groups. However, as the study designs in this thesis are nested case-control studies within the source cohort, all controls were alive when the corresponding case occurred and all participants were alive through follow-up.

Information bias occurs as a result of the systematic tendency to erroneously place participants in different exposure/outcome categories. This leads to misclassification of exposure and/or outcome. There are several biases that may result in misclassification, including: detection bias, observer/interview bias, recall bias, and reporting bias [218]. In our studies, observer/interview bias was minimized as measurements used in our analyses were taken by trained personnel. Body measurements to calculate body mass index and blood samples used to measure TCC, MBL, and CRP were collected by trained personnel. VTE events were well-validated by objective criteria, thereby limiting misclassification. Random errors in laboratory measurements may also result in misclassification. Other baseline variables were collected through self-administered questionnaires. While this method is cost-effective and efficient, it is prone to **reporting bias.** Participants may fear judgement about socially undesirable behaviors such as drinking or smoking and thus underreport their usage [218]. Participants may also refuse to answer questions they find sensitive or embarrassing. As questionnaires were filled out at inclusion in the study before the

thrombotic event occurred, there is no risk of **recall bias**, where individuals who experienced an event are more likely to remember an exposure than those who did not.

Misclassification can be non-differential or differential [218]. **Differential misclassification** bias occurs when the degree of misclassification of the exposure or the outcome is different between the groups being compared. It can occur when the misclassification of the exposure is dependent on the outcome, for example if the sensitivity of identifying an exposure in cases is higher than in controls [211]. Similarly, it can occur when the outcome is related to the exposure, for example if a disease is more frequently detected due to an exposure, and true cases in the unexposed group remain undetected and thereby misclassified [219]. Differential misclassification may lead to bias toward or away from the null. For example, recall bias is a classic example of differential exposure misclassification as cases are more likely to remember relevant exposures compared to individuals who did not suffer the outcome of interest. **Non-differential misclassification** bias occurs when the misclassification is the same across comparison groups for all participants and can be due to misclassification of exposure or outcome status. When the variable of interest is binary, the estimate is biased towards the null and the association is underestimated, but if there are more than two exposure categories, bias away from the null is also possible [211]. The end-point evaluating committee was blinded to the baseline risk of the participants in the Tromsø Study to avoid potential differential misclassification due to knowledge of exposure status affecting diagnosis probability. Any misclassification that may have occurred was therefore likely non-differential, as it was likely to be similar between the cases and controls. Non- differential misclassification is common in cohort studies, and can result from measurement errors or incomplete self-administered questionnaires. Special care must be taken on wording of questionnaires to avoid misunderstanding and minimize potential misclassification.

Regression dilution bias is a type of information bias that affects longitudinal studies assessing the relationship between baseline measurements of a continuous variable and an outcome. Regression dilution bias is an example of non-differential misclassification. It is caused by random measurement errors, either due to technical errors due to imprecise measurement devices or biological variation [220, 221]. In the nested case-control studies used in this thesis, the participants were followed for up to 12 years. In paper I, the variable

of interest (TCC) was subject to variation over the long follow-up period, making our results prone to underestimation of the real association between exposure (TCC) and outcome (VTE) [222]. In papers II and III, variation in MBL levels during the follow-up period could lead to lower than the true risk estimates. We therefore performed sensitivity analysis by restricting the maximum time from blood sampling in Tromsø 4 to the VTE events while keeping all the controls in the analysis. Logistic regression analysis on time restriction was set to require a minimum of 10 VTE events, and ORs were estimated at every 0.1 year increase in time since blood sampling and plotted as a function of maximum time. In paper I, the risk of VTE by plasma levels of TCC declined substantially with time between blood sampling and event. Paper II showed that the ORs for VTE, DVT, and particularly PE by low MBL levels decreased with shortened time between blood sampling and the respective events. In paper III, ORs for PE by high plasma MBL levels were higher with shortened time between blood sampling and event. These sensitivity analyses show that all our results were affected by regression dilution bias to some extent, giving us attenuated OR estimates.

5.1.3. Confounding and mediation

A **confounder** is a variable that is associated with both the exposure and the outcome but is not in the causal pathway [211]. This leads to **confounding,** or mixing of effects. A confounder distorts the association between exposure and outcome. There are different approaches to correct for the presence of confounding, including stratification, matching, and regression models [223]. Regression is the most common method to minimize confounding in observational studies [209]. Multivariable analysis allows for the demonstration of an independent association between a variable of interest and an outcome after adjusting for potential confounding factors [224]. By using regression analysis models rather than stratification to correct for confounding, the statistical power of the study remains the same as data from all participants is used [211].

As previously discussed, age is a strong risk factor for VTE [13, 14]. Obesity is another major risk factor for VTE, accounting for 33% of unprovoked incident VTE [10]. While the risk is higher in females of childbearing age compared to men of similar age [15, 225], the overall age-adjusted incidence rate is higher for men than for women [14]. Age, sex, and BMI are

associated with risk of VTE, but their association with MBL levels is less clear. Immune system function is known to decline with age. Whether this also applies to the complement system remains unknown [226, 227], though one study found that classical and alternative pathway functional activity was enhanced with age, and terminal complement components (C5, C8, and C9) increased with age [226]. Interestingly, concentrations of complement activation products analyzed did not differ between age groups stratified by decade (between 20-70 years old) [226]. The same study found females have significantly lower median serum MBL concentrations compared to men, but no significant differences between the sexes were found in overall lectin pathway activity [226]. In our nested case-control study in Paper II and III, there was no correlation between MBL levels and age, and females had a significantly lower mean MBL level than men (1317 ng/mL vs 1463 ng/mL, p=0.03). Though neither we nor others have found that MBL levels differ in individuals with normal BMI and obesity [228], we included BMI as a covariate, along with age and sex, in our regression models to exclude the possibility that these factors were confounding the apparent association we found between plasma MBL levels and risk of VTE.

C-reactive protein (CRP) is an acute phase protein, with levels increasing during the inflammatory response [229]. Several studies have found that CRP levels are associated with increased risk of VTE [230-232]. MBL and CRP are both acute phase proteins, and activators of complement pathways [233]. Nevertheless, CRP has been found to have additional effects on MBL-mediated complement activation, both enhancing the lectin pathway or inhibiting MBL-mediated cytolysis through the inhibition of the alternative pathway [233]. In papers II and III, CRP was included as a covariate in our multivariable models for VTE. Nevertheless, comparing estimated ORs to a logistic regression model without CRP as a covariate suggests that CRP had little effect and did not act as a confounder in our studies.

A phenomenon that closely resembles confounding is mediation. Like a confounder, a **mediator** is an additional variable associated with a predictor of interest and the outcome. However, a mediator must be a causal consequence of the predictor of interest [234]. In papers II and III, we did sensitivity analysis to remove the possibility that MBL levels may predispose to any co-morbidities that are established triggers for VTE. These mediators include myocardial infarction [105], stroke [107], acute infection [115], and cancer [91]

occurring within 3 months of a VTE event. In these studies, we excluded participants with any of these conditions from our analysis to remove their potentially mediating effects.

It is important to consider that despite careful consideration in selection of covariates for multivariable analysis, the possibility that our studies may be affected by **residual confounding** remains. Residual confounding occurs when unrecognized confounders exist that are excluded from multivariable analysis or the categories of the confounder adjusted for are too broad and thereby result in imperfect adjustment [211]. Thus the associations we found in our studies could potentially be a result of unrecognized confounding variables that were not taken into account. Residual confounding is a common threat to large observational studies.

5.1.4. Missing data

Missing data is common and may introduce bias into an epidemiological study. Missing data can be a result of failure to respond to certain questions in a questionnaire, loss to follow-up, equipment failure, incorrect handling or loss of samples for laboratory measurements, and many other reasons [235]. It is thus imperative that studies are carefully planned and executed to minimize data loss. There are several solutions, but no optimal approach, to handling missing data. These include omission of variables with missing values, omission of individuals with incomplete data, or imputation (estimation) of missing values [235]. If a large proportion of data is omitted to account for missing data, statistical power may be reduced as a result.

In the papers in this thesis, plasma samples with insufficient quality were excluded from the studies. As the proportion of missing data was low in both case and control groups (10% and 8%, respectively) and likely non-differential, we could exclude these individuals without a major loss of statistical power.

In all papers in this work, other binary variables denoting basic characteristics such as smoking and CVD that contained missing values were treated as not having that trait. For example, if an individual's information about smoking was missing, he or she was classified as a non-smoker. This data was missing from very few individuals (2 (0.2%) individuals for

smoking, 3 (0.2%) for CVD, 5 (0.4%) for CRP, 3 (0.2%) for BMI, and 4 (0.3%) for diabetes), and were therefore unlikely to have a great impact our findings.

5.2. Discussion of the main results

5.2.1. Complement activation and risk of venous thromboembolism (Paper I)

In Paper I, we investigated whether activation of the complement system, measured by plasma levels of the soluble terminal complement complex (TCC), was associated with risk of venous thromboembolism. In this population-based nested case-control study, we found plasma TCC levels in the highest quartile were associated with a 74% higher risk of unprovoked VTE compared to the lowest quartile. No genetic variants across the whole genome or the complement-related genome were significantly associated with TCC levels.

This study was the first to investigate the association between plasma TCC levels as a reflection of overall complement activation *in vivo* and risk of VTE. Prior to this study, there have been few studies investigating the role of the complement system in the development of VTE. In a large, prospective study in the general population in Copenhagen, Nørgaard and colleagues reported that participants with plasma C3 levels in the highest tertile had a 58% higher risk for VTE compared to those in the lowest tertile [236]. The risk estimate was attenuated after adjustment for CRP and BMI, but remained 31% elevated compared to the lowest tertile. The authors speculated that C3 and complement activation is likely a mediator in the pathogenesis of venous thrombosis, although they could not exclude the possibility that C3 was a bystander and merely functioned as a marker of inflammation in a similar manner to CRP.

Subramaniam et al. used an inferior vena cava ligation model in mice to induce DVT and study the contributions of complement factors to platelet activation and fibrin formation [237]. They showed that C3-deficient mice had a reduced incidence of thrombus formation and size compared to wild-type mice. Additionally, platelet activation was diminished, and deposition of platelets and fibrin were also reduced compared to control animals. C5-deficient mice also had reduced thrombus size. This study implicated complement as a mediator of thrombus formation.

In Paper I, we assessed *in vivo* complement activation by measuring soluble TCC levels. The TCC, or the membrane attack complex (MAC) if assembled on a membrane surface, is the final activation product of complement activation. It is composed of C5b, C6, C7, C8, and multiple C9s (C5b-9), forming a pore-like structure that aids in cell lysis. While

studies suggest that TCC is biologically inert, it has proven a useful tool for measuring complement activation [157]. TCC has been investigated as a procoagulant molecule in several *in vitro* studies. Sublytic C5b-9 was found to increase PS expression on platelets and to increase FVa and Xa binding to the plasma membrane, culminating in increased platelet prothrombinase activity [238, 239]. C5b-9 also induces TF expression in endothelial cells [240, 241] as well as secretion of vWF from Weibel-Palade bodies [242]. Indeed, C5b-9 has also been associated with arterial CVD [243]. Our study shows for the first time that TCC is also associated with VTE.

The lack of genetic variants associated with TCC levels shows the importance of environmental factors in complement activation. As complement activation is a tightly controlled process, there need to be clear initiating mechanisms. Triggers for activation include hypoxia, and it has been shown that the classical pathway is activated upon hypoxia-reoxygenation of human umbilical vein endothelial cells [244, 245]. Human endothelial oxidative stress also activates complement via MBL and the lectin pathway [246]. Venous thrombi develop in the venous valvular sinuses [40, 42], areas of severe local hypoxia [41]. Local endothelial cells are subjected to oxidative stress under these hypoxic conditions, likely increasing binding of MBL and thereby lectin pathway activation as in experimental settings. While MBL does not usually bind to host cells, it has been shown to bind to apoptotic cells [247, 248]. Hypoxia results in changes in cell protein expression and glycosylation [249] of endothelial cells, which may explain MBL recognition of altered self-cells. The lectin pathway therefore deserved to be investigated as a prothrombotic pathway in the development of VTE, and was used in the studies performed in Paper II and III.

There remain several important points to consider in our study. Sensitivity analysis restricting time from blood sampling to VTE event shows that the associations we found were affected by regression dilution bias due to the long follow-up period. Therefore, the ORs for the true association for risk of VTE are underestimated. Overall, the implications of our study are in agreement with the findings of other studies investigating complement and venous thrombosis, though our results need to be externally validated. The mechanisms initiating complement activation remain undiscovered, and future functional studies will be met with great interest.

5.2.2. Plasma mannose-binding lectin levels and risk of venous thromboembolism (Paper II)

In paper II, we examined whether plasma levels of mannose-binding lectin were associated with risk of venous thromboembolism in a nested case-control study derived from a population-based cohort study (The Tromsø Study). Individuals in the lowest quartile of plasma levels of MBL had a reduced OR for VTE (OR 0.79, 95%CI: 0.56-1.10) and DVT (OR 0.70, 95%CI: 0.47-1.04) compared to individuals with MBL levels in the highest quartile after adjusting for age, sex, BMI, and CRP.

Studies that have examined the association between MBL levels and the risk of VTE are sparse. Two of these studies were done on SLE patients using MBL genotyping rather than plasma MBL measurements, and their results were discordant, with one group having found that *MBL2*-deficient genotypes had an increased risk of VTE in a cross-sectional study [206], while no association with risk of VTE was found in a prospective cohort [205]. The discrepant results could be due to different study design, different variant alleles that were analyzed in the two studies, or a difference in outcome prevalence in the different study populations. Additionally, as plasma MBL levels vary up to 1000-fold among healthy individuals [250], and more than 10-fold even among those of the same haplotype [173], the results of these studies will likely have yielded different results if they had measured plasma levels instead of genotypes. It is also important to consider that the populations of interest in these studies were patients with SLE, an autoimmune disease with a known increased risk for both arterial and venous thrombosis [251]. Another study examined the gene expression of various complement factors, including MBL, in peripheral blood mononuclear cells (PBMC) in patients with symptomatic PE and age- and sex-matched controls [252]. The MBL mRNA gene expression in PBMCs was lower in PE patients than controls. However, the study design was rated poor in a recent systematic review of the lectin pathway in thrombotic conditions due to a high estimated risk of bias [253]. The final study investigated the association between circulating MBL levels and VTE in a small case-control study conducted by our group [254]. We found that MBL deficiency (plasma levels $\leq$100 ng/mL) and low lectin pathway potential activity were associated with an increased risk of unprovoked VTE (OR 3.5 and 2.7, respectively). A greater proportion of VTE patients (33.3%) had MBL-deficiency compared to age- and sex-matched controls (12.5%). As the study was small and underpowered, and the possibility of reverse causation could not be excluded, we

conducted the study in Paper II to examine the association between plasma MBL levels and risk of VTE in a larger population-based study with a prospective design.

Several studies have suggested involvement of MBL and the lectin pathway in complement activation in response to hypoxic conditions [246, 249]. Hypoxia changes the protein expression [255] and glycosylation pattern [256] of endothelial cells [249]. These changes may explain why MBL binds to apoptotic cells [247, 248], as MBL usually does not bind to healthy host cells. Hypoxia characterizes the local environment in the venous valvular sinuses, where venous thrombi have been shown to originate, and it is likely to contribute to the pathological mechanism of VTE. The recognition of previously unexposed glycosylation patterns on host cells leads to binding of MBL and activation of the lectin pathway under these hypoxic conditions.

The lectin pathway has been studied as a prothrombotic pathway. Several studies have investigated the role of MBL in arterial thrombosis. Indeed, *in vivo* animal studies suggest MBL and its associated serine proteases have a role in coagulation. Both MBL null and MASP-1/3 null mice show a prolonged bleeding time on tail tip excision [257]. The prolonged bleeding time in MBL null mice was reversed on reconstitution with recombinant human (rh)MBL. In the same study, the bleeding time in C3 null mice was similar to that of wild type controls, suggesting that the hemostatic problem occurred before the point of C3 in complement activation and only involved components before the common pathway. A later study by the same group showed that both MBL null and MASP-1/3 null mice showed reduced $FeCl_3$-induced thrombogenesis compared to wild type controls [258]. Reconstitution with rhMBL restored thrombogenesis to a similar degree as wild type mice. This study indicates that MBL and MASPs are both significant contributors to clot formation. Furthermore, in a model where mice expressed the human *MBL2* gene, 3F8 monoclonal MBL inhibiting antibody prevented arterial thrombosis and limited myocardial injury in infarction [259]. The antibody 3F8 also prevented $FeCl_3$-induced arterial thrombosis in the same *MBL2*-expressing mice. Moreover, several studies have found that inhibiting the lectin pathway by using monoclonal antibody against MBL [260] or MBL knockout mice [261, 262] have decreased injury following myocardial ischemia-reperfusion. Recently, one research group proposed that MBL is a key factor in the pathogenic events following vascular ischemia in a

ischemic stroke model in mice, first by activating platelets and then by activating complement [263].

Though these animal studies have focused on arterial CVD, *in vitro* studies also support a prothrombotic role for MBL and the lectin pathway. Indeed, the MASPs are promiscuous proteins with thrombin-like substrate specificity in addition to their functions of cleaving complement components C2 and C4. MASP-1 can cleave fibrinogen and activate FXIII [264], high molecular weight kininogen (HMWK) [265], and TAFI [201]. Additionally, MASP-1 can activate PAR4 [266]. MASP-1 and -2 have both been shown to cleave prothrombin to thrombin [171, 172]. Thus, the prothrombotic potential of MBL and the MASPs should also be considered important in the development of venous thromboembolic disease.

Overall, despite the clear role established by *in vitro* studies and animal studies, studies in humans show a somewhat inconsistent association between MBL levels and arterial CVD [253]. Some studies have found an association between low MBL genotypes or MBL deficiency and increased risk of atherosclerosis [267], carotid plaques [268], and myocardial infarction [269, 270]. These studies found that low MBL levels also predicted an earlier onset and more progressive disease [267]. As MBL-deficiency is also associated with susceptibility to infection, infection by *Chlamydia pneumonia* has been proposed as a link to the development of atherosclerosis [271, 272]. Other studies, however, found that high MBL genotypes or plasma levels were associated with an increased risk of acute coronary syndromes [273] and stroke [253, 274]. These studies also found high MBL levels were also associated with greater severity and a worse outcome [253, 274, 275], while individuals with MBL deficiency had smaller cerebral infarcts and a more favorable outcome [276, 277]. These discrepant results may be due to the different study designs and the populations examined in the studies.

In order to eliminate the possibility that the risk estimates we found by plasma MBL were influenced by mediation of CVD, which increases the risk for VTE, we performed sensitivity analysis. Previous studies have found that CVD, such as ischemic stroke [107] and myocardial infarction [105], is a risk factor for VTE, especially within a short period immediately following the event. Therefore, we removed individuals who suffered a stroke or acute MI within 3 months prior to their VTE event from our analysis. We also excluded

patients with acute infections within 3 months before their VTE event, as low MBL may be a risk factor for infection, especially in early childhood [278, 279] or in adult patients with concomitant disease or immunodeficiency [280]. Acute infection is itself a risk factor for VTE, with the highest risk for an event occurring 3 months after infection [115]. Our sensitivity analysis gave results that were essentially similar to our overall findings, suggesting that the effects of plasma MBL levels were not affected by the presence of these comorbidities.

Previous studies have shown that MBL-deficient individuals have a high prevalence of associated disease with increased susceptibility to various types of infectious disease as well as noninfectious disorders, including autoimmune disorders [176, 182, 183]. While MBL-deficiency has been associated with the development of SLE and autoimmune disorders, we did not have data on these conditions and were thereby unable to do sensitivity testing on patients with these conditions. Autoimmune diseases such as SLE and rheumatoid arthritis are also known to be associated with increased risk of VTE [111, 251]. Nevertheless, our findings are not likely to be affected by the presence of individuals with these disorders, especially since we included CRP in our adjustment models.

Our findings in this study strengthen the evidence for a role for the complement system, and specifically the lectin pathway, in the development of VTE. At present, our findings are unchallenged and await confirmation by other studies. Future studies investigating the molecular mechanisms of the lectin pathway in the pathophysiology of VTE are warranted.

5.2.3. ABO blood type, plasma mannose-binding lectin levels, and risk of venous thromboembolism (Paper III)

In Paper II, we observed an association between plasma MBL levels and risk of VTE, with individuals with plasma MBL levels in the lowest quartile having a reduced OR for overall VTE (OR 0.79, 95%CI: 0.56-1.10) and DVT (OR 0.70, 95% CI: 0.47-1.04) compared to individuals with MBL levels in the highest quartile. This protective effect was even more pronounced (55% reduced risk) when restricting the time between blood sampling and VTE event to 4 years. In Paper III, we set out to explore whether high plasma MBL levels were associated

with any genetic variants that could influence the association of high plasma MBL levels and risk of VTE.

Circulating MBL levels are largely genetically determined. Polymorphisms in exon 1 and its associated promotor region of the *MBL2* gene give varying levels of functional MBL [169]. The great variability in MBL levels even in individuals of the same haplotype suggests that there are still some undiscovered factors affecting circulating MBL levels [169]. We therefore performed a pQTL analysis to find genetic polymorphisms that are associated with high plasma levels of MBL. As high levels of MBL were associated with a greater risk, we looked for polymorphisms associated with the highest quartile of MBL levels. Among the SNPs that were significantly associated with high levels of MBL was a known polymorphism of the *MBL2* gene (rs1800450). Unexpectedly, the rs8176719 SNP of the *ABO* gene, known to differentiate between O- and non-O blood types [64], was also significantly associated with high MBL levels. This association between blood type and MBL levels has not been previously described.

Non-O blood type is the most common risk factor for VTE [61, 281] with a population attributable risk of 20-30% [62, 282]. Many studies have found an association between blood type and risk of VTE, with non-O type individuals having the greatest risk (pooled OR 1.79, 95%CI 1.56-2.05)[63]. The *ABO* gene has many polymorphisms that determine blood type, among which a homozygous deletion at the rs8176719 SNP site results in blood type O [64].

The mechanism by which there is an increased risk of VTE by non-O blood group is not fully understood, though it is partly attributable to vWF and Factor VIII. Non-O blood types have 25% higher levels of vWF and factor VIII compared to blood type O [65, 283]. This is similar to the 40% increased MBL levels in non-O blood types compared to type O blood we found in Paper III. Increased vWF levels are associated with an increased risk of VTE [66, 284]. When adjusting for blood type, risk of VTE by both vWF [66, 285] and MBL levels (paper III) were attenuated. On the other hand, several studies have found that the risk of VTE by non-O blood type is not completely attenuated after adjusting for vWF and FVIII levels [66, 68] suggesting that non-O blood type has additional prothrombotic effects. Indeed, the *ABO* gene has been associated with many other proteins, many of which are also related to risk of VTE: galectin-3 [286, 287], P-selectin [288, 289], E-selectin [290-292], IL-6 [293, 294] and ICAM-1 [288, 295]. Erythrocyte traits such as hematocrit, hemoglobin, and

red blood cell count that are associated with risk of VTE [296] are also associated with genetic variants of the *ABO* gene [286, 297, 298].

Unlike vWF which carries ABO blood group determinants [65], it has not been shown that MBL contains blood group moieties. Therefore, we created new subgroups of MBL levels and ABO blood type and examined the risk of VTE. We found that high levels of MBL in type O individuals still had increased risk of VTE, with OR estimates similar to risk by non-O blood type alone. Presumably this is not true for vWF, as vWF carries ABO blood type carbohydrates and thus likely has a different relationship with blood type than MBL.

High MBL levels gave the greatest risk of PE (OR 2.37), especially provoked PE (OR 3.45), in individuals with O blood type. Our findings indicate that high MBL levels are an important risk factor for VTE in type O individuals. The risk of VTE by non-O groups remained similar regardless of MBL levels, with the exception of an attenuated risk for provoked PE in the high MBL/non-O blood group. Thus non-O blood type seemed to obscure the effect of high MBL levels. Whether MBL and the unknown effects of non-O blood type are part of the same prothrombotic mechanism or whether these two risk factors have separate effects that attenuate each other is not yet known.

As vWF is a highly glycosylated protein, it can also interact with several carbohydrate-binding proteins such as lectins [299]. Indeed, the C-type lectin CLEC4M has been shown to bind and internalize vWF [300] and FVIII [301], thereby affecting plasma levels of these proteins. Two other C-type lectins, the macrophage galactose-type lectin (MGL) [302] and the Ashwell-Morrell Receptor (AMR) [303] were shown to be clearance receptors for hyposialylated vWF. Low density lipoprotein receptor-related protein 1 (LRP1) on macrophages has also been proposed as a mechanism of vWF clearance [304], and interestingly, a polymorphism of the receptor has also been associated with increased risk of VTE [305]. Notably, like vWF, MBL has also been found to interact with LRP1, both directly [306] or indirectly through calreticulin [247]. Galectin-1 and -3 have been shown to bind to vWF and possibly mediate thrombus formation through this interaction [307]. Whether MBL, a C-type lectin, can bind to or interact with vWF or whether these proteins share a common mechanism or clearance pathway affected by blood type is unknown and demands further investigation.

The increased risk of VTE in individuals with high plasma MBL levels and type O blood was almost entirely limited to provoked events. Trauma, sepsis, and surgery are among provoking factors for VTE [308, 309], and complement activation is known to play a role in all of these conditions. High MBL levels may predispose to increased potential lectin pathway activity. Indeed, MBL levels have been found to correlate with potential lectin pathway activity *in vitro* [226, 254]. Lectin pathway activation occurring during provoking events may contribute to clot formation, especially given the pleiotropic effects of MASPs. The prothrombotic effects of MASP-1 include cleavage of fibrinogen to fibrin [264], prothrombin to thrombin [171], FXIII [264], TAFI [201], and HMWK [265], and the activation of PAR4 receptors [266]. These enzymatic effects are similar to that of thrombin.

The increased risk for PE by high MBL in type O individuals may be due to frequent embolization. Clots formed by MASP-1 have been shown to have thicker fibrin fibers and be less dense than clots prepared with thrombin [201], making them more susceptible to embolization. Alternately, although PE is often considered a sequel of DVT, up to half of cases find no concomitant DVT, suggesting that *de novo* formation of PE may occur locally in the lung vasculature [1]. In addition, recent studies have suggested they may have separate etiologies [57, 310].

The association of the non-O SNP with high levels of MBL is a novel finding, one which is complicated by the combined effects on risk of VTE. The risk of VTE is not additive when both factors are present. The association between high MBL and VTE appears more important in persons of type O blood, as persons of non-O blood type have a similarly increased risk regardless of MBL levels. Our hypothesis of increased lectin pathway activity and MASP-1 contribution to thrombus formation and embolization is plausible. Other studies are needed to confirm our findings. In the future, functional studies should examine possible mechanisms of MBL and the lectin pathway in the pathogenesis of VTE, and to establish whether there is an association between vWF and MBL.

6. Conclusions

- Complement activation, assessed by soluble terminal complement complex (TCC) levels, was associated with risk of incident venous thromboembolism, especially unprovoked events. Neither genome-wide nor complement-related genetic variants were significantly associated with plasma TCC levels, suggesting that predominantly environmental factors are involved in determination of plasma TCC levels.

- Low plasma levels of mannose-binding lectin were associated with a decreased risk of venous thromboembolism, and especially deep vein thrombosis. The risk of VTE was not influenced by the presence of co-morbidities that are commonly associated with low MBL levels, such as arterial cardiovascular disease and infection, or cancer.

- High plasma MBL levels were associated with an increased risk of VTE. The rs8176719 SNP determining O and non-O blood groups affected plasma MBL levels, and individuals with non-O blood type have on average 40% higher mean plasma MBL level compared to those with type O blood. High plasma MBL levels in type O individuals conferred an increased risk of VTE to a similar degree as non-O blood type.

7. Final remarks and future perspectives

The findings in this thesis support a role of the complement system in the pathogenesis of VTE. Overall complement activation, as measured by the TCC, was associated with risk of venous thromboembolism. Furthermore, the lectin pathway, investigated by plasma MBL levels, was also associated with VTE risk, and blood type. Our findings reveal a dynamic interaction between genetic determination of MBL and blood type, particularly in those with blood type O, as well as the environmental influence of overall complement activation, reflecting the interplay of many factors in this multicausal disease.

While the findings in this thesis suggest a role for complement in the pathogenesis of VTE, functional studies are needed to investigate the molecular mechanisms behind the associations. Future studies should validate our results, as well as investigate the effects of MBL and MASPs on thrombin generation in experimental studies. Similar studies on the other PRMs of the lectin pathway (ficolins and collectins) and on the MASPs are also warranted.

Our finding an association between circulating MBL levels and blood type is intriguing, and underscores the importance of an established yet still mysterious risk factor for thrombosis. Non-O blood group is associated with 25% higher vWF and FVIII levels [65, 283], as well as 40% higher MBL levels in plasma. The increased risk for VTE conferred by this blood group remains after adjusting for these factors [66, 68], and the mechanisms for this remain unknown. More research is needed to elucidate possible mechanisms for the increased thrombotic risk by non-O blood groups. *In vitro* studies exploring possible interactions between vWF, FVIII, and MBL will be met with great interest.

Unravelling the mechanisms involved in the pathogenesis of venous thrombosis is paramount for improving prediction models and treatment options.

8. References

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Paper I

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ORIGINAL ARTICLE

Complement activation assessed by the plasma terminal complement complex and future risk of venous thromboembolism

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Abstract

Background: It remains uncertain whether activation of the complement system, assessed by the soluble terminal C5b-9 complement complex (plasma TCC), is associated with future risk of incident venous thromboembolism (VTE).

Objectives: To investigate the association between plasma levels of TCC and future risk of incident VTE in a nested case-control study, and to explore genetic variants associated with TCC using protein quantitative trait loci analysis of exome sequencing data.

Methods: We sampled 415 VTE cases and 848 age- and sex-matched controls from a population-based cohort, the Tromsø study. Logistic regression models were used to calculate odds ratios with 95% confidence intervals for VTE across quartiles of plasma levels of TCC. Whole exome sequencing was conducted using the Agilent SureSelect 50 Mb capture kit.

Results: The risk of VTE increased across increasing quartiles of plasma TCC, particularly for unprovoked VTE. Participants with TCC in the highest quartile (>1.40 complement arbitrary units/mL) had an odds ratio for unprovoked VTE of 1.74 (95% confidence interval: 1.10–2.78) compared with those with TCC in the lowest quartile (≤0.80 complement arbitrary units/mL) in analyses adjusted for age, sex, and body mass index. A substantially higher risk for VTE was observed in samples taken shortly before VTE event. We found no association between genome-wide or complement-related gene variants and plasma levels of TCC.

Conclusions: We found that high levels of plasma TCC were associated with VTE risk, and unprovoked events in particular. There was no genome-wide association between gene variants and plasma levels of TCC.

KEYWORDS

complement system, protein quantitative trait loci analysis, terminal complement complex, venous thromboembolism, whole exome sequencing

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1 | INTRODUCTION

Venous thromboembolism (VTE) is a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE is a complex disease, affecting 1–2 per 1000 individuals annually, with serious short- and long-term complications, including recurrence and death.[1,2] The incidence of VTE has slightly increased during the past two decades,[3,4] and it is likely that the incidence will continue to rise because the prevalence of major risk factors for VTE, such as high age, obesity and cancer, are increasing in the population.[5–7] VTE has become a major challenge to public health and health care systems because of frequent hospitalizations, monitoring of treatment to avoid bleeding complications, severe complications, and a high mortality rate after 30 days.[8] It is therefore pivotal to identify novel biomarkers and unravel underlying disease mechanisms of VTE to facilitate targeted prevention and treatment.

The complement system is an important part of the innate immune system organized in a cascade of proteolytic serine proteases with a number of biological functions.[9] Growing evidence from observational and animal studies suggests that the complement system is involved in the early steps of the pathogenesis of VTE, consistent with an extensive cross-talk between the complement and hemostatic systems.[10] Complement factor C3 is an acute-phase reactant and a central component in activation of the complement system.[11] In a large population-based cohort, subjects with plasma complement C3 levels in the highest tertile had a 58% higher risk of VTE than those in the lowest tertile; the risk estimate declined to 31% after further adjustment for C-reactive protein (CRP) and body mass index (BMI).[12] However, it is not known whether complement C3 is a marker or a mediator of VTE risk.

Activation of any of the three complement pathways merge at C3, which subsequently activates C5 and the terminal complement pathway, with final formation of C5a and the terminal C5b-9 complement complex (TCC).[13] The TCC exists in two forms, surface-bound C5b-9 forming the membrane attack complex and a soluble form where C5b-9 binds to vitronectin and clusterin and becomes non-lytic.[14] Soluble TCC is generally accepted as a marker of *in vivo* complement activation and can be measured in plasma.[15,16]

No previous study has, to the best of our knowledge, investigated the association between plasma levels of TCC and future risk of VTE in individuals recruited from the general population. The aims of the present study were therefore to: (i) investigate whether plasma levels of TCC were associated with incident VTE in a nested case control study, (ii) explore whether genetic variants were associated with plasma levels of TCC using protein quantitative trait loci (pQTL) analysis of exome sequencing data; and (iii) investigate whether identified gene variants (if present) were associated with risk of VTE.

Essentials

- It is unknown if terminal complement complex (TCC) is associated with venous thromboembolism (VTE).
- Genome-wide and complement-related gene variants were not associated with plasma levels of TCC.
- High plasma levels of TCC were associated with future risk of VTE, particularly unprovoked events.
- The association between plasma TCC and risk of VTE increased with shorter follow-up time.

2 | METHODS

2.1 | Study population

The Tromsø Study is a single-center, population-based cohort, with repeated health surveys of inhabitants of Tromsø, Norway. All inhabitants aged ≥25 years living in the municipality of Tromsø were invited to participate in the fourth survey, conducted in 1994–1995. A total of 27 158 subjects 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). All first lifetime VTE events were identified using the hospital discharge diagnosis registry and the autopsy registry and the radiology procedure registry at the University Hospital of North Norway, which is the sole hospital in the Tromsø region. Trained personnel adjudicated and recorded each VTE by extensive review of medical records. A VTE was confirmed if presence of signs and symptoms of PE or DVT (proximal or distal) were combined with objective confirmation by radiological procedures (i.e., compression ultrasonography, venography, spiral computed tomography, perfusion-ventilation scan, pulmonary angiography) or autopsy, and resulted in treatment initiation (unless contraindications were specified) as previously described.[17] A VTE occurring in the presence of one or more provoking factors was classified as provoked. The following were regarded as provoking factors: surgery or trauma (within 8 weeks before the event), acute medical conditions (acute myocardial infarction, acute ischemic stroke, acute infections), immobilization (bed rest >3 days or confinement to wheelchair within the past 8 weeks) or other factor specifically described as provoking by a physician in the medical record (e.g., intravascular catheter).

During the follow-up period (1994–2007), 462 individuals experienced a VTE event. For each case, two age- and sex-matched controls, who were alive at the index date of the VTE event, were randomly sampled from the source cohort (n = 924). In total, 47 cases and 76 controls did not have available plasma samples of sufficient quality for the analyses. Thus, our final nested case control study consisted of 415 cases and 848 controls.

2.2 | Baseline measurements

Height (to the nearest centimeter) and weight (to the nearest 0.5 kilograms) were measured in participants wearing light clothing and no shoes. BMI was calculated as weight divided by the square of height in meters. A self-administered questionnaire was used to collect detailed baseline information on smoking status and previous

cardiovascular events (stroke, angina pectoris, transient ischemic accident, and myocardial infarction).

2.3 | Blood and DNA samples

Nonfasting blood was collected from an antecubital vein into 5-mL vacutainers (Becton Dickinson, Meylan Cedex, France) containing ethylenediaminetetraacetic acid (K_3-ethylenediaminetetraacetic acid 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 laboratechnik, Nürtringen, Germany) in 1-mL aliquots and stored at −80°C until further analysis. DNA isolated from blood was stored at the National CONOR Biobank.[18]

2.4 | Exome sequencing

Whole exome sequencing at high-coverage (≈100×) was conducted in a subgroup of the study population (355 VTE patients and 354 control subjects) using the Agilent SureSelect 50 Mb capture kit. The resulting genotypes were effectively filtered[19] and imputations were carried out as previously described in detail.[20]

2.5 | Measurement of the soluble terminal complement complex

Plasma samples were thawed on crushed ice, and plasma levels of TCC were measured using an in-house enzyme-linked immunosorbent assay as described previously.[15,21] In brief, enzyme-linked immunosorbent assay plates (Nunc, Immunoplate II, Copenhagen, Denmark) were coated with the monoclonal antibody, aE11 specific for a neoantigen exposed in C9 after activation and incorporation into TCC, the poly (C9) unit of the TCC. Biotinylated anti-C6 monoclonal antibody (9C4) was used as detection antibody and streptavidin horseradish peroxidase (GE Healthcare UK) was added as the final step. Optical density was measured at 405 nm (Dynatech MR580; Dynatech Laboratories Inc., Alexandria, VA, USA). Results are given in complement arbitrary units (CAU)/mL. The intra-assay coefficient of variation was negligible, and the inter-assay coefficient of variation was 10.3%.[15]

2.6 | Statistical analysis

Statistical analyses were carried out using R, version 3.4.4 (The R Foundation for Statistical Computing, Institute for Statistics and Mathematics, Vienna, Austria). Plasma TCC was categorized according to quartile cutoffs in the control population (≤0.80, 0.80–1.04, 1.04–1.40, >1.40 CAU/mL). Means and proportions of baseline characteristics across categories of TCC were calculated using descriptive statistics. Logistic regression models were used to calculate odds ratio (OR) for VTE with 95% confidence interval (CI) according to quartiles of TCC adjusted for age, sex, and BMI. We did not adjust for CRP because it is likely to be in the causal pathway of complement activation and VTE. The lowest quartile of TCC was used as the reference group. We also calculated the *P* value for linear trend of ORs across increasing quartiles of TCC.

Because the follow-up time in the source cohort was long (≥12 years for many persons), the results based on baseline TCC measurements could be influenced by regression dilution bias.[22] To investigate this, we performed analyses in which we 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 generated at every 0.1 year increase in time because blood sampling and plotted as a function of this maximum time.

The 1 034 910 genome-wide variants derived from the whole-exome sequencing were used to investigate whether plasma TCC was regulated by any alternative genetic variants. This pQTL analysis was performed both in a genome-wide setting, and restricted to the loci within ± 500 kb of the different genes involved in the complement system (Table S1). To adjust for multiple testing, the commonly used significance threshold of 5×10^{-8} was used in the genome-wide setting. As the cis analysis in total contained 11 806 variants, a Bonferroni-based adjustment for multiple testing corresponded to a significance threshold of $0.05/11.806 = 4.23 \times 10^{-6}$. The plasma TCC values were transformed to follow a perfect standard normal distribution before entering the QTL analysis. The QTL analysis was done by the EPACTS (Efficient and Parallelizable Association Container Toolbox) software.[23] The EMMAX (Efficient Mixed Model Association eXpedited)[24] test within EPACTS was used, which uses a mixed model to test for associations between a quantitative trait (TCC in this study) and genetic variants while adjusting for covariates and genetic relatedness structure in the cohort.

3 | RESULTS

The distribution of baseline characteristics across quartiles of plasma TCC is shown in Table 1. The mean age, sex distribution, and mean BMI were essentially similar across quartiles of plasma TCC. The proportion of subjects with a history of cardiovascular disease was slightly lower and the proportion of smokers higher in the lowest TCC quartile. As expected, plasma levels of high-sensitivity CRP increased across quartiles of TCC from 1.32 ± 1.08 mg/L in the lowest quartile to 1.96 ± 1.65 mg/L in the highest quartile.

The characteristics of the VTE patients are shown in Table 2. The mean age at the time of VTE was 67.4 years, and 48.4% of the cases were men. In total, 37.8% of the VTE events were PEs and 62.2% were DVTs, and 42.2% of the cases were unprovoked. The most common provoking factors were surgery/trauma (22.4%) and active cancer (21.4%).

The ORs of VTE across quartiles of plasma TCC levels are shown in Table 3. For overall VTE, the OR increased across quartiles of plasma TCC (*P* for trend = 0.06), and subjects with plasma TCC > 1.40 CAU/mL had a 35% higher risk of VTE compared with

TABLE 1 Characteristics across quartiles of plasma levels of the terminal complement complex (TCC)

	Plasma TCC (CAU/mL)			
	≤0.8 (n = 313)	<0.80–1.04 (n = 297)	>1.04–1.40 (n = 322)	>1.40 (n = 331)
Age, years	58.7 ± 13.5	61.3 ± 12.8	61.4 ± 14.0	59.8 ± 14.4
Sex, % men (n)	50.8 (159)	48.1 (143)	42.2 (136)	48.0 (159)
BMI, kg/m^2	26.3 ± 4.2	26.6 ± 4.0	26.2 ± 4.3	26.6 ± 4.4
hsCRP, mg/L	1.32 ± 1.08	1.50 ± 1.28	1.71 ± 1.30	1.96 ± 1.65
CVD[a]	13.7 (43)	17.5 (52)	16.1 (52)	15.1 (50)
Daily smoking	34.2 (107)	30.3 (90)	32.9 (106)	27.5 (91)

Note: [a]Self-reported history of cardiovascular disease (myocardial infarction, angina, stroke).

those with TCC ≤ 0.80 CAU/mL (OR: 1.35; 95% CI: 0.97–1.88). In subgroup analyses restricted to unprovoked VTE, the OR for the upper (>1.40 CAU/mL) vs. the lower quartile of plasma TCC (≤0.80 CAU/mL) was 1.74 (95% CI: 1.10–2.78), and there was a statistically significant linear trend in the ORs across increasing quartiles of TCC (*P* for trend = 0.02). When restricting the analysis to provoked VTE, no difference in risk estimates of VTE across quartiles of TCC was observed. Further adjustment for BMI did not influence the estimates (Table 3). To consider the possibility of underestimating ORs resulting from regression dilution bias, we estimated ORs for VTE among subjects with high (highest quartile) vs. low (lowest quartile) plasma TCC as a function of time between blood sampling and the VTE events (Figure 1). The OR for VTE by high plasma TCC was substantially higher with shortened time between the blood sampling and the VTE events.

The ORs for DVT and PE across quartiles of plasma TCC are shown in Table 4. The OR for DVT increased across quartiles of plasma TCC (*P* for trend = 0.06). Subjects with plasma TCC values >1.40 CAU/mL had a 38% higher risk of DVT compared with those with TCC ≤ 0.80 CAU/mL (OR: 1.38; 95% CI: 0.93–2.06). Although not statistically significant, the OR of unprovoked PE was higher in subjects with plasma TCC in the highest quartile compared with those in the lowest quartile (OR: 1.58; 95% CI: 0.82–3.10). The estimates did not change after adjustment for BMI (Table 4). The ORs for DVT and PE among subjects with high (highest quartile) vs. low (lowest quartile) plasma TCC were calculated as a function of time between blood sampling and VTE (Figure 2). The ORs for DVT and PE showed essentially similar patterns as the ORs for overall VTE (Figure 1, left panel), and increased substantially with shortened time between blood sampling and the respective events.

To identify genetic variation associated with plasma levels of TCC, we tested for association between genome-wide and complement-related variants and plasma levels of TCC. Figure 3 shows a modified Manhattan plot based on the results of the pQTL analysis with the genome-wide variants marked by gray circles and the complement-related analysis (variants within ±500 kb of the genes involved in the complement system) marked by blue triangles. Age, sex, BMI, and VTE status were included as covariates in the models. No gene variant had a *P* value that was statistically significant either in the genome-wide or in the complement-related analysis.

TABLE 2 Characteristics of the venous thromboembolism (VTE) events (n = 415)

	% (n)
Age at VTE, years	67.4 ± 13.6
Sex, male	48.4 (201)
Deep vein thrombosis	62.2 (258)
Proximal	72.5 (187)
Distal	27.5 (71)
Pulmonary embolism	37.8 (157)
Unprovoked VTE	42.2 (175)
Provoked VTE	57.8 (240)
Surgery/trauma	22.4 (93)
Active cancer	21.4 (89)
Acute medical condition	15.4 (64)
Immobilization	15.9 (66)
Estrogens	7.7 (32)
Other factor	3.9 (16)

4 | DISCUSSION

We investigated the association between complement activation, assessed by plasma levels of TCC, and future risk of VTE in a population-based nested case control study. The risk of VTE increased across quartiles of plasma TCC, and subjects with plasma TCC >1.40 CAU/mL (highest quartile) had a 35% higher risk of overall VTE and 74% higher risk of unprovoked VTE compared with those with TCC ≤0.80 CAU/mL (lowest quartile). The ORs for VTE by plasma TCC increased substantially with shortened time between blood sampling and the VTE events. Further, we found no association between genome-wide and complement-related gene variants and plasma levels of TCC. Our findings indicate that plasma levels of TCC are independent of gene regulation, and that high levels of plasma TCC are associated with VTE risk, and unprovoked events in particular.

Few studies have investigated the link between complement factors and the risk of VTE. In a large population-based cohort (the

TABLE 3 Odds ratios (OR) with 95% confidence intervals (CI) for venous thromboembolism (VTE) according to quartiles of plasma levels of the terminal complement complex (TCC)

Quartiles of plasma TCC (CAU/mL)	Controls	Cases	Model 1 OR (95% CI)	Model 2 OR (95% CI)
All VTE				
≤0.80	220	93	Ref	Ref
0.80–1.04	204	93	1.08 (0.76–1.53)	1.06 (0.75–1.51)
1.04–1.40	213	109	1.22 (0.87–1.71)	1.23 (0.88–1.73)
>1.40	211	120	1.35 (0.97–1.88)	1.33 (0.96–1.86)
P for trend			0.06	0.06
Per 1 SD increase	848	415	1.09 (0.97–1.22)	1.08 (0.97-1.22)
Unprovoked VTE				
≤0.80	220	35	Ref	Ref
0.80–1.04	204	39	1.23 (0.75–2.02)	1.17 (0.71–1.94)
1.04–1.40	213	43	1.31 (0.81–2.15)	1.34 (0.82–2.19)
>1.40	211	58	1.74 (1.10–2.78)	1.72 (1.08–2.75)
P for trend			0.02	0.02
Per 1 SD increase	848	175	1.13 (0.99–1.29)	1.13 (0.99–1.29)
Provoked VTE				
≤0.80	220	58	Ref	Ref
0.80–1.04	204	54	0.99 (0.65–1.51)	0.98 (0.64–1.49)
1.04–1.40	213	66	1.17 (0.78–1.74)	1.15 (0.77–1.73)
>1.40	211	62	1.11 (0.74–1.67)	1.11 (0.74–1.67)
P for trend			0.47	0.47
Per 1 SD increase	848	240	1.03 (0.88–1.19)	1.04 (0.88–1.20)

Notes: Model 1: adjusted for age and sex.
Model 2: adjusted for age, sex and body mass index.

Copenhagen General Population Study), 80 517 Danes were followed on average 4.9 years, in which 1176 developed VTE. Subjects with plasma complement C3 levels in the highest tertile had a 58% higher risk of VTE compared with those in lowest tertile, which declined to 31% higher risk of VTE after additional adjustment for CRP and BMI.[12] Even though the risk estimate was attenuated by adjustment for CRP and BMI, the remaining risk and the pivotal role of complement C3,[12] may suggest that complement C3 is not merely a marker, but a mediator of VTE risk. The latter concept is supported by experiments in C3- and C5-deficient mice which had a lower incidence of VTE and reduced thrombus size compared to wild-type mice in a tissue factor (TF)-dependent model of flow restriction-induced VTE.[25] Recently, we reported that high activity of the classical complement pathway was associated with unprovoked VTE in a small case control study.[26] Furthermore, systemic lupus erythematosus (SLE) patients with a history of VTE had increased deposition of C1q, C3, and C4d on platelets compared with SLE patients without VTE, suggesting that activation of the classical pathway of the complement system may play a role in the pathogenesis of VTE in SLE patients.[27] The authors speculated that the effect was mediated by formation of TCC at the surface membrane of platelets. Accordingly, we found that plasma TCC, the end stage of complement activation, measured in plasma was associated with increased risk of VTE, especially with unprovoked VTE. In contrast to plasma TCC and other activation products, which directly reflect complement activation, plasma levels of individual complement components such as C3 are not a reliable indicators of the degree of complement activation in vivo. Thus, our study strongly support the concept that the degree of complement activation, and not merely concentrations of parent molecules per se, is related to future risk of VTE.

Our study is, to the best of our knowledge, the first to provide actual evidence for an association between complement activation, assessed by plasma TCC, and future risk of VTE. In prospective studies, the biomarker levels are usually measured at the time of inclusion and related to an outcome that occur several years later. Plasma levels of modifiable biomarkers are expected to change over time. Fluctuations in exposure during follow-up will lead to a phenomenon called regression dilution bias,[22] which results in an underestimation of the true association between exposure and outcome. Accordingly, we found that the risk of VTE by plasma levels of TCC declined substantially with time between blood sampling and VTE.

In our study, genome-wide and complement-related gene variants showed no association with plasma levels of TCC. These findings suggest that the plasma levels of TCC are mainly influenced by environmental rather than genetic factors. Autopsy and imaging studies indicate that venous thrombi originate in the valvular sinuses of large veins. The milieu in the valvular sinuses is characterized by

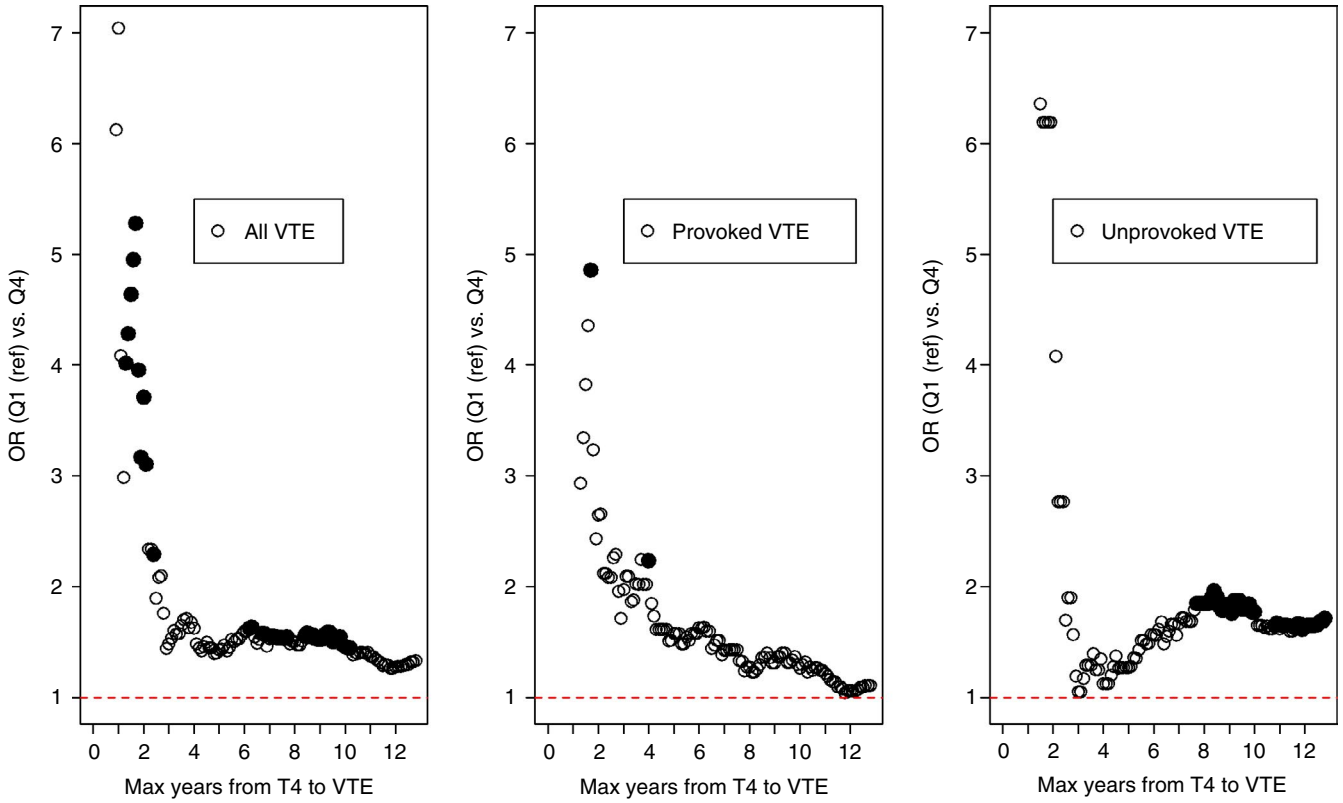

FIGURE 1 Plots of estimated odds ratio (OR) for venous thromboembolism (VTE) as a function of time from blood sampling in Tromsø 4 (1994–1995) and the VTE event (all, provoked, and unprovoked events) in analyses adjusted for age, sex, and body mass index. Large, solid circles indicate ORs with *P* values <0.05

severe hypoxia and stasis,[28,29] conditions known to induce cellular immune responses. Because the complement system is activated by hypoxic cells and tissues,[30] it is reasonable to assume that complement is also activated in the hypoxic milieu in the valvular sinuses. It may be speculated that subjects susceptible to VTE exhibit a certain pathophysiological milieu in the valvular sinuses, which makes them prone to complement activation and thereby development of VTE. Alternatively, other inflammatory conditions associated with increased plasma levels of TCC[31,32] may be differentially distributed between subjects who will and will not develop VTE. However, because the inflammatory response, in our study assessed by plasma CRP levels, secondary to such conditions would be in the causal pathway between plasma TCC and VTE risk, it would be methodological incorrect to adjust for plasma CRP levels in the statistical analyses. In fact, complement activation is upstream of inflammatory responses such as CRP formation,[33] but when CRP is produced and bound to surfaces it is able to activate the classical pathway of the complement system.[34,35]

A clear temporal sequence between exposure and outcome, such as in our nested case control study, is a prerequisite to establish plasma levels of TCC as a risk factor of VTE. Circumstantial evidence support the concept that complement activation, assessed by plasma TCC, may be a mediator of VTE risk through platelet and coagulation activation because it reflects the degree of membrane-inserted C5b-9. First, incorporation of the C5b-9-complex into the cell membrane activates platelets and results in (i) the exposure of negatively charged phosphatidylserine,[36] which may assemble and amplify coagulation reactions[37]; (ii) the formation of procoagulant microvesicles,[38] which is associated with risk of VTE[39]; and (iii) secretion of procoagulant granules from the cytoplasm of platelets.[40] Second, the complement system has direct procoagulant activities, including the ability to cleave and activate coagulation factors and increase TF expression in various cell types. In addition, C5a has the ability to upregulate the synthesis and release of plasminogen activator inhibitor-1, the main endogenous inhibitor of the fibrinolytic system, from mast cells and basophils,[41] which further shifts the prothrombotic-antithrombotic balance in favor of a prothrombotic condition.[42]

Strengths of our study include the recruitment of VTE patients from a population-based cohort and age- and sex-matched, apparently healthy controls from the same source population. It is a large prospective study, with robust measurements of plasma TCC. That blood samples were collected before VTE makes it possible to make assumptions on the direction of the association between exposure (complement activation) and outcome (VTE). Some limitations of the study need to be considered. The subgroup analyses of unprovoked/provoked PE and DVT were underpowered, and the results from these analyses should therefore be interpreted with caution. The blood samples, in which the analysis of plasma TCC was conducted, were drawn in 1994–1995

TABLE 4 Odds ratios (OR) with 95% confidence intervals (CI) for deep vein thrombosis (DVT) and pulmonary embolism (PE) according to quartiles of plasma levels of the terminal complement complex (TCC)

Quartiles of plasma TCC (CAU/mL)	Controls	Cases	Model 1 OR (95% CI)	Model 2 OR (95% CI)
All DVT				
≤0.80	220	56	Ref	Ref
0.80–1.04	204	55	1.07 (0.70–1.62)	1.04 (0.68–1.58)
1.04–1.40	213	73	1.36 (0.91–2.02)	1.34 (0.90–2.00)
>1.40	211	74	1.38 (0.93–2.06)	1.36 (0.91–2.02)
P for trend			0.06	0.07
Per 1 SD increase	848	258	1.05 (0.90–1.20)	1.05 (0.90–1.21)
Unprovoked DVT				
≤0.80	220	19	Ref	Ref
0.80–1.04	204	22	1.28 (0.67–2.46)	1.22 (0.64–2.34)
1.04–1.40	213	28	1.58 (0.86–2.96)	1.59 (0.86–2.99)
>1.40	211	34	1.87 (1.05–3.45)	1.83 (1.02–3.38)
P for trend			0.03	0.03
Per 1 SD increase	848	103	1.05 (0.84–1.25)	1.06 (0.85–1.26)
Provoked DVT				
≤0.80	220	37	Ref	Ref
0.80–1.04	204	33	0.95 (0.57–1.59)	0.93 (0.56–1.55)
1.04–1.40	213	45	1.25 (0.78–2.02)	1.22 (0.75–1.97)
>1.40	211	40	1.12 (0.69–1.83)	1.12 (0.69–1.82)
P for trend			0.43	0.45
Per 1 SD increase	848	155	1.04 (0.86–1.22)	1.04 (0.86–1.22)
All PE				
≤0.80	220	37	Ref	Ref
0.80–1.04	204	38	1.10 (0.67–1.80)	1.07 (0.65–1.77)
1.04–1.40	213	36	1.01 (0.61–1.66)	1.04 (0.62–1.71)
>1.40	211	46	1.30 (0.81–2.09)	1.33 (0.82–2.15)
P for trend			0.35	0.28
Per 1 SD increase	848	157	1.12 (0.97–1.28)	1.12 (0.98–1.28)
Unprovoked PE				
≤0.80	220	16	Ref	Ref
0.80–1.04	204	17	1.16 (0.57–2.39)	1.10 (0.54–2.27)
1.04–1.40	213	15	0.99 (0.47–2.08)	1.00 (0.48–2.10)
>1.40	211	24	1.58 (0.82–3.10)	1.60 (0.83–3.15)
P for trend			0.23	0.20
Per 1 SD increase	848	72	1.19 (1.01–1.37)	1.19 (1.01–1.37)
Provoked PE				
≤0.80	220	21	Ref	Ref
0.80–1.04	204	21	1.05 (0.55–1.99)	1.02 (0.54–1.95)
1.04–1.40	213	21	1.01 (0.53–1.92)	1.03 (0.54–1.97)
>1.40	211	22	1.09 (0.58–2.05)	1.12 (0.59–2.11)
P for trend			0.83	0.74
Per 1 SD increase	848	85	1.02 (0.77–1.25)	1.03 (0.78–1.26)

Notes: Model 1: adjusted for age and sex.
Model 2: adjusted for age, sex, and body mass index.

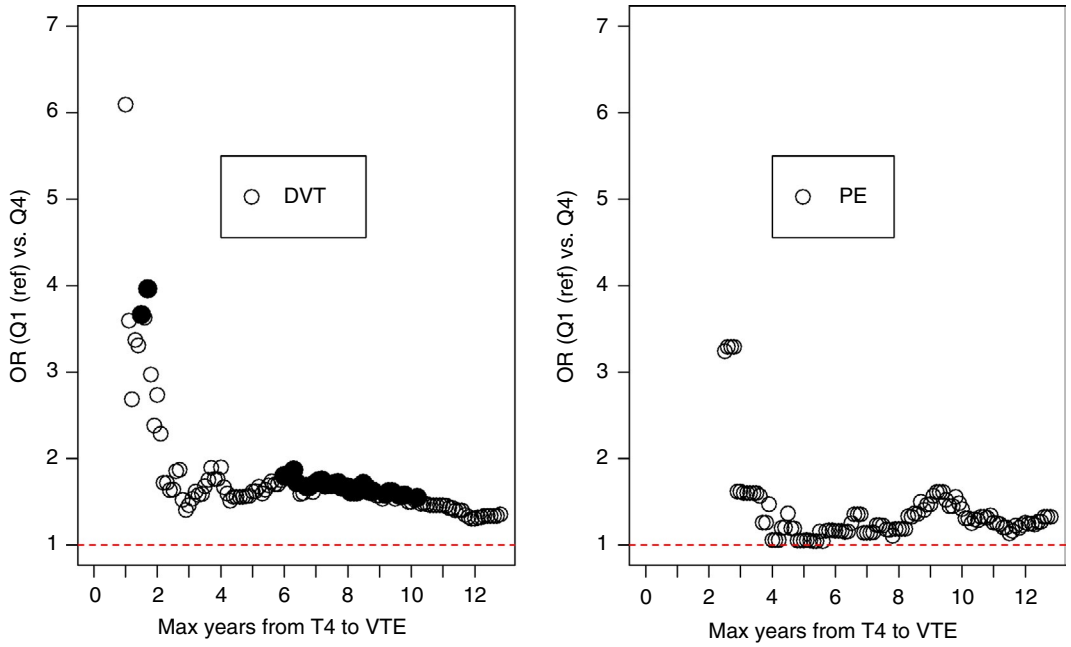

FIGURE 2 Plots of estimated odds ratio (OR) for deep vein thrombosis (DVT) and pulmonary embolism (PE) as a function of time from blood sampling in Tromsø 4 (1994–1995) and event in analyses adjusted for age, sex. and body mass index. Large, solid circles indicate ORs with $P < 0.05$

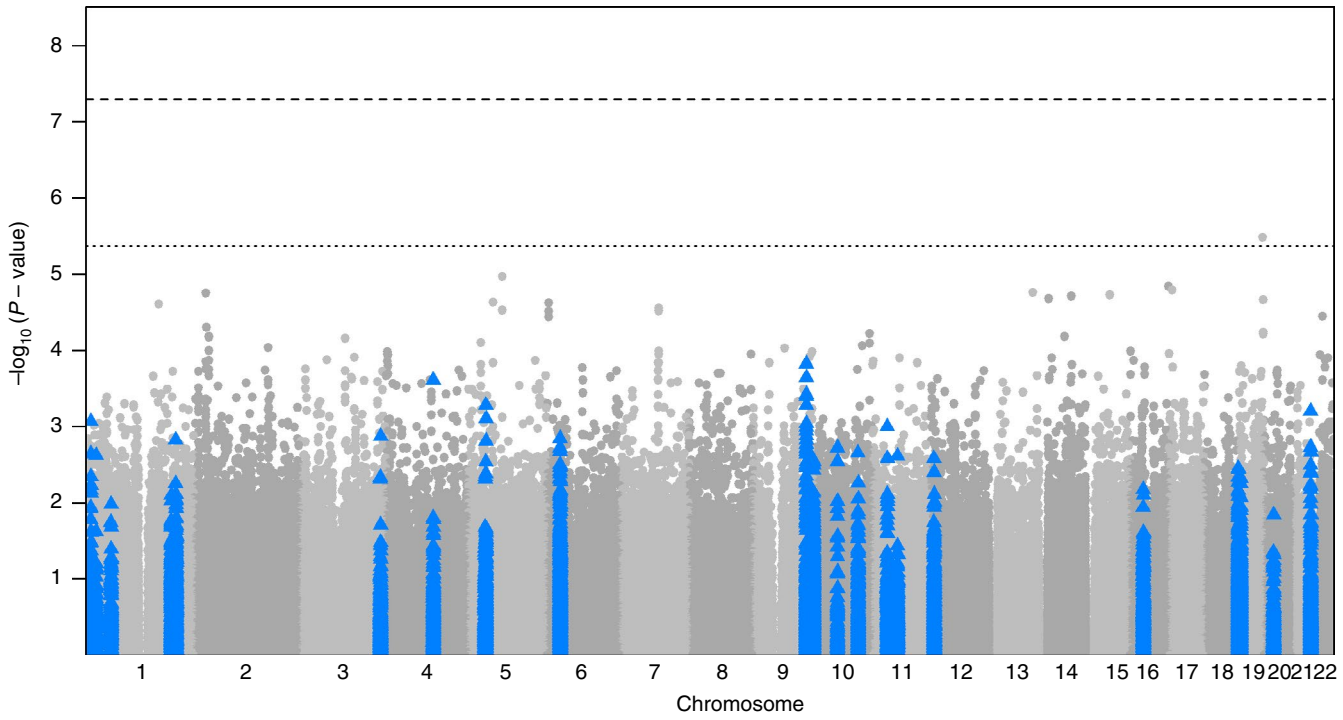

FIGURE 3 Modified Manhattan plot of quantitative trait loci analysis results. The upper, dashed horizontal line indicates the 5 × 108 *P* value significance threshold. As the cis analysis has 11 806 variants being tested, a strict Bonferroni-based control for multiple testing translates to a cis significance threshold of −log_10⁡0.00000423 = 5.37. This is indicated by the lower, dotted horizontal line in the figure. The variants of the cis regions are marked with blue triangles

and stored at −80°C for up to 22 years. The long storage time could potentially affect the plasma levels of TCC; however, it is unlikely that this would change the results because this storage effect would be similar in all samples. Additionally, plasma TCC was only measured at baseline, and changes in TCC levels during follow-up (up to 12 years) could result in underestimation of the true association.[43] Accordingly, we found that the ORs for VTE by plasma TCC decreased substantially with prolonged time between

blood sampling and the VTE events. Finally, plasma TCC is a very stable activation product compared with upstream activation products.[21] Plasma levels of TCC remain unchanged after several freezing and thawing cycles [21] and storage at −70°C for 10 years (T.E Mollnes, unpublished data).

In conclusion, results from our nested case control study imply that complement activation, assessed by plasma levels of TCC, is associated with increased risk of VTE, and unprovoked events in particular. Genome-wide and complement-related gene variants were not associated with plasma levels of TCC, suggesting that local or systemic environmental factors are the dominating determinants of complement activation leading to increased plasma TCC. Functional studies are warranted to investigate the molecular mechanisms behind the association between plasma TCC and VTE risk.

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DISCLOSURE OF CONFLICT OF INTERESTS

The authors state that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

I. I. Høiland analyzed data and wrote and revised the manuscript. R. A. Liang analyzed data and revised the manuscript. S. K. Brækkan analyzed data and participated in the statistical analysis. N. Latysheva, K. Pettersen, and J. K. Ludviksen performed the laboratory analysis. O. Snir analyzed data and participated in writing and revising the manuscript. T. Ueland analyzed data and participated in writing and revising the manuscript. K. Hindberg analyzed data, performed the protein quantitative trait loci analysis, and participated in writing and revising the manuscript. T. E. Mollnes analyzed data and participated in writing and revising the manuscript J.-B. Hansen conceived and designed the study, analyzed data, and participated in writing and revising the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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ORIGINAL ARTICLE

Plasma levels of mannose-binding lectin and future risk of venous thromboembolism

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Abstract

Background: Animal and observational studies have suggested a pathophysiological role for complement in venous thromboembolism (VTE), but the initiating mechanisms are unknown. Mannose-binding lectin (MBL) bound to altered host cells leads to activation of the lectin complement pathway, and both high and low MBL levels have been implicated in the pathophysiology of cardiovascular disease.

Objectives: To investigate the association between plasma MBL levels and future risk of incident VTE.

Methods: We conducted a nested case-control study in 417 VTE patients and 849 age-matched and sex-matched controls derived from the general population (Tromsø Study). Plasma MBL levels were measured using enzyme-linked immunosorbent assay. Logistic regression models were used to estimate odds ratio (OR) for VTE across quartiles of plasma MBL levels.

Results: Subjects with plasma MBL levels in the lowest quartile (<435 ng/mL) had a reduced OR for overall VTE (OR 0.79, 95% confidence interval [CI]: 0.56-1.10) and for DVT (OR 0.70, 95% CI: 0.47-1.04) compared to those with MBL in the highest

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quartile (≥2423 ng/mL) after multivariable adjustments. For VTE, DVT, and pulmonary embolism (PE) the ORs decreased substantially with decreasing time between blood sampling and VTE event.

Conclusions: Our findings suggest that low plasma MBL levels are associated with reduced risk of VTE, and DVT in particular.

KEYWORDS

complement, deep vein thrombosis, mannose-binding lectin, pulmonary embolism, venous thromboembolism

1 | INTRODUCTION

Venous thromboembolism (VTE), including DVT and pulmonary embolism, affects 1 to 2 per 1000 individuals each year. It is a major public health challenge because of short-term and long-term complications, such as frequent recurrence and potentially death.[1–4] Inherited and environmental risk factors along with changes in blood flow, hypercoagulability, or dysfunction of the vessel wall affect individual thrombosis potential.[5,6] Despite improved awareness and prevention, the incidence of VTE has remained unchanged or even increased marginally over the past decades.[2,7] In order to diminish the health burden of VTE, it is imperative to identify novel biomarkers and unravel underlying disease mechanisms in order to improve risk prediction and provide targeted prevention and treatment.

Recent studies have implicated a role for the complement system in the pathogenesis of VTE due to an extensive cross-talk between the complement and hemostatic systems.[8–10] Complement factor C3 is an acute-phase reactant and a central component in the activation of the complement system.[11] Results from a large population-based cohort in Copenhagen showed that participants with plasma complement C3 levels in the highest tertile had a 58% higher risk of VTE compared to those in the lowest tertile. The risk estimate declined to 31% but was still significant after further adjustment for C-reactive protein (CRP) and body mass index (BMI).[12] In an inferior vena cava stenosis model, C3-deficient mice had a lower incidence of venous thrombosis and developed thrombi that were smaller in weight and size compared to those of wild-type controls.[13] The latter findings may suggest that complement C3 is a mediator rather than only a marker of VTE risk.

Mannose-binding lectin (MBL) is a pattern recognition molecule that binds to carbohydrates such as mannose on pathogens or damaged host cells and thereby activates the lectin pathway of the complement system.[14,15] The MBL circulates in molecular complexes with serine proteases called MBL-associated serine protease-1, MBL-associated serine protease-2, and MBL-associated serine protease-3 (MASPs-1,-2,-3).[14,15] The MASP-1 and MASP-2 are activated when MBL binds to specific carbohydrate structures on microbial and cell surfaces. This leads to cleavage of complement factors C4 and C2 and the formation of C4b2b convertase, with subsequent activation of C3 and the common complement pathway.[16] *In vitro* studies have shown that MASP-1 has thrombinlike activity and can cleave factor XIII (FXIII), fibrinogen, high-molecular-weight kininogen, and thrombin-activatable fibrinolysis inhibitor, while MASP-2 can cleave prothrombin to thrombin.[15,17] The MASPs can activate and stabilize clot formation,[15] and *in vivo* animal studies show that MASPs likely have a role in thrombogenesis.[18,19]

Essentials

- The initiating mechanisms for the role of complement in VTE is unknown.
- Mannose-binding lectin (MBL) leads to activation of the lectin complement pathway.
- Low plasma MBL was associated with a reduced risk of VTE, especially DVT.
- The OR for VTE decreased with decreasing time between blood sampling and event.

Plasma levels of MBL are largely determined by genotypes of the *MBL2* gene[20] and remain rather stable within individuals despite a moderate increase during an acute-phase response.[21,22] The MBL levels vary markedly between individuals because of the variation in the *MBL2* gene,[23] and approximately 5% to 20% of the population is MBL-deficient with functional levels below 100 ng/mL.[24–27] Thus, low levels of MBL have been suggested as a reliable surrogate marker of variation in the *MBL2* gene. The association between plasma levels of MBL and risk of VTE has not been thoroughly investigated. Given the procoagulant effects of MASPs *in vitro* and in animal models, it is likely that low levels of MBL would protect against development of VTE. However, in patients with systemic lupus erythematosus, *MBL2*-deficient genotypes were associated with increased[28] or unchanged[29] risk of VTE, whereas low plasma levels of MBL (<100 ng/mL) were associated with increased VTE risk in a small case-control study recruited from the general population.[30] The conflicting results may partly be explained by chance because of the low number of participants included in these studies, inconsistent patient selections, or the retrospective nature of the case-control study with the potential risk of reverse causation. The aim of the present study was

therefore to investigate the association between plasma levels of MBL and risk of VTE in a nested case-control study derived from the general population.

2 | METHODS

2.1 | Study population

The Tromsø Study is a single-center, population-based cohort, with repeated health surveys of inhabitants of Tromsø, Norway. Members of the population aged ≥25 years living in the municipality of Tromsø were invited to participate in the fourth survey, conducted in 1994-1995. A total of 27 158 subjects participated (77% of those invited) and were followed from the date of inclusion until an adjudicated incident VTE event, migration, death, or end of follow-up (1 September 2007). All first lifetime events of VTE occurring among the participants in this period were identified using the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry from University Hospital of North Norway (UNN), which is the sole provider of diagnostic radiology and treatment of VTE in the Tromsø area. Participants with a history of VTE before baseline were excluded. Trained personnel adjudicated and recorded each VTE by extensively reviewing medical records. The identification and adjudication process of VTEs has previously been described in detail.[31] In short, the adjudication criteria for VTE were presence of signs and symptoms of DVT or PE combined with objective confirmation by radiological procedures, which resulted in initiation of treatment (unless contraindications were specified). A VTE occurring in the presence of one or more provoking factors was classified as provoked. Provoking factors were surgery or trauma (within 8 weeks before the event), acute medical condition (acute myocardial infarction, acute ischemic stroke, acute infections), immobilization (bed rest >3 days or confinement to wheelchair within the last 8 weeks, or long-distance travel ≥4 h within the last 14 days), or other factors specifically described as provoking by a physician in the medical record (e.g., intravascular catheter).

There were 462 individuals who experienced a VTE event during the follow-up period (1994-2007). For each case, two age-matched and sex-matched controls, who were alive at the index date of the VTE event, were randomly sampled from the source cohort (n = 924). In total, 45 cases and 75 controls did not have plasma samples of sufficient quality available for the analyses. Thus, our final nested case-control study consisted of 417 cases and 849 controls. The regional committee for medical and health research ethics approved the study, and all participants provided written consent.

2.2 | Baseline measurements

Height (to the nearest centimeter) and weight (to the nearest 0.5 kg) were measured in participants wearing light clothing and no shoes. Body mass index (BMI) was calculated as weight divided by the square of height in meters (kg/m^2). A self-administered questionnaire was used to collect a detailed history of previous cardiovascular disease (CVD) events (stroke, angina pectoris, transient ischemic attack, and myocardial infarction), recurrent VTE, diabetes mellitus, and other concurrent diseases. The questionnaire also included questions about dietary habits, physical exercise, smoking, and alcohol consumption.

2.3 | Blood sample collection and storage of blood products

At inclusion in Tromsø 4 (1994-1995), non-fasting 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. Platelet poor plasma was prepared by centrifugation at 3000 g for 10 min at room temperature, after which the supernatant was transferred into cryovials (Greiner Labortechnik, Nürtingen, Germany) in 1-mL aliquots and stored at −80°C.

For biomarker measurements in plasma, samples were thawed in a water bath at 37°C for 5 min, followed by centrifugation for 2 min at 13 000 g to obtain platelet-freeplasma.

2.4 | Measurements of plasma levels of CRP and MBL

Plasma levels of high-sensitivity C-reactive protein were measured in duplicates using commercially available reagents by enzyme immunoassay (R&D Systems, Minneapolis, MN) in a 384 format using the combination of a SELMA (Jena, Germany) pipetting robot and a BioTek (Winooski, VT) dispenser/washer (EL406). Absorption was read at 450 nm with a wavelength correction set to 540 nm using an EIA plate reader (Synergy H1 Hybrid, BioTek, Vinooski, VT). The intraindividual and interindividual coefficients of variation were 2.6% and 9.1%, respectively. Oligomerized MBL was measured using enzyme-linked immunosorbent assay (Bioporto Diagnostics A/S, Hellerup, Denmark) according to the manufacturer's instructions. The coefficient of variation was in the range of 3.8% to 5.5%.

2.5 | Statistical analysis

Statistical analyses were carried out using Stata version 15 (StataCorp LLC, College Station, TX, USA) andR version 3.5.2 (The R Foundation for Statistical Computing, Vienna, Austria). The MBL was categorized according to quartile cutoffs in the control population (<435, 435-1367, 1368-2422, ≥2423 ng/mL). Means and proportions of baseline characteristics across quartiles of MBL were calculated using descriptive statistics. Logistic regression models were used to calculate OR of VTE with 95% CI according to quartiles of MBL. The highest MBL quartile was used as the reference group. We also calculated the P value for linear trend across decreasing quartiles of MBL. Separate analyses were also conducted with unprovoked VTE, DVT, and PE as the outcomes.

The results were based on a single baseline measurement with long follow-up (>12 years for many individuals) and could be

TABLE 1 Distribution of baseline characteristics according to quartiles of plasma levels of MBL

	Quartiles MBL			
	Q1 (<435 ng/mL)	Q2 (435-1367 ng/mL)	Q3 (1368-2422 ng/mL)	Q4 (≥2423 ng/mL)
n	310	320	311	325
Age (years)	62 ± 13	60 ± 13	61 ± 14	59 ± 15
Sex, % men (*n*)	44.5 (138)	45.6 (146)	43.7 (136)	54.7 (178)
BMI, kg/m^2	26.8 ± 4.3	26.7 ± 4.4	26.7 ± 4.3	25.4 ± 3.8
Smoking, % (*n*)	28.7 (89)	28.4 (91)	28.3 (88)	38.8 (126)
hsCRP, mg/L	1.71 ± 1.5	1.67 ± 1.4	1.50 ± 1.2	1.63 ± 1.4
CVD[a], % (*n*)	16.1 (50)	13.1 (42)	18.0 (56)	16.3 (53)
Cancer[b], % (*n*)	3.1 (10)	6.8 (21)	4.4 (14)	3.9 (12)
Diabetes[c], % (*n*)	2.60 (8)	5.31 (17)	3.87 (12)	4.01 (13)

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; hsCRP, high-sensitivity C-reactive protein; MBL, mannose-binding lectin.
[a]Self-reported history of cardiovascular disease (myocardial infarction, angina, stroke).
[b]History of cancer before baseline.
[c]Information on diabetes status was missing in four persons.

TABLE 2 Characteristics of the VTE events (*n* = 417)

	% (n)
Age at VTE (years)	67.3 ± 13.7
Sex (males)	48.2 (201)
Deep vein thrombosis	62.4 (260)
Pulmonary embolism	37.6 (157)
Unprovoked VTE	42.2 (176)
Provoked VTE	57.8 (241)
Surgery/trauma	22.3 (93)
Acute medical condition	15.6 (65)
Cancer	21.3 (89)
Immobilization	18.0 (75)
Other factors	4.1 (17)

Abbreviation: VTE, venous thromboembolism.

influenced by regression dilution bias. To address this, we performed analyses that restricted maximum time from blood sampling in Survey 4 of the Tromsø Study (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 generated at every 0.1-year increase in time since blood sampling and plotted as a function of the maximum time.

3 | RESULTS

The distribution of baseline characteristics of study participants according to quartiles of MBL is shown in Table 1. The mean age (ranging from 59 to 62 years) was similar across quartiles. The mean BMI was lowest (25.4 kg/m^2) in the highest quartile of MBL. The proportions of males and smokers were highest in the highest quartile (54.7% and 38.8%, respectively). The proportion of participants with cancer was highest (6.8%) in the second lowest quartile. There was no obvious trend in the mean high-sensitivity C-reactive protein measurements and the proportion of participants with a history of CVD across quartiles.

The characteristics of the VTE patients are shown in Table 2. The mean age at the time of VTE was 67.3 years, and 48.2% were men. In total, 62.4% of the events were DVTs and 37.6% of the events were PEs, and 42.2% of the events were unprovoked. Surgery/trauma was the most common provoking factor (22.3%), followed by cancer (21.3%), immobilization (18.0%), and acute medical conditions (15.6%).

The risk of VTE, DVT, and PE across quartiles of plasma levels of MBL is shown in Table 3. Subjects with plasma MBL levels in the lowest quartile (<435 ng/mL) had a lower OR for VTE (OR 0.87, 95% CI: 0.62-1.21) compared to those with MBL in the highest quartile (≥2423 ng/mL) in a model adjusted for age and sex. The OR for VTE was slightly lower with further adjustment for BMI and CRP (OR 0.79, 95% CI: 0.56-1.10). The association was stronger for DVT than PE. Subjects with plasma MBL levels in the lowest quartile (<435 ng/mL) had a lower OR for DVT (OR 0.76, 95% CI: 0.51-1.13) compared to those with MBL in the highest quartile (≥2423 ng/mL) in a model adjusted for age and sex, and the OR decreased further (OR 0.70, 95% CI: 0.47-1.04) after additional adjustment for BMI and C-reactive protein. There was no clear association between plasma levels of MBL and risk of PE. The ORs for unprovoked events were essentially similar to the ORs of all (provoked and unprovoked) events (Table 4).

To consider the possibility of underestimating ORs because of regression dilution bias, we estimated ORs for VTE and subgroups (DVT and PE) among subjects with lowest (lowest quartile) versus highest (highest quartile) plasma MBL as a function of time between blood sampling and the VTE events (Figure 1). The OR by low plasma MBL was substantially lower with shortened time between the blood sampling and the VTE events. The ORs for DVT

TABLE 3 Odds ratios with 95% confidence intervals for venous thromboembolism and VTE subgroups (DVT and PE) according to quartiles of plasma levels of mannose-binding lectin

Quartiles of MBL (ng/mL)	Controls	Cases	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Overall VTE				
≥2423	212	113	Reference	Reference
1368-2422	213	98	0.87 (0.62-1.21)	0.81 (0.58-1.13)
435-1367	212	108	0.96 (0.69-1.33)	0.88 (0.63-1.22)
<435	212	98	0.87 (0.62-1.21)	0.79 (0.56-1.10)
P for trend			0.6	0.2
DVT				
≥2423	212	75	Reference	Reference
1368-2422	213	63	0.84 (0.57-1.23)	0.79 (0.53-1.17)
435-1367	212	65	0.87 (0.59-1.27)	0.80 (0.54-1.18)
<435	212	57	0.76 (0.51-1.13)	0.70 (0.47-1.04)
P for trend			0.2	0.1
PE				
≥2423	212	38	Reference	Reference
1368-2422	213	35	0.92 (0.56-1.52)	0.85 (0.51-1.41)
435-1367	212	43	1.14 (0.71-1.84)	1.04 (0.64-1.69)
<435	212	41	1.09 (0.67-1.76)	0.96 (0.59-1.57)
P for trend			0.6	0.9

Note: Model 1: adjusted for age and sex. Model 2: adjusted for age, sex, body mass index, and C-reactive protein.
Abbreviations: CI, confidence interval; DVT, deep vein thrombosis; OR, odds ratio; PE, pulmonary embolism; VTE, venous thromboembolism.

and PE showed essentially similar patterns to the ORs for overall VTE (Figure 1) and decreased substantially, particularly for PE, with shortened time between blood sampling and the respective events.

In the sensitivity analyses, we tested whether the association between low plasma MBL levels and low OR for VTE was influenced by comorbidities that could occur as a consequence of low MBL levels and were established triggers for VTE (Tables S1 and S2). The ORs are shown for VTE and subgroups (DVT and PE) in quartiles of MBL in participants without cancer (Table S1) and without those who developed myocardial infarction or stroke or had acute infections that required hospitalization during the last 3 months before the VTE event (Table S2). The results were essentially similar to those of the total study population, indicating that the association between plasma MBL and VTE risk was not influenced by other comorbidities such as cancer, arterial CVD, and acute infection.

4 | DISCUSSION

In the present study, we investigated the association between plasma MBL levels and future risk of VTE in a large nested case-control study derived from the general population. We found that approximately 13% of the participants had low levels of plasma MBL (100-499 ng/mL) and that 12% of participants were MBL-deficient (<100 ng/mL), results that are similar to findings from previous studies of Scandinavian populations.[32,33] The risk of VTE, and DVT in particular, was lower in subjects with low plasma levels of MBL. Subjects with plasma MBL levels in the lowest quartile had a 30% lower OR for DVT (OR: 0.70; 95% CI: 0.47-1.04) compared to those with plasma MBL in the highest quartile. The ORs for VTE, and PE in particular, by plasma MBL decreased substantially with shortened time between blood sampling and the VTE events and were not influenced by other comorbidities such as cancer, arterial CVD, or acute infection. Our findings support the hypothesis that low plasma levels of MBL protect against VTE.

Our study is, to the best of our knowledge, the first to investigate the association between plasma levels of MBL and future risk of VTE in the general population. Subjects with MBL levels in the lowest quartile had a 21% and 30% lower OR of VTE and DVT, respectively, compared to those in the highest quartile. Even though plasma levels of MBL are mainly determined by the *MBL2* genotype,[20,34] they are also influenced by age, sex, and hormonal status and may increase 2-fold to 3-fold upon inflammatory responses.[21,35] Plasma levels of modifiable biomarkers are expected to change over time. Fluctuations in exposure during follow-up will lead to a phenomenon called regression dilution bias,[36] which usually results in an underestimation of the true association between exposure and outcome. Accordingly, we found that the risk of VTE by plasma levels of MBL declined substantially with shortened time between blood sampling and VTE (Figure 1).

Previously, few studies have investigated the association between MBL and VTE risk. In a cohort of 91 Danish patients with systemic lupus erythematosus followed for 9 years, 14 developed VTE and the *MBL2* genotype was not associated with risk of VTE.[29] In a cross-sectional

TABLE 4 Odds ratios with 95% confidence intervals for unprovoked venous thromboembolic events and unprovoked events in venous thromboembolic event subgroups (DVT and PE) according to quartiles of plasma levels of mannose-binding lectin

Quartiles of MBL (ng/mL)	Controls	Cases	Model 1 OR (95% CI)	Model 2 OR (95% CI)
Unprovoked VTE				
≥2423	212	46	Reference	Reference
1368-2422	213	42	0.93 (0.59-1.47)	0.85 (0.53-1.36)
435-1367	212	47	1.04 (0.66-1.63)	0.94 (0.60-1.49)
<435	212	41	0.92 (0.58-1.46)	0.80 (0.50-1.29)
P for trend			0.9	0.5
Unprovoked DVT				
≥2423	212	28	Ref	Ref
1368-2422	213	29	1.05 (0.60-1.84)	0.98 (0.55-1.72)
435-1367	212	26	0.95 (0.53-1.67)	0.86 (0.48-1.53)
<435	212	21	0.78 (0.43-1.42)	0.67 (0.36-1.24)
P for trend			0.4	0.2
Unprovoked PE				
≥2423	212	18	Ref	Ref
1368-2422	213	13	0.74 (0.35-1.54)	0.68 (0.32-1.43)
435-1367	212	21	1.19 (0.62-2.30)	1.09 (0.56-2.12)
<435	212	20	1.15 (0.59-2.24)	1.01 (0.52-2.00)
P for trend			0.4	0.6

Note: Model 1: adjusted for age and sex. Model 2: adjusted for age, sex, body mass index, and C-reactive protein.

Abbreviations: CI, confidence interval; DVT, deep vein thrombosis; MBL, mannose-binding lectin; PE, pulmonary embolism; VTE, venous thromboembolism.

study of 114 Spanish SLE patients, the patients with *MBL2*-low genotypes had a higher prevalence of VTE than those with normal MBL genotypes (22% vs. 4%, respectively, $P = 0.016$).[28] However, the increased VTE risk was, according to the authors, at least in part attributed to the coexistence of antiphospholipid syndrome. There are several possible explanations for the apparent conflict with our results showing a protective effect of low plasma MBL levels on future risk of VTE. First, MBL deficiency is a predisposing factor for the incidence[37,38] and severity of systemic lupus erythematosus,[39] as well as the frequency of infectious complications. Systemic lupus erythematosus[40] and acute infectious diseases[41] are associated with increased risk of VTE and may therefore counterbalance the beneficial effect of low MBL levels. Second, although mainly determined by the *MBL2* genotype, there is no stringent relationship between *MBL2* genotypes tested in previous studies and plasma MBL levels. In a merged population consisting of 1642 healthy individuals, *MBL2*-deficient genotypes had sensitivity of 82%, specificity of 82%, and negative predictive value of 98% to predict serum levels of MBL <500 ng/mL.[42] The established *MBL2*-deficient genotypes will therefore lead to non-differential misclassification of plasma MBL levels that could lead to an underestimation of the true association between low MBL and VTE risk.[36,43] In a small retrospective case-control study including 24 patients with unprovoked VTE without comorbidities and 24 age-matched and sex matched controls,[30] we found that the prevalence of MBL-deficiency (MBL <100 ng/mL) was higher in VTE patients (33.3%) than in age-matched and sex-matched controls (12.5%). The higher prevalence of MBL deficiency in VTE patients in the case-control study was surprising and unexpected and encouraged us to perform a larger prospective study with sufficient power and to avoid the possibility of reverse causation.

We hypothesized that low MBL levels would protect against development of VTE. The procoagulant effects of MASPs on coagulation factors, endothelial cells, and platelets link MBL and the lectin pathway to thrombogenesis.[15,17–19,44] Both MASP-1 and MASP-2 have been shown to cleave prothrombin to thrombin.[17,45] The MASP-1 has a thrombinlike substrate specificity and cleaves fibrinogen, FXIII, high-molecular-weight kininogen, and thrombin-activatable fibrinolysis inhibitor, thereby contributing to both clot formation and stabilization.[15,17,18,46] Like thrombin, MASP-1 can activate PAR4, a receptor responsible for the activation and aggregation of platelets as well as proinflammatory processes such as leukocyte recruitment to endothelial cells.[44] Human umbilical vein endothelial cells exposed to oxidative stress, such as hypoxia-reperfusion, are able to bind MBL and thereby activate the complement system through the lectin pathway.[47–49] *In vivo* animal models have shown that the lectin pathway is indeed activated in ischemia-reperfusion and furthermore in thrombus formation. In a model where knock-in mice expressed human MBL, the monoclonal antibody 3F8 inhibiting MBL prevented arterial thrombosis and limited the injury in infarction.[50] A rat model of ischemia-reperfusion injury showed that anti-MBL antibodies protected the myocardium against tissue injury.[51] The MBL-MASP complexes, particularly with MASP-1, were found to play a role in arterial thrombus formation both *in vitro* and *in vivo* in a mouse thrombosis

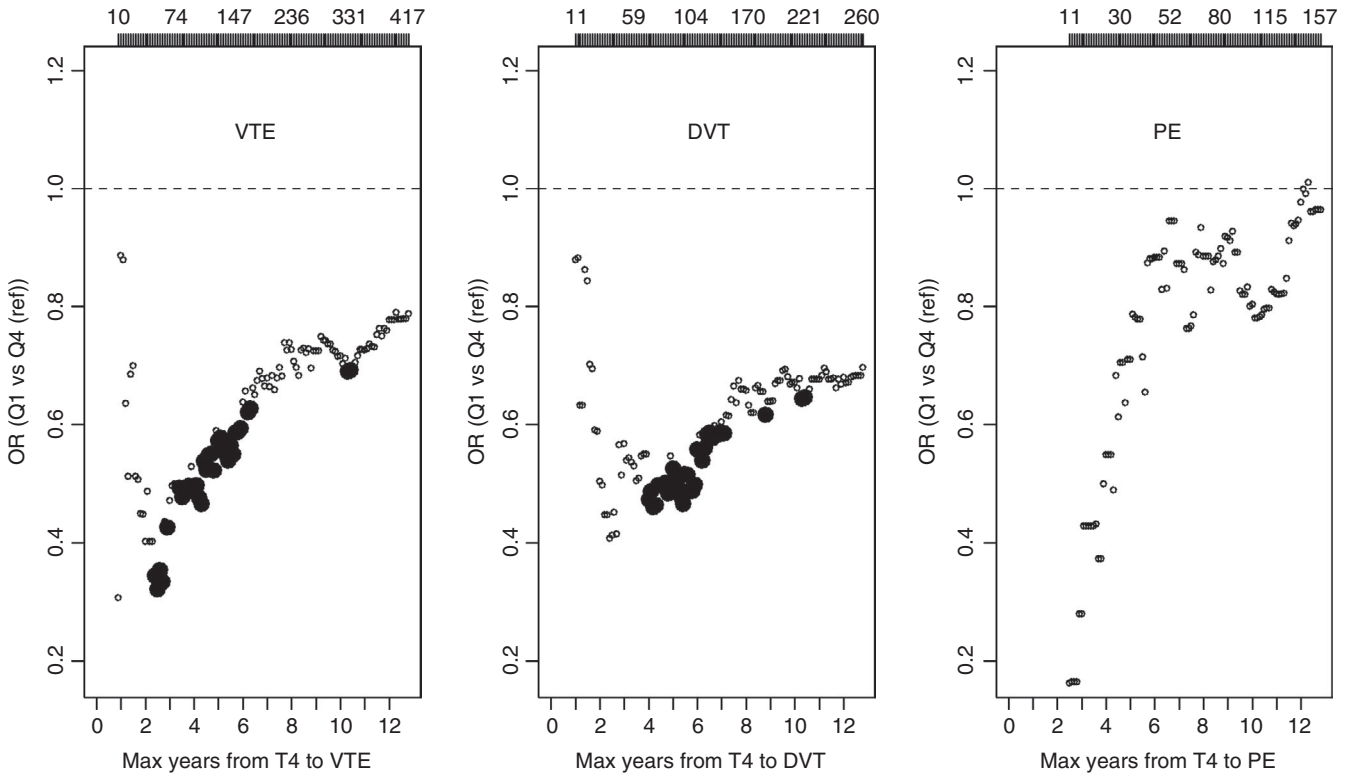

FIGURE 1 Plots of estimated ORs for overall VTE, DVT, and PE as a function of time from blood sampling in the fourth survey of the Tromsø Study (1994-1995) and event in analyses adjusted for age, sex, BMI, and hsCRP. Large, solid circles indicate ORs with *P* values < 0.05. CI, confidence interval; DVT, deep vein thrombosis; OR, odds ratio; PE, pulmonary embolism; VTE, venous thromboembolism

model.[19] We would expect a similar activation of the lectin pathway in the valvular sinuses of the deep veins, where DVT has been shown to originate,[52] because of the severe local hypoxia.[53] As plasma MBL levels correlate well with lectin pathway activity,[30,54] it is reasonable to presume that low MBL levels to some extent would suppress lectin pathway activity and in this way limit thrombus formation.

Mannose-binding lectin-deficient individuals are susceptible to other diseases, such as various types of infectious disease, autoimmune disorders, and arterial CVD.[35,55] These diseases are known to be associated with VTE risk[40,41,56,57] and could thereby counterbalance the potential beneficial effect of MBL deficiency. Mannose-binding lectin deficiency has been associated with advanced atherosclerosis[58,59] and a higher risk of myocardial infarction, independent of other traditional risk factors.[60,61] In contrast, other studies have reported an association between high levels of MBL and risk of ischemic stroke[62–64] and coronary artery disease.[65,66] In our study, low plasma levels of MBL protected against future risk of VTE, and the risk estimates remained similar in the sensitivity analyses accounting for other diseases (Tables S1 and S2).

The strengths of our study include the recruitment of VTE patients from a population-based cohort with age-matched and sex-matched controls from the same source population. It is a large prospective study where blood samples were collected before VTE, allowing assumptions on the direction of the association between exposure (plasma levels of MBL) and outcome (VTE). The blood samples used for plasma MBL analysis were drawn in 1994-1995 and stored at −80°C for up to 22 years. The long storage time could potentially affect the plasma levels of MBL. However, it is unlikely that it would affect the results, as the potential storage effect would be similar in cases and controls. Plasma samples were thawed and refrozen at least twice in preparation for analysis. Nonetheless, this did not likely affect our results as plasma MBL measurements have been shown to remain stable for at least seven freeze/thaw cycles.[67] Plasma MBL was only measured at baseline, and changes in MBL level during follow-up (up to 12 years) could result in underestimation of the true association.[36] Accordingly, we found that the favorable effect of low plasma MBL levels on VTE risk diminished substantially with prolonged time between blood sampling and the VTE event. Of note, the majority of our results did not reach statistical significance and should therefore be interpreted with caution.

In conclusion, the results from our nested case-controls study indicate that low plasma MBL levels were associated with reduced risk of VTE, and DVT in particular. Our findings should be validated and extended to investigate whether *MBL2*-deficient genotypes are associated with reduced VTE risk in population-based cohorts.

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CONFLICT OF INTERESTS

The authors state that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Robin A. Liang analyzed data, wrote, and revised the manuscript. Ina I. Høiland wrote and revised the manuscript. Thor Ueland and Pål Aukrust performed the laboratory analysis and revised the manuscript. Kristian Hindberg and Sigrid K. Brækkan analyzed data and participated in the revision of the manuscript. Omri Snir and Peter Garred provided intellectual input and revised the manuscript. John-Bjarne Hansen and Tom Eirik Mollnes designed the study and participated in the writing and revision of the manuscript. All the authors read and approved the final manuscript.

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Paper III

Stiftelsen Kristian Gerhard Jebsen

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