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Role of the Complement System in the Pathogenesis of Venous Thromboembolism

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Contents

Acknowledgments

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Summary

Venous thromboembolism (VTE) is a common disease with serious short- and long-term complications. VTE is a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE) and is the third most common cardiovascular disease, causing significant morbidity and mortality. Despite preventive strategies, the incidence of VTE has been stable or increasing slightly during the last decades, affecting 1-2 per 1000 individuals each year. Identifying new biomarkers and unraveling underlying mechanisms might help diminish the health burden of VTE. The complement system is a cascade system similar to the coagulation system. The two systems have a high degree of crosstalk and are activated together in many conditions. Polyphosphates (polyPs) are naturally occurring, highly anionic linear polymers of monophosphate units. Short-Chained (SC)-PolyPs are stored and secreted from platelets upon stimulation and are reported to inhibit complement activation and to facilitate propagation of coagulation activation under experimental conditions. This indicates that SC-polyP might modulate the possible link between the two systems, and play a role in the pathogenesis of VTE.

The main goals of this thesis were to assess the association between complement activation and VTE risk, and to investigate the role of SC-polyPs in the activation of these two systems. In paper I, we performed a case-control study to investigate the association between potential complement activity and VTE risk. We found that individuals with high potential activity of the classical pathway of the complement system and individuals with MBL deficiency had higher risk for VTE. In paper II, we conducted a nested-case-control study derived from a population-based cohort (The Tromsø Study) to investigate the relationship between complement activation and future risk of VTE. We found that high degree of complement activation, assessed by plasma terminal complement complex (TCC), was associated with

increased risk of VTE, and especially unprovoked VTE. Results from the whole blood model in Paper III showed that SC-polyPs had the ability to activate FXII and did not alter *E.coli*-induced complement activation. Our findings suggest that complement activation is involved in the pathogenesis of VTE, and that SC-polyP might initiate coagulation activation via FXII without affect complement activation.

Sammendrag

Venøs blodpropp eller venøs tromboembolisme (VTE) er en sykdom med alvorlige komplikasjoner. VTE er en fellesbetegnelse for dyp venetrombose (DVT) og lungeemboli (LE) og er verdens tredje vanligste hjerte- og karsykdom. Til tross for økt fokus på forebygging har forekomsten vært stabil (om lag 1.5 per 1000 innbyggere årlig) eller økende de siste tiårene. Identifiseringen av nye biomarkører kan gi økt forståelse av sykdomsmekanismene ved VTE og bidra til bedre risikostratifisering av sykdommen, som igjen kan redusere de helsemessige konsekvensene. Komplementsystemet er viktig del av vårt immunsystemet. Det er et kaskade system som kan sammenlignes med koagulasjonssystemet, og aktiveringer av de to systemene påvirker hverandre i mange situasjoner. Polyfosfater er naturlige negativ ladede polymerere bestående av monofosfat. Kort kjedede polyfosfater blir utskilt av aktiverte blod plater. Disse er rapportert å hemme komplementsystemet og å fremme koagulasjons aktivering. Dette tilsier at polyfosfater kan modifisere samspillet mellom komplement- og koagulasjonssystemene.

Hovedmålet med denne avhandlingen var å utforske sammenhengen mellom komplementsystemet og risikoen for VTE, i tillegg til å utforske betydningen av kort kjeda polyfosfat i aktiveringen av de to systemene. I artikkel I gjorde vi en kasus-kontroll studie for å undersøke om den potensielle aktiveringen av de ulike aktiveringsveiene i komplementsystemet var assosiert med risikoen for VTE. Vi fant at individer med høy potensiell aktivitet i den klassiske veien av komplement systemet og individer med MBL defekt hadde en høyere risiko for VTE. I artikkel II gjorde vi en nøsta kasus kontroll studie, med deltagere fra Tromsøundersøkelsen, for å utforske om komplement aktivering var assosiert med VTE risiko. Vi fant at individer med høy komplement aktivering (TCC) hadde en høyere risiko for VTE. Fra fullblods modellen i artikkel III fant vi at kort kjeda

polyfosfater aktiverte FXII av koagulasjons systemet, men påvirket ikke *E.coli*-indusert komplement aktivering. Våre resultater tyder på at komplementsystemet kan spille en rolle i sykdomsmekanismene ved VTE, og at kort kjeda polyfosfater aktiverer koagulasjonssystemet via FXII.

List of papers

The thesis is based on the following papers:

Paper I

Impact of complement pathways activity, mannose-binding lectin and tissue-factor induced thrombin generation on the risk of unprovoked venous thromboembolism

Høiland II, Liang RA, Hindberg K, Latysheva N, Brekke OL, Mollnes TE, Hansen JB

Thrombosis Research, 2018 September 169;50-56

Paper II

Complement Activation Assessed by the Soluble 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

Submitted to Journal of Thrombosis and Hemostasis, 2018 December

Paper III

The Effect of Short-Chained Polyphosphates on Coagulation and Complement Activation in a Human Whole Blood Model

Høiland II, Sovershaev T, Liang RA , Latysheva N, Jensen SB, Østerud B, Morrissey JH, Smith SA, Brekke OL, Mollnes TE, Hansen JB

Manuscript

Abbreviations

APC: activated protein C

BMI: body mass index

C: complement

CTPH: chronic thromboembolic pulmonary hypertension

DVT: deep vein thrombosis

EVs: extracellular vesicles

F: factor

FVL: factor V Leiden

HK: high molecular weight kininogen

MPs: microparticles

PAI-1: plasminogen activator inhibitor-1

PE: pulmonary embolism

PK: prekallikrein

PolyP: polyphosphate

PS: phosphatidylserine

PTS: post-thrombotic syndrome

SC: short chained

TAFI: thrombin-activatable fibrinolysis inhibitor

TF: tissue factor

TFPI: tissue factor pathway inhibitor

tPA: tissue plasminogen activator

uPA: urokinase plasminogen activator

VTE: venous thromboembolism

1. General introduction

Venous thromboembolism (VTE) is a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE). DVT is caused by a thrombus that forms in the deep venous system, usually in the lower limbs. A DVT can obstruct the blood flow and cause pain, swelling and redness in the affected part. PE primarily occurs when all, or parts, of a DVT brake off and travels with the blood stream to the lungs. The clot can lodge in the lungs and block the blood flow in the lung [1]. Traditionally PE was thought to be a complication of DVT, however, the origin of the PE remains unknown in 50% of patients [2, 3]. DVT is the most common of these conditions, accounting for up to two thirds of all VTE events [4].

The pathophysiology of VTE is still not fully elucidated. However, autopsy- and imaging studies have shown that venous thrombus formation originates in the valvular sinuses of large veins with an intact vascular endothelium in a milieu characterized by severe hypoxia and stasis [1, 5, 6]. Studies have shown that the complement is important in ischemic reperfusion injury, suggesting that hypoxia may activate the complement system. The complement system, which is an important part of the innate and the adaptive immune system, is cross-linked to the coagulation system in several ways. Few studies have investigated the association between complement components and VTE and found that components of the complement system are associated with a higher risk of VTE. These studies have raised the question of if the complement system is a bystander or a mediator of the VTE risk. It is likely that the complement system is involved in early steps in the pathogenesis of the disease. Platelets have also been found to play a pivotal role in thrombus formation. Activated platelets release short-chained polyphosphates from their dense granules [7]. These inorganic polyphosphates have received increased attention in recent years due to their ability to

activate the coagulation system. There is a need to further explore the pathophysiology of VTE and to discover new players in the formation of the blood clot.

1.1. Epidemiology of Venous thromboembolism

Venous thromboembolism (VTE), occurs in 1-2 per 1000 adults in Western countries each year, making it the third most common cardiovascular disease [8]. Although the incidences of arterial cardiovascular diseases, e.g. myocardial infarction and stroke, have declined by 25-40% during the last two decades [9], the incidence of VTE remains stable or has even increased during the same time period [10, 11].

VTE events can be classified into provoked and unprovoked, depending on the presence of predisposing factors during the 3 months before a VTE event [12]. A provoked VTE event can occur in the presence of transient or persistent risk factors, and transient risk factors can be major or minor [13]. A provoked VTE can occur in the presence of major transient risk factors such as surgery with general anesthesia >30min or Cesarean section in the 3 months before the event, or in the presence of minor risk factors (i.e. surgery with general anesthesia <30min, estrogen therapy, pregnancy) in the 2 months before the event [13]. VTE events can also be provoked by persistent risk factors such as paralysis, wheelchair use, active cancer or any other ongoing non-malignant condition associated with at least a 2-fold increased risk of recurrence after stopping anticoagulant therapy [13, 14]. Population-based studies have estimated that about 50-60% of VTE are associated with the presence of provoking factors [15-17]. In the absence of a provoking factor, the VTE is said to be unprovoked without any obvious cause. The risk of recurrence after a provoked VTE event is generally lower than in the case of a unprovoked event[18].

VTE has major implications for the individual and for society. Short-term complications of VTE include thrombus extension, recurrence in the weeks to months after the first event, and death. The one-month case-fatality rate after VTE diagnosis is reported to be between 6 and 11% [15, 19], and the one-year case-fatality rate is between 17 and 23% [15, 19]. A large Canadian study reported in 2010, 30-day and one-year case-fatality rates of 10.6% (95% CI 10.4-10.8) and 23.0% (95% CI 22.8-23.3) respectively [19]. These numbers was confirmed in a recent Norwegian study including 710 subjects with incident VTE where the overall cumulative mortality rate was 8.5% (95% CI 6.7-11.0) at 30 days and 24.2% (95% CI 21.2-27.6) at 1 year [20]. In cancer patients, the one-year case-fatality rate is as high as 63-88% [15, 21].

Long-term complications of VTE include post-thrombotic syndrome (PTS), chronic thromboembolic pulmonary hypertension (CTPH) and late recurrence. The most common complication of venous thromboembolism is PTS, affecting 20-50% of patients with DVTs and 10-20% of patients with PEs [22]. PTS typically causes chronic pain, swelling and changes in the skin in the affected leg [23], but the pathophysiology is not fully understood and several mechanisms for PTS have been proposed [24]. The end-point of PTS is venous ulcer, which occurs in 5-10% of those with PTS and can be difficult to treat [22, 25]. Risk factors for PTS include older age, obesity, proximal DVT location, recurrent DVTs, varicose veins, and insufficient anticoagulant therapy, whereas traditional risks factors for VTE such as active cancer, surgery, plaster casts or inherited thrombophilias have not been found to influence the risk [23, 26]. CTPH is a rare (0.5-4% of PEs), but serious, complication [27, 28]. CTPH is caused by a chronic obstruction of major pulmonary arteries when a thrombus fails to resolve and undergoes fibrotic transformation [28]. CTPH is characterized by dyspnea, discomfort in the chest, and signs similar to right-sided heart failure [29, 30]. Recurrent VTE

affects up to one-third of patients during a ten-year period after the first event, with the second event tending to occur on the same location as the first (e.g. DVT or PE)[31]. The risk of suffering from a recurrent VTE is most likely in the weeks after initial hospitalization after a DVT [32].

In addition to the obvious personal consequences for a patient suffering complications after experiencing a VTE event, there is also a major economic burden to the society. In a Norwegian study using data from two large population based studies, participants with VTE had a 62% higher risk of disability pension compared to those not experiencing a VTE [33].

1.2 Pathophysiology of VTE

In 1856, the German scientist Rudolph Virchow proposed a triad for the formation of thrombosis. Virchow's triad proposed that thrombus formation was caused by either changes in blood coaguability, changes in the vessel wall, or stasis [6]. Although advances in research have provided us with a deeper understanding of thrombosis, this triad still represents a cornerstone in our understanding of the pathophysiological mechanisms of VTE.

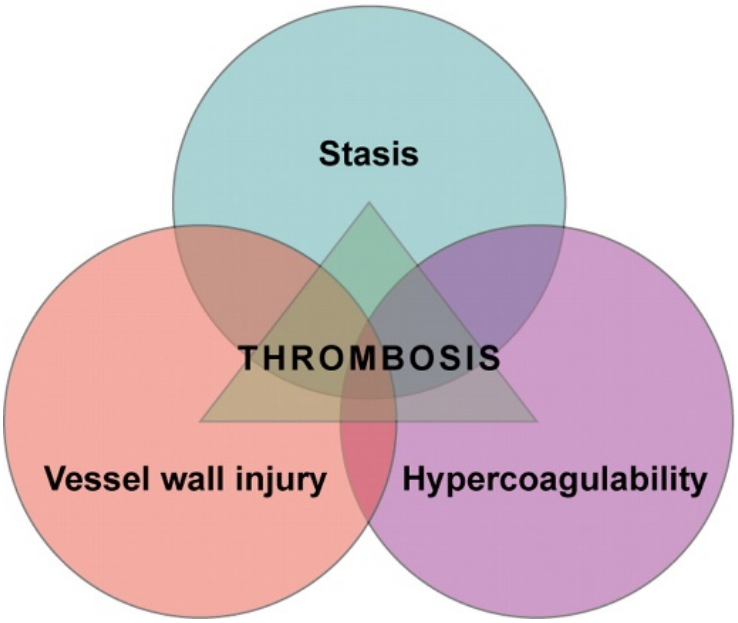

Figure 1. Virchow's triad. Three main factors contributing to thrombus formation: stasis, vessel wall injury, and hypercoagulability. (Adapted from Kyrle & Sabinger, Blood 2009)

Hypercoagulability or an abnormally increased tendency towards blood clotting plays an important part in the pathogenesis of VTE. This can be due to inherited genetic defects such as single nucleotide polymorphisms (e.g. Factor V Leiden and prothrombin G20210A) and deficiencies in natural anticoagulants (e.g antithrombin, protein C and S)[34]. A hypercoagulable state can also be acquired by external factors, and includes obesity, pregnancy, oral contraceptives or hormone replacement therapy [35]. Pregnancy, oral contraceptives and hormone therapy can lead to a state of hyperestrogenemia, which leads to increased hepatic synthesis of procoagulant proteins and decrease the synthesis of anticoagulants [35].

An undamaged endothelium expresses various anticoagulants, such as tissue factor pathway inhibitor (TFPI), endothelial protein C receptor, thrombomodulin, and heparin-like proteoglycans [36]. Damage to the endothelium can lead to tissue factor (TF) expression by a variety of vascular cells, which will activate the coagulation cascade [37]. Together factor (F) VIIa and TF activate the extrinsic pathway of blood coagulation via activation of FIX and FX. A relationship between arterial thrombosis and vessel wall injury is well established, but the role of endothelial damage or dysfunction in venous thrombosis is debatable. Sevitt *et.al.* investigated 50 thrombi taken from autopsies, and did not find evidence of endothelial damage accompanying thrombi, unless associated with acute insults (i.e. surgery or trauma)[5].

The deep recess of the valvular sinuses is recognized as the initiation site of venous thrombus formation as confirmed by autopsy and phlebography studies [1, 6]. Prolonged stasis in a vein, particular in the deepest recess of the valvular sinuses, causes lowered oxygen tension

and cellular hypoxia [1, 38]. This hypoxic state and stasis leads to the upregulation of multiple stress-response genes, including hypoxia inducible factor 1-alpha, P-selectin (CD62), and other adhesion receptors at the endothelial surface as a response to the oxidative stress [39]. The upregulation of stress-response genes leads to a proinflammatory state of the endothelium and recruitment and activation of leukocytes (particularly monocytes and neutrophils), platelets and the formation of extracellular vesicles (EVs). In addition, it can lead to the exposure of TF and initiate the extrinsic pathway of coagulation [38, 40]. Endothelial injury may also activate FXII through the exposure of matrix proteins, and contribute to blood coagulation through the intrinsic pathway of coagulation [41-43]. FXII can be activated by neutrophil extracellular traps (NETs) and in addition, activated platelets can release inorganic polyphosphates which can activate the intrinsic pathway [7, 44].

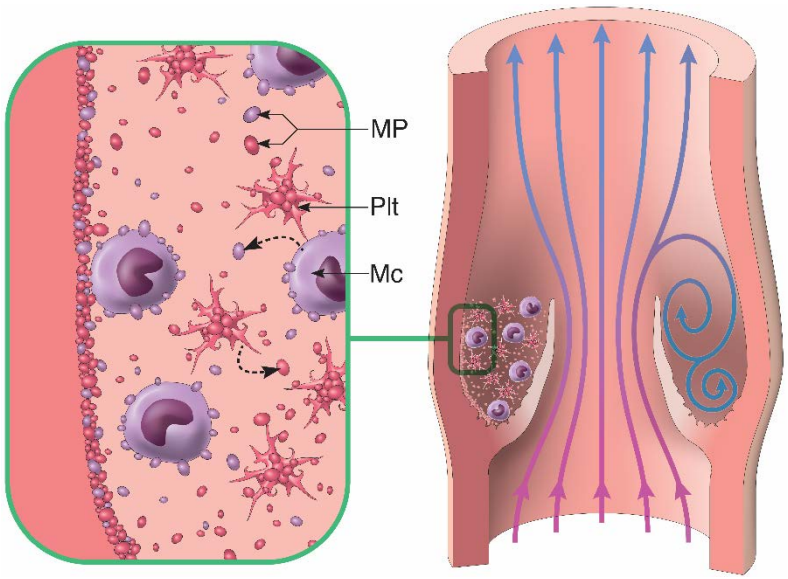

Figure 2. The pathophysiology of venous thrombosis. Blood is trapped by a vortex in the valve pocket, and gets desaturated and creates a hypoxic environment. This activates endothelial cells and white blood cells, such as monocytes (Mc) and platelets (Plt) are recruited. When these cells are activated, tissue factor bearing extracellular vesicles, also called micro particles (MP), bud off and contribute to coagulation and thrombus formation.

1.3 Risk factors for VTE

Several acquired and inherited risk factors are associated with VTE. A risk factor is defined as any characteristic, attribute, or exposure of an individual that increases the chance of developing a disease. For VTE, most often more than one risk factor has to be present for an event to occur. This is explained by the thrombosis potential model, which shows how combinations of hereditary risk factors, advancing age and provoking factors may yield a thrombosis potential exceeding an individual's thrombosis threshold potential (Figure 3).

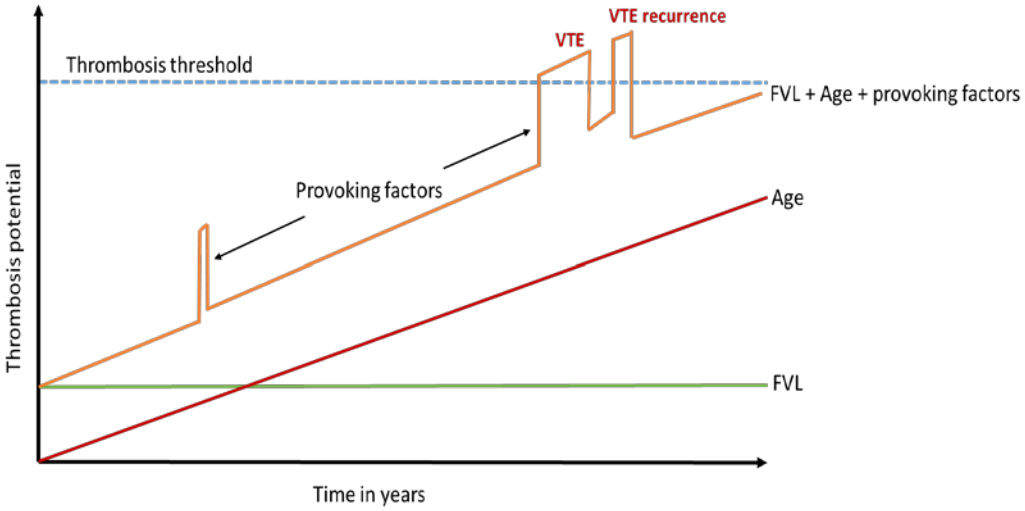

Figure 3. The thrombosis potential model. The green line represents intrinsic factors that are stable over time such as Factor V Leiden (FVL), and the red line represents the effect of a risk factor that increases over time, like age. The orange line demonstrates the joint effects of FVL and age in combination with provoking factors. Provoking factors early in life may not be enough to reach the thrombosis threshold. However, a provoking factor later in life may exceed the thrombosis threshold and results in a VTE. If the thrombosis potential remains increased following a VTE event, a provoking factor may exceed the thrombosis threshold again and result in a recurrent VTE (adapted from Rosendaal, Lancet 1999).

1.3.1 Heritable risk factors for VTE

Family and twin studies clearly show that hereditary factors are important for the risk of VTE. It is estimated that the heritability of VTE is around 50% [45-47]. Non-O blood groups is a prevalent genetic trait (present in 60-65%) and individuals with non-O blood group have a 1.5-2-fold increased risk of VTE compared to individuals with O blood group [48-51]. In a

large meta-analysis, non-O blood group (A_1 and B) was present in 70% of VTE cases and 54% of controls [50]. The increased risk of VTE associated with non-O blood group is partly mediated through increased levels of von Willebrand factor (vWF) and FVIII [49, 52]. However, when vWF and FVIII were taken in to account in the risk analysis, non-O blood group were still associated with increased risk of VTE, suggesting that other mechanisms are also involved [53, 54].

Factor V Leiden (FVL) is a mutation in the FV gene, which leads to reduced ability of FV to be inactivated by activated protein C (APC) [55, 56], and is associated with a 3-fold increased risk of having a VTE [47]. The mutation is present in approximately 5% of the Caucasian population in heterozygous form [48, 56, 57]. Homozygous form of FVL is far less common [57], but individuals with homozygous FVL have a higher risk of VTE compared to heterozygous individuals [58]. Those with homozygous form of FVL experience thrombosis at a younger age and they have a higher rate of recurrence of VTE than controls [59]. The "FV Leiden paradox" refers to the fact that FVL is associated with a higher risk of DVT than PE [60]. FVL has a synergistic effect with other factors such as oral contraceptives [61, 62], pregnancy [63], smoking [64], obesity [64] and cancer [65] on the risk of VTE.

Increased levels of coagulation factors are associated with a higher risk of VTE [66]. Prothrombin 20210A is a mutation is present in approximately 2% of the Caucasian population [48, 67], leads to increased levels of plasma prothrombin levels [68] and is associated with a 3-fold increased risk of VTE [68]. The mutation is due to a single base pair substitution at nucleotide position 20210 located at the 3' untranslated region of the non-coding region of the prothrombin gene on chromosome 11 [68].

Protein S and C serves as regulators of the coagulation cascade, and deficiencies in these are present in less than 1% of the general population [69]. Activated protein C (APC) and its cofactor protein S inactivate FVIII and FV [48]. Several gene mutations can lead to protein C and S deficiencies, and heterozygous carriers have up to a 10-fold increased risk of VTE [69]. Gene mutations that lead to antithrombin deficiencies are associated with a 10- to 30-fold increased VTE risk [70]. Such mutations are very rare and are present in 0.02 to 0.2% of the general population [70-72].

In the 2000s genome-wide association studies became available and since then additional gene variants associated with VTE have been discovered. By 2015, 17 genes with genetic variations associated with VTE risk had been identified [47]. In the future, novel genetic factors are expected to be identified. These factors may potentially include weak genetic factors as well as genetic factors associated with a high risk of VTE.

1.3.2 Acquired risk factors for VTE

Several acquired risk factors exist for the development of VTE. Age, obesity, height, cancer, immobilization, surgery, trauma, pregnancy, puerperium, and use of oral contraceptives are among the acquired risk factors of VTE. Some of these are classified as being provoking factors, either transient (e.g. pregnancy, surgery) or persistent (e.g. active cancer, inflammatory bowel disease), while male sex and older age are risk factors that are not considered as provoking factors [13].

A major acquired risk factor for VTE is **age,** and the risk of VTE increases exponentially with increasing age. The annual incidence of VTE in young adults (20-30 years) is 1 in 10000, in middle aged (50 years) is 1 in 1000 and in elderly (>75 years) is 1 in 100 [1, 73]. It is not

clear why the risk of VTE increase exponentially with age, but a possible explanation is age-related increase in procoagulant proteins such as fibrinogen, FVIII and FIX [74]. Older people also have increased levels of interleukin (IL)-6 and C-reactive protein (CRP) which indicates an increased inflammatory state [74]. Age related changes in the vessel wall and valves may also be involved [1]. The elderly are also less physically active [75], which leads to more immobility, and as a consequence the muscle strength can get weaker which can contribute to the increased VTE risk.

Obesity, defined as a body mass index (BMI) of >30kg/m^2 is associated with a 2-3 fold increased risk of VTE [76]. Mendelian randomization studies have found that there is a causal relationship between high BMI and VTE risk [77] and that 30% of the VTE risk can be explained by obesity. Other measurements of obesity have also been investigated, and in the Tromsø study, waist circumference yielded the highest risk estimate for VTE, and identified most subjects at risk [78]. Not only obesity, but also weight gain is a risk factor for VTE [79]. In many populations, BMI is high, and obesity continues to increase, this constitutes a major challenge [80, 81]. Possible mechanisms behind the association between obesity and increased VTE include venous stasis due to increased intraabdominal pressure and inflammatory properties of adipose tissue [82].

Height is another anthropometric measure associated with VTE risk [83, 84]. In the Tromsø Study, the risk of VTE increased by 34% per 10cm increase in height in men [83], and recent Mendelian randomization studies have confirmed this estimate [85]. Possible mechanisms for this association might be that taller people have more valves and greater venous surface compared to shorter people, and that there is greater hydrostatic pressure and venous stasis in taller people [85, 86].

Cancer is recognized as a major risk factor for VTE, associated with a 5-7 fold increased risk, and overall, cancer is associated with 20-25% of all incident VTEs [15, 16, 87, 88]. The risk of cancer-associated VTE is highest the first months after cancer diagnosis but remains elevated for years [89]. The VTE risk differs among histological subtype, cancer stage, and time since cancer diagnosis. Hematological malignancies and cancers of the lung, gastrointestinal tract, and brain are high risk sites [4, 89], and metastatic cancers yield a higher VTE risk that localized cancers [89]. The high risk of VTE in cancer patients might be due to tumor-derived procoagulant factors, such as EVs positive for TF, inflammation, and activation of neutrophils [90]. In addition, cancer patients are often hospitalized, undergo surgery, and are prone to infections and to be immobilized. Treatment related risk factors include chemotherapy, radiotherapy, and central venous catheters.

Immobilization is a consequence of many medical conditions and has been associated with a 2-fold increased risk of VTE [91] and a 6-fold increased risk of DVT [92]. A recent study using the Tromsø Study found a 73-fold increased risk of VTE in immobilized patients [93] . Venous stasis is thought to be the reason for the increased risk of VTE when immobilized. Hospitalization is also a major risk factor for VTE, and hospitalized patients have >100 fold higher risk of VTE compared to residences in the community [94].

Major surgery is a strong risk factor for VTE and is associated with a 4-22 fold increased VTE risk [95, 96]. Surgical procedures associated with a high VTE risk include neurosurgery, major orthopedic surgery of the leg, cancer surgery of the thorax, abdomen or pelvis, and renal transplantation [97].

When screening systematically for VTE in **trauma** patients not receiving thrombosis prophylaxis, VTE was found in more than 50% [98]. One study found that even with thromboprophylaxis 1/3 of patients developed DVT after major trauma [98, 99].

Serious medical conditions including rheumatic disorders, ischemic stroke, myocardial infarction, congestive heart failure, and respiratory disease [100] are recognized as risk factors for VTE. **Infection** is also identified as an independent risk factor for VTE. A recent study showed that acute infection in hospitalized patients was a strong trigger for VTE (20-fold increase), and that immobilization and acute infection had a more than additive effect on VTE risk (141-fold increased VTE risk) [93].

1.4 The coagulation system

In 1964, two groups simultaneously proposed a cascade or waterfall model of blood coagulation. The models consisted of a series of steps in which activation of one coagulation factor lead to the activation of another, finally resulting in thrombin generation and fibrin clot formation [101, 102]. They proposed that each coagulation factor existed as a proenzyme that could be converted to an active enzyme upon activation. This concept has later been modified, and clotting factors that were thought to be enzymes have later been found to work as cofactors and do not possess enzymatic activity (FVIIIa was found to be a cofactor for FIXa and FVa for FXa) [103]. Two pathways of coagulation have been described: the extrinsic/tissue factor pathway and the intrinsic/contact activation pathway. These two pathways converge, forming the common pathway, which results in the activation of FX to FXa [104]. FXa converts prothrombin to thrombin [105] and finally thrombin converts fibrinogen to fibrin which is the main component of the venous clot [1]. Thrombin cleaves plasma soluble fibrinogen into Aα and Bβ polypeptides which in turn release fibrinopeptide A

and B. The release of fibrinopeptides results in the formation of fibrin monomers. The fibrin monomers then polymerizes to form a gel of fibrin polymers that traps blood cells [106] . In addition, thrombin activates FXIII to FXIIIa, which in turn mediates the covalent cross-linking of the fibrin polymers to form a stable fibrin clot [107].

The extrinsic pathway, also called the tissue factor pathway, is activated at the site of injury due to the exposure TF on extravascular cells. TF (also known as FIII, CD142, or thromboplastin) is a transmembrane protein which acts as a receptor and a cofactor for FVII/FVIIa and is recognized as the main contributor for initiating hemostasis, [108] the process that protects the body for injury due to bleeding. Upon injury to the endothelium followed by expression of TF, plasma containing FVII/FVIIa comes into contact with TF

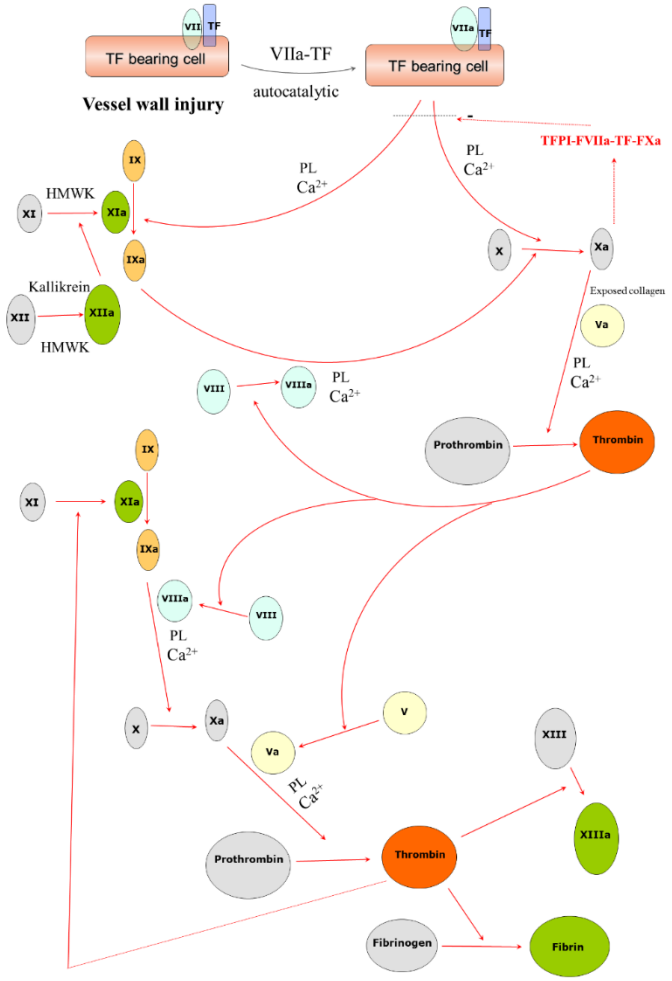

Figure 4. Outline of the coagulation cascade in hemostasis. The first traces of FXa form a complex with TF-FVIIa-TFPI, which turns off the function of TF. FVa generated from platelets upon collagen exposure from platelets drives thrombin generation

[109]. FVIIa at natural biological concentrations is not sufficient for activating FIX or FX, TF binding is necessary for activation of FIX and FX [110]. Once bound to TF, FVII is rapidly converted to FVIIa [111] and the TF-FVII/FVIIa complex activates FIX and FX [37] leading to the common pathway of coagulation. The majority of FVII circulates as a zymogen, but there are also small amounts of plasma FVIIa in healthy individuals [112], thus there are two possible ways to form the TF-FVIIa complex, either by direct capture of circulation FVIIa, or by capture of FVII by TF followed by conversion of bound FVII to FVIIa. Once sufficient amounts of FXa are formed and the TFPI-FVIIa-TF-Xa complex is formed, the TF pathway is turned off and FXa is responsible for maintaining hemostasis (Figure 4).

The cell-based model of blood coagulation [113] emphasizes the interaction between coagulation factors and specific cell surfaces (e.g. platelets and monocytes). This model was developed, in part, for cell-based experimental models that used monocytes [114-116], or fibroblasts [113] as a source of TF and activated platelets as a surface for thrombin generation [117]. In this model, coagulation occurs in three overlapping steps; initiation, amplification, and propagation, and requires two types of cells: TF-bearing cells and platelets. [117]. Small amounts of thrombin generated on TF-bearing cells in the initiating phase of coagulation amplify the initial procoagulant signal by enhancing platelet adhesion [118], and activation [116]. When platelets are activated they release FVa from α-granules onto their surface and the enzyme complex of FXa and prothrombin assemble to form the initial prothrombinase [119]. Thrombin cleaves vWF bound FVIII thereby releasing it from vWF and activating it [113]. By the end of the amplification phase, activated platelets with bound FXIa [120] , FVa [121] and FVIIIa [113] set the stage for thrombin activation in the propagation phase, which occurs on the surface on activated platelets expressing phosphatidylserine (PS). During the propagation phase, FIXa binds to FVIIIa on the platelet surface, and the FIXa/FVIIIa complex

activates FX to FXa. The binding of FX by FIXa/FVIIIa complex is dependent on PS. FXa can then bind to its cofactor FVa, and the FXa/FVa complex bound to PS on the platelet surface, can produce thrombin in sufficient amounts to clot fibrinogen and feedback activate FXI [113]. A similar mechanism is probably involved in the hemostatic mechanism when there is a vessel wall injury whereby TF, located in adventitia is exposed to blood, and platelets are activated by interaction with collagen at the site of injury.

The cellular origin of TF in circulating blood is a matter of debate [122]. Studies have reported TF expression in platelets [122], neutrophils [122] and eosinophils [123]. Other studies have failed to detect TF expression in platelets [122, 124, 125], neutrophils [122, 125, 126] and eosinophils[127]. The discrepancies of TF in these cells may be due to non-specific assays [128] or non-specific binding of the TF-antibody used [129]. However, activated monocytes are shown to be the main source of TF in blood [130], and constitutively express small amounts of TF under basal conditions [122, 125, 126]. EVs can display TF on their surface and can be a source of TF in plasma [131].

In 1967, Peter Wolf identified what he called “platelet dust” while studying coagulation [132]. This was later identified as extracellular vesicles (EVs) which is a general term enveloping exosomes and microvesicles released from blood cells and tissues upon activation or apoptosis [133]. Studies have shown that elevated plasma levels of EVs are associated with several diseases, including: antiphospholipid syndrome [134], heparin-induced thrombocytopenia [135], hypertension [136], myocardial infarction [137] acute ischemic stroke [138], sepsis [139] and HIV infection [140]. EVs are highly procoagulant due to negatively charged phospholipids, PS and polyphosphates (polyPs) together with TF on their

surface [131]. Growing evidence from observational- [141, 142], and experimental studies [143-145] suggest that EVs are involved in the pathogenesis of VTE.

The intrinsic pathway is activated when FXII comes into contact with negatively charged surfaces such as long-chained inorganic polyPs, materials in medical devices [146, 147], microbial cell wall and surfaces [148], damaged tissues [149], and activated platelets [7]. This pathway commences with the contact phase, in which factors prekallikrein (PK), high molecular weight kininogen (HK), FXII, and FXI are exposed to a negatively charged surface. FXII then undergoes a conformational change with HK as a cofactor, which generates small amounts of activated FXII (FXIIa). Once small amounts of FXIIa accumulate, it cleaves plasma PK to plasma kallikrein, which again activates FXII and generates a positive feedback loop of FXII activation. FXIIa together with HK cleaves FXI, to generate activated FXI (FXIa). FXIa initiates a series of Ca^{2+}-dependent sequential proteolytic cleavage events that lead to thrombin generation, fibrin formation, and production of a fibrin clot in plasma [150]. Thrombin is responsible for the conversion of fibrinogen to fibrin [37, 151], which makes up the majority of a venous blood clot.

The intrinsic pathway may be the most important player in thrombus formation. Recent animal studies of experimental thrombogenesis suggest that this pathway of blood coagulation may play a pathogenic role in human thrombosis [152]. Cheng *et al.* found that FXII-deficient mice were more resistant to the development of thrombosis than both FXI- and FIX-deficient mice [153]. This suggests that FXII may exert additional effects on thrombosis, independent from activation of FXI. The role of FXII in thrombosis has been supported by several additional studies. Cai *et al*. demonstrated that total protection against thrombosis (by arteriovenous shunt and the $FeCl_3$-mediated arterial thrombosis models) could be attained in

FXII knockout rats [154]. Xu *et al.* showed that the administration of infestin-4 (a competitive FXIIa inhibitor) produced a considerable and dose-dependent reduction of clot weight in an arteriovenous shunt thrombosis model in rats and rabbits [155]. Finally, Larsson *et al.* developed a FXII function-neutralizing antibody and showed that inhibition of FXIIa-mediated blood coagulation was effective to abolish thrombus formation under flow in both rabbits and mice [146]. Factors FXII and FXI have been proposed as new targets for anticoagulant therapy, with FXI being the most promising of the two. By blocking FXII or FXI the risk of VTE is reduced and at the same time major bleeding is avoided [156].

Coagulation is resolved by fibrinolysis, which involves an enzymatic cascade that leads to the removal of fibrin deposits [157]. The key component of fibrinolysis is plasminogen, zymogen of the serine proteinase plasmin. Plasminogen is converted to plasmin by tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) [157, 158]. Through positive feedback, plasmin cleaves both tPA and uPA, making them more active two-chained polypeptides [159]. Once formed, plasmin cleaves fibrin to soluble degradation products, exposing carboxy-terminal lysine at which tPA and plasminogen can bind. This leads to enhanced plasmin generation and fibrin removal. The binding of tPA and plasminogen to fibrin can be blocked by thrombin-activatable fibrinolysis inhibitor (TAFI) [159]. As the name implies, TAFI is activated by thrombin, and activation leads to the removal of carboxy-terminal lysine and the attenuation of plasmin generation and stabilizing the fibrin clot [159]. Plasminogen inhibitors such as plasminogen activator inhibitor-1 (PAI-1), and inhibitors of plasmin (α2-plasmin inhibitor) also regulate fibrin dissolution [159]. Hemostasis involves a combination of both coagulation and fibrinolysis.

The coagulation system is regulated at several steps in the cascade. Tissue factor pathway inhibitor (TFPI) inhibits the extrinsic pathway by binding directly to, and inhibiting the TF–FVII/FVIIa complex [160]. The effect of TFPI is dependent on the formation of the FXa-TFPI-FVIIa-TF complex, thus FXa is necessary for TFPI function. TFPI also inhibits and interacts with FXa alone. A threshold effect between low levels of TFPI and increased risk of VTE has been proposed [161, 162], and heterozygote TFPI deficiency has been shown to be more common in thrombotic patients compared to controls [163]. Antithrombin inhibits thrombin and FXa, and is one of the most important natural proteins responsible for the prevention of spontaneous intravascular clot formation [164]. Deficiency of antithrombin results in an increased risk of venous thromboembolism. The protein C anticoagulation pathway is one of the most important antithrombotic pathways. Activated protein C (APC) functions as an anticoagulant by inactivation of FVa and FVIIIa in the presence of protein S [165].

1.5 The complement system

In the late 1800s the bacteriologists, Paul Ehrlich, Jules Bordet, and George Nuttal identified a heat liable substance in serum, which killed bacteria. They called it “alexine” which means “defender of man”. Several years later Ehrlich replaced “alexine” with “complement” [166]. It took more than 50 years after the first discovery until Pillmer discovered the next part of the complement system in 1954. He found properdin, when isolating C3 from serum, which later was proven to be an important part of the alternative pathway [167] . Kawasaki *et al.* found the MBL protein in 1978, but its function and the lectin pathway, was discovered as late as in 1989, by Super *et al.*, and later elaborated by Matsushita *et al.* [166, 168].

The complement system consists of more than 50 components, including complement recognition molecules, proteases, enzyme complexes and biologically active split products as

well as soluble and membrane bound receptors and regulators. The major source of complement proteins found in plasma is the liver, with the exception of C1q, properdin and C7, which are predominantly synthetized in bone marrow derived cells, and factor D, which is synthetized in adipocytes [169-171]. The complement system is an important component of the innate immunity and is a link between the innate- and adaptive immune system. It is a cascade system comparable to the coagulation, fibrinolytic, and the kallikrein-kinin systems, with its components found both in circulation and in tissues. It is highly effective in the destruction of invading microorganisms, the elimination of immune complexes, and the clearance of damaged host cells [172].

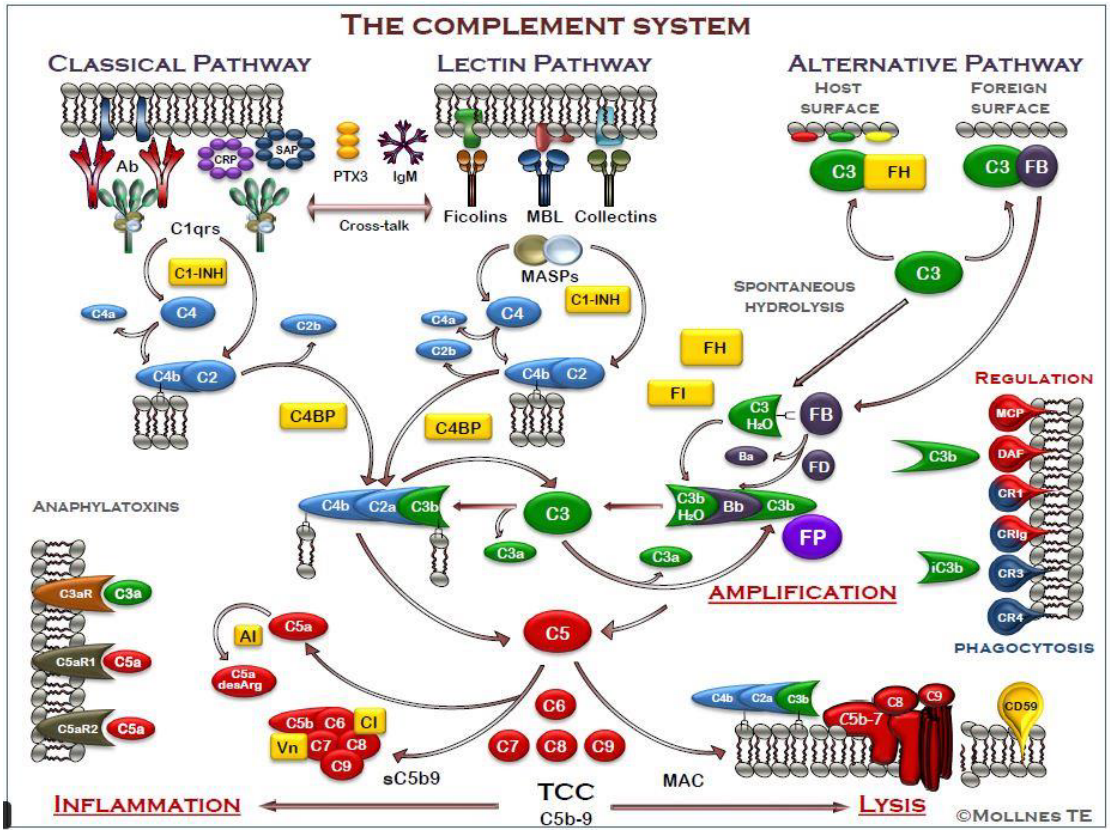

Figure 5. An overview of the complement system. The classical, lectin, and alternative pathways converge into a final common pathway when C3 convertase cleaves C3 into C3a and C3b. Details are explained in the text. Ab = antibody, CRP= C-reactive protein, SAP = serum amyloid protein, C1-INH = C1 inhibitor, MBL = mannose-binding lectin, MASP = MBL-associated serine protease, TCC= Terminal Complement Complex. (Kindly provided by Prof. T.E Mollnes).

The complement system is activated by three pathways: the classical pathway, the lectin pathway, and the alternative pathway (Figure 5).

The classical pathway is activated by binding of C1q to antibodies on a pathogenic surface and by antibody-independent mechanisms. C1q binds efficiently to single IgM molecules, but requires hexamers of IgG molecules for similar efficient binding [173]. Antibody-independent activation of the classical pathway can occur via bacterial products, pentraxines (including CRP), and apoptotic and necrotic cell components like annexins, DNA, and histones [174]. C1q circulates in a surveillance mode in complex with C1r and C1s. When C1q binds to its target, it undergoes a conformational change and auto activates the serine proteases C1r and C1s [175]. C1s cleaves C4 into C4a and C4b, and leads to further association and cleavage of C2, leading to assembly of the C3-convertase: C4b2a, which cleaves C3 to C3a and C3b which leads to the formation of the C5 convertase C4b2a3b [176].

The lectin pathway is activated by the recognition of pathogens via pattern-recognition molecules (PRM) of the lectin type via pattern-recognition receptors (PRRs) [177]. The first PRM discovered was mannose-binding lectin (MBL) and later several other PRMs including ficolin 1-, 2-, 3 and several collectins were found [178]. The lectin pathway can also be involved in the clearance of apoptotic cells [179], and bind self-structures as mitochondria [180] and DNA [181]. MBL is analogous to C1q and triggers three MBL-associated proteases (MASPs). MASP-1 and -2 cleave C4 and C2 in a calcium-dependent manner and the subsequent steps of the lectin pathway are identical to the classical pathway with assembly of the C3-convertase C4b2a and C5-convertase C4b2a3b [176].

The alternative pathway is activated by spontaneous hydrolysis of the internal thioester bond in C3[177], which creates C3(H_2O), a C3b-like molecule. Factor B is recruited and binds factor D in a magnesium-dependent manner. Factor B is cleaved by factor D to Bb and Ba, resulting in the formation of the alternative pathway C3-convertase C3(H_2O)Bb. The alternative pathway can also use C3b produced by either pathway to bind factor B, and forming the C3 convertase C3bBb. Properdin, the only positive regulator of complement, binds to C3bBb and stabilizes this complex which cleaves C3 and binds to C3b to form the C5 convertase $C3b_2$BbP, which cleaves C5 in the same manner as the classical- and lectin pathway C5 convertase [176]. The spontaneous hydrolysis of C3 can be termed a "tick-over" and ensures that the complement system is always ready to react [182]. The alternative pathway is an amplification loop of complement activation. Positive feedback from C3bBbP results in further cleavage C3 and production of additional C3bBb, consequently, this pathway is responsible for the vast majority of C3b and C5b-9 formation, independent of the initiating pathway [183].

Irrespectively of which pathway of complement that is activated, the three activation pathways converge at the formation of C3 convertase, the formation of opsonins (C3b), anaphylatoxins (C3a and C5a) and the terminal C5b-9 complex (TCC). [177].

The anaphylatoxins C3a and C5a are small peptides with important signaling properties. They bind to their respective receptors, C3aR, C5aR1 and C5aR2 on immune cells, and several other cell types [184]. The effect of binding of these anaphylatoxins depends on the cell type expressing the receptors. C3a was previously thought of as a strictly proinflammatory molecule, but was recently shown to also exert anti-inflammatory properties especially in neutrophils while still showing proinflammatory effects on monocytes, eosinophils and mast

cells [185]. C5a is regarded as the dominant proinflammatory effector-signaling molecule of the complement system. It acts as a chemotactic agent on neutrophils and monocytes and induces oxidative burst, induces upregulation of adhesion molecules and the release of enzyme-containing granules and cytokines [186]. C5a has also anti-inflammatory and immune regulatory effects when binding to C5aR2 [187].

The end stage of complement activation is the formation of the terminal TCC complex C5b-9. The TCC assembles when C5b associates with C6, C7, C8, and multiple C9 molecules. If assembled on a surface, the binding of C7 leads the addition of several C9 molecules which forms a pore through the membrane. This is the Terminal complement complex (TCC), also called the membrane attack complex (MAC). This pore can lyse cells and bacterial membranes [188]. If the terminal pathway is activated in the fluid phase, the TCC complex will build up in a soluble form, sC5b-9, that can be measured in plasma as an indicator complement activation, although no biological function is known for sC5b-9. Notably, lysis is not the only effector function of the TCC. Sublytic C5b-9 inserted into a cell membrane can induce proinflammatory responses including NLRP3-inflammasome activation and degranulation [189, 190].

To protect the host tissue and cells from damage, the complement system is tightly regulated. Several surface-expressed complement inhibitors that limit the activation of this system to the site of infection are found on host cells. Thus, complement regulators mainly acting as inhibitory molecules on the activation are found both as soluble and membrane bound molecules.

C1-INH is a serine protease inhibitor, which inactivates C1r and C1s of the classical pathway and the MASPs in the lectin pathway. C1-INH also inactivates serine proteases of other cascades, such as the contact pathway of the coagulation system and the fibrinolytic system, and is therefore not strictly a complement inhibitor [191].

The regulation of the formation of C3 convertase, and thus the cleavage of C3 is essential [192] in complement activation, and factor H is a key player in the regulation of this step [192, 193]. Factor H binds and protects self-surfaces from complement deposition by binding to C3b, accelerating the decay of the alternative pathway C3 convertase (C3bBb) and acts as a cofactor for factor I mediated proteolytic degradation of C3b [194]. Factor I (FI) is a fluid phase serine protease which cleaves and inactivates C4b and C3b [195] and the inactivated products (C4c, C4dg, iC3b, C3c and C3dg) can no longer assemble to the C3-convertases to further drive the cascade. C4BP is a major soluble inhibitor of both the classical and the lectin pathways of complement [196]. It exerts its inhibitory action by binding to and limiting the function of activated complement component C4b. C4BP acts as a cofactor for FI in the inactivation of soluble and cell-bound C4b [197]. Membrane cofactor protein MCP (CD46) also acts as a cofactor for FI-mediated cleavage of C4b and C3b. As its name implies, this is a membrane bound regulator, expressed on all cell types except erythrocytes [198].

Decay accelerating factor (DAF; CD55), is a membrane bound regulator, which binds to and dissociates the alternative pathway C3-convertase. CD59 prevents final assembly C5b-9 on cell membranes, thus preventing lysis [198](37).

In addition to the immune and inflammatory roles, the complement system has been found to play a role in metabolism, hematopoiesis, reproduction, central nervous system development,

liver regeneration, apoptosis, and of particular interest here, coagulation [199]. Conditions which leads to a hyperactive complement system, are increasingly recognized as contributors to vascular and thromboembolic diseases, such as paroxysmal nocturnal hemoglobinuria, atypical hemolytic uremic syndrome [200] , and antiphospholipid syndrome [201].

1.5.1 Crosstalk between the complement and coagulation system

The convergence between complement and coagulation extends far beyond the chemical nature of the complement and coagulation components (Figure 6), both of which form proteolytic cascades [202]. The endothelium and circulating cells all have the machinery to initiate, activate and respond to effectors generated from activation of both systems [203]. The two systems system appear to be triggered simultaneously by several conditions, e.g. severe tissue injury [202, 204], acute trauma [205], or during systemic inflammation [206], which is beneficial for the host. On the other hand, dysregulation of one system, might result in escalating activation of both, which might end in tissue damage from inflammation or thrombosis [207]. The term thromboinflammation is important to mention when discussing the crosstalk between the complement and coagulation systems. Thromboinflammation is part of the repair process after damage, and describes the crosstalk between complement, coagulation, and platelets on one side, and leukocytes and the endothelium on the other [208]. Thromboinflammation is associated with thrombotic events such as cardiac infarction, stroke, and other cardiovascular conditions [209].

The endpoint of complement activation, with release of C5a and TCC formation can enhance blood clotting by several mechanisms. Incorporation of TCC as MAC into the platelet membrane activates platelets and results in 1) the exposure of negatively charged lipids [210] and TF [202] on the platelet surface, 2) the release of TF-positive MVs [211], and 3) dense

granule secretion from the platelet cytoplasm [212]. TCC also induces permeability changes in the vessel wall [202, 213]. C5a modifies the activity of mast cells and basophils to expose plasminogen activator inhibitor-1 (PAI-1) which inhibits fibrinolysis [214, 215]. C5a induces a "switch" in mast cells from a profibrinolytic (t-PA release) to a prothrombotic phenotype (PAI-1 release) and induces neutrophils and monocytes to expose TF [202] (Figure 6), thus modifying the balance between coagulation and fibrinolysis.

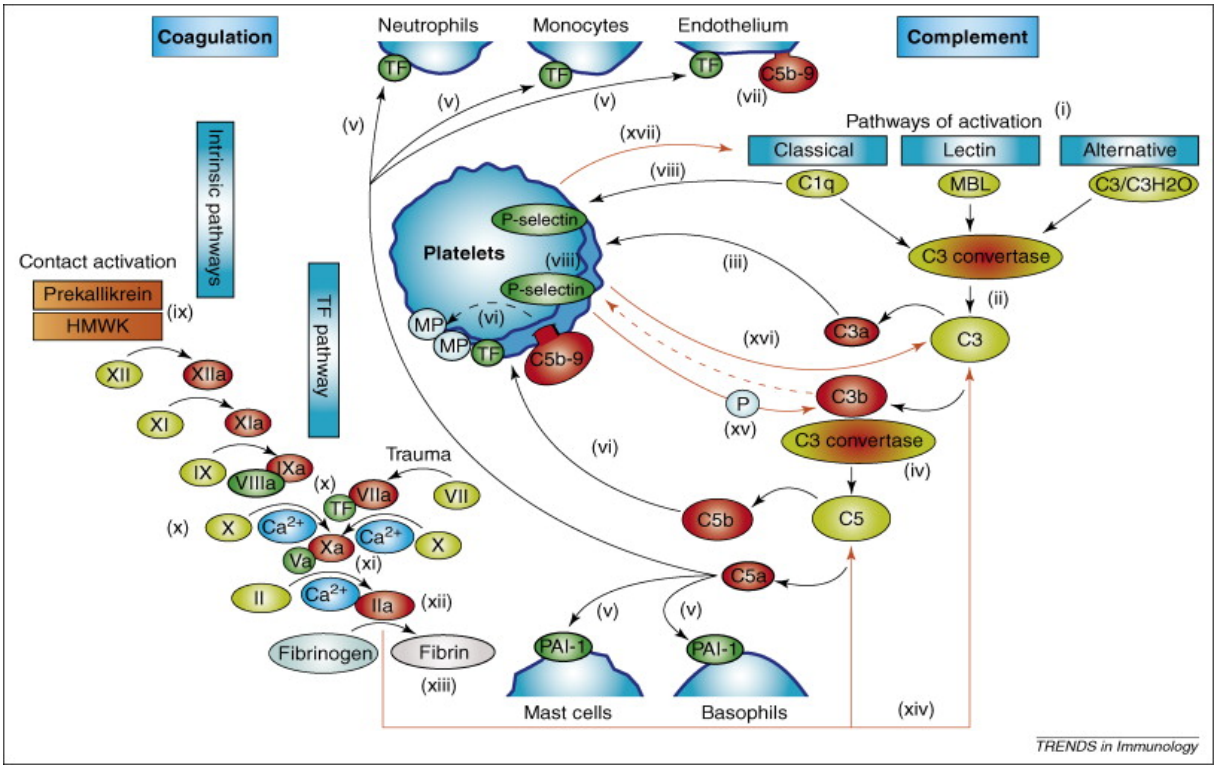

Figure 6. Overview of complement–coagulation and platelet crosstalk (Markiewski, Trends in immunology 2007).

Factors of the coagulation system have complement activating properties. Kalowski et al. reported in 1975 that thrombin and thromboplastin injected into rabbits led to activation of the complement system through C3 [216]; they concluded that complement activation occurs concomitantly with intravascular clotting when induced *in vivo*. This was confirmed by evidence of thrombin-mediated cleavage of C3 into in a dose- and time-dependent manner in

human serum [217]. In addition, kallikrein and plasmin, has been shown to directly activate C3 and C5 [218, 219]. Several studies have shown that enzymes other than complement can activate C5 independently of C3 [220-222], among these, thrombin has been proposed as a potential potent C5 convertase, in the absence of C3 [222]. Finally, factor XIIa of the intrinsic coagulation pathway is able to activate the classical complement cascade through the activation of C1q [219].

The complement systems possible effect on platelet activation has been extensively studied [203]. Binding of iC3b, C3d and C3dg to complement receptor (CR)-2 activates platelets [223] and several studies have showed the presence of the C1q receptor gC1qR/p33 and cC1qR on platelets, and found that binding of C1q mediates platelet aggregation and activation [224-226]. *In vitro* studies have found the presence of receptors for C3a and C5a on the platelet surface [227, 228]. The discovery of several complement inhibitors on human platelets, including Factor H [229, 230], points towards the complement system's importance for platelet function. Platelets can also activate the complement system through P-selectin, and activated platelets generate C3a [231]. In addition, thrombin receptor-activating peptide (TRAP)-6 activated platelets activate the complement system by releasing chondroitin sulfate [232].

The effect of complement on platelets is interesting in the case of VTE, since both elevated platelet count [233, 234] and mean platelet volume [234, 235] are associated with increased risk of VTE. The classical function of platelets is to cover and close endothelial and tissue wounds [236, 237]. In addition to wound closure, which is followed by stable thrombus formation, platelets contribute to long-term healing and regenerative mechanisms [238, 239]. However, if platelet activation occurs without proper regulation or at improper locations,

thrombus formation by activated platelets can result in life-threatening events such as myocardial infarction, stroke, or atherosclerotic plaques rupture [240-242].

1.5.2 The complement system and risk of VTE

Although many risk factors for VTE have been identified, there are still a considerable number of events that are considered to be unprovoked without known predisposing factors [32]. The cellular and molecular mechanisms behind the initiation of thrombus formation are still not fully understood. In light of the close interactions between coagulation and complement, it is reasonable to think that the complement system plays a role in the pathogenesis of VTE.

Autopsy- and imaging studies suggest that formation of venous thrombi originate in the valvular sinuses of large veins. The milieu in the valvular sinuses is characterized by severe hypoxia and stasis, conditions known to induce cellular immune responses [1, 5, 6]. As the complement system is an important player in the immune system and is also activated by hypoxic cells and tissues, one can assume that the complement system is activated in the hypoxic milieu in the valvular sinuses. Activated complement can promote activation of the coagulation system, which can lead to thrombus formation if not counterbalanced by regulatory mechanisms. Results from observational and animal studies suggest that the complement system is involved in the early steps in the pathogenesis of VTE. In a large population-based cohort study, subjects with plasma complement C3 concentration in the highest tertile had 31% higher risk of VTE compared to those in lowest tertile [243]. This study led to the question if C3 was a bystander or a mediator of the VTE risk. The role of C3 as a mediator of VTE risk was demonstrated in a TF-dependent mouse model of flow restriction-induced venous thrombosis [244]. C3-deficient

mice had lower incidence of venous thrombosis and reduced thrombus size, compared to wild-type mice. In addition, C5-deficient mice had a significantly reduced clot burden after 48 hours compared to wild type [244]. Further, in a cross-sectional study of patients with systemic lupus erythematosus (SLE), patients with a previous history of VTE showed increased deposition of complement factors C1q, C4, and C3 on platelets [245].

In light of these studies one can draw the conclusions that C3 and C5 are involved in thrombus formation, but the mechanisms are not understood. C3 and C5 are central molecules and do not show which pathway is activated, or if the complement system activation is sufficient to generate the TCC. These questions, if answered can future explore the link between the complement system and its role in VTE. To date there are no studies that have investigated complement pathway activity or, the overall complement activation and risk of VTE.

1.6 Polyphosphates

Inorganic polyphosphates (polyPs) are linear polymers of orthophosphate, linked by phosphoanhydride bonds. They are found in all mammalian cells and lower organisms and they are shown to have proinflammatory and prothrombotic effects [246]. The polymer varies in length from cell to cell and in different organisms, ranging from 60 to 100 units in human platelets and up to thousands of phosphate units in bacteria [246]. Platelets release polyPs from their dense granules upon activation, which, play a role in a variety of hemostatic and thrombotic mechanisms. In addition, as discussed above, activated platelets are associated with increased risk of VTE. As a result, polyphosphate has been proposed as a potential druggable target to prevent thrombosis.

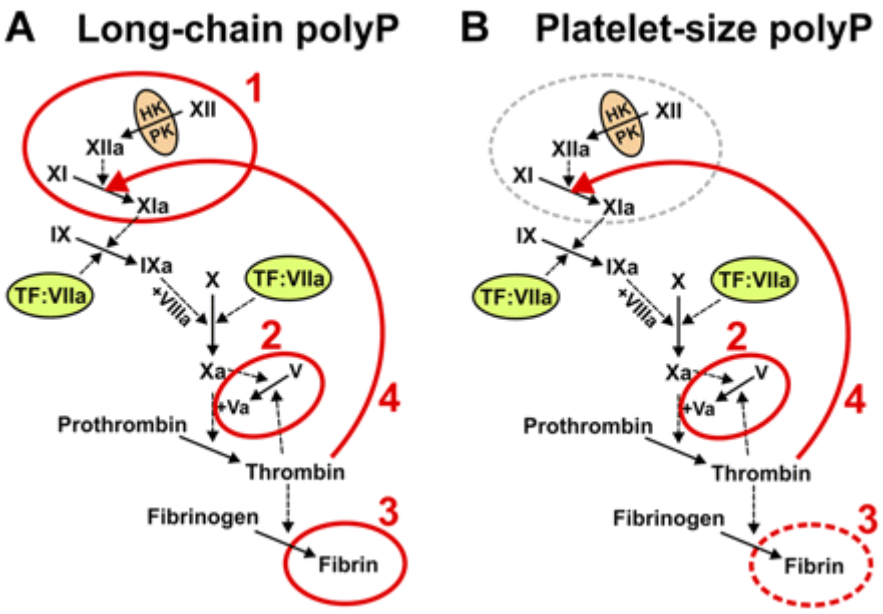

Figure 7. The roles of polyP in blood clotting vary depending on polymer length. (A) Bacterial long-chain polyP acts at 4 points in the clotting cascade, indicated in red: 1 initiates the contact pathway of blood clotting via FXII; 2, accelerates factor V activation and abrogates TFPI function; 3, enhances fibrin polymerization; and 4, accelerates factor XI back-activation by thrombin. (B) Short chained platelet-sized polyP acts most potently at 3 points in the clotting cascade, indicated in red: 2 accelerates factor V activation and abrogated TFPI function; 3, overlaps the minimal size necessary to enhance fibrin polymerization; and 4, accelerates factor XI back-activation by thrombin (Morrissey, Blood 2012).

The role of polyPs in mammalian systems is rapidly emerging although to date, most studies have been done on polyPs from prokaryotes and unicellular eukaryotes. Interest in polyPs in human cells started with its detection in dense granules of platelets, which suggested a potential role in coagulation. Now it is recognized that polyP plays a role in several hemostatic and thrombotic mechanisms. PolyP acts at several steps in the coagulation cascade that influence thrombin generation; it enhances the generation of FXIa, [247] , accelerates the activation of FV to FVa by both FXa and thrombin [248], and it opposes the anticoagulant function of TFPI [249] (Figure 7). PolyP also influences fibrin clot structure and inhibits fibrinolysis [250]. Clots formed in the presence of polyP are more turbid, contain thicker fibrin fibers, are more resistant

to elastic stretching and are more resistant to fibrinolysis [251]. The mechanisms behind this are not known, but one possible explanation is that polyPs are incorporated in to the clots [246]. In addition, PolyPs enhances the binding of platelets to von Willebrand Factor (VWF) which promotes platelet aggregation [252]. This has led to the consideration of polyphosphate as a potential target to prevent thrombosis [253, 254].

Bacterial long chained (LC-) polyPs (>200 units) are recognized as a surface for assembly of the coagulation proteins necessary for activation of the contact pathway of coagulation [43]. Studies have shown that LC-polyP is a potent trigger of the contact pathway [255, 256]. Although LC-polyPs clearly activate coagulation through the contact pathway via FXII, the role of platelet-sized polyPs in FXII activation are less clear.

Smith *et al*. showed that short-chained (SC-) polyPs from platelets modulated blood coagulation and fibrinolysis [255]. This was confirmed by the finding that platelet-derived polyPs were strong activators of the contact pathway through activation of FXII [7] in plasma. In addition, platelet-derived polyP triggered FXII activation *in vivo*, as its injection induced fatal pulmonary embolism and increased vascular permeability in wild-type mice, but not in FXII-deficient mice. These findings provide a plausible explanation for the bleeding diathesis in patients with Hermansky-Pudlak syndrome, whose platelets are deficient in dense granules and contain lower concentrations of polyP than normal platelets [257]. Despite these observations, however, it remained unclear if platelet polyP does activate FXII. LC-polyP has been proven to be a more potent activator of FXII compared to SC-polyP [256]. Faxälv *et al*. found that high concentrations of SC-polyP gave a weak and not physiologically relevant FXIIa generation compared to equal amount of kaolin in plasma. In addition, SC-polyP did not shorten clotting time in recalcified human whole blood. They conclude that platelet-

derived polyPs do not activate the contact pathway via FXII, but might still play an important role in other steps of the cascade [258]. Recently, it was proposed that platelet polyPs are mobilized on the platelet surface, and activate the contact system by forming nanoparticles on the platelet surface [259]. The diverging results in the studies done on SC-polyPs and coagulation activation are difficult to explain and more studies are needed.

Interestingly, polyPs have been shown to inhibit the complement system. In an experimental system with 2% serum monitoring complement activation by erythrocyte lysis, Wat *at al.* showed that polyP inhibited complement activation by blocking the C5 from assembling to the C5b-9 complex. The authors showed that both platelet sized and long-chained polyP had an inhibitory effect on the formation of the TCC, though the long-chained polyP showed a stronger effect than the platelet-sized [260]. In addition, Wijeyewickrema and colleagues found that SC-polyP acts as a cofactor for the C1 inhibitor. In the presence of SC-polyP together with C1-INH, C1s cleavage of C4 decreased [261] compared to the situation without polyPs. The inhibitory effect of SC-polyPs on the complement system are in contrast to the fact that the coagulation- and complement system act together. One explanation of these observations might be due to the experimental systems used. Both of the studies presented above used artificial systems to investigate the effect of polyPs on the complement system. Purified systems, such as buffer systems lack a number of regulatory proteins that will influence on the crosstalk *in vivo*.

Since polyPs have been found to be involved in both the coagulation- and the complement system it is interesting to study them in light of VTE. If SC-polyPs from platelets modulate both systems they might be involved in the pathogenesis of VTE. FXII has been proposed as a target for VTE prevention. To date no studies on SC-polyP effect on FXII activation or on the

possible inhibitory effect on the complement system in whole blood have been conducted. Such studies would give us insight in to the effect of SC-polyPs on coagulation and complement in presence of blood cells and other factors in the blood.

2. Aims of the thesis

The aims of the PhD project were:

1. To investigate the relation between the activation potential of the three complement pathways, and to explore their impact on VTE risk and thrombin generation in a population-based case-control study.

2. To investigate whether total complement activation, assessed by plasma levels of the terminal complement complex (TCC), is associated with risk of VTE in a nested case-control study. Moreover, to explore whether genetic variants were associated with plasma levels of TCC and investigate whether identified gene variants (if present) were associated with risk of VTE.

3. To study the impact of exogenously added short chained polyphosphates (SC-polyP) on complement- and coagulation activations and explore underlying mechanism(s) in a human whole blood model.

3. Methods

3.1 Study population

3.1.1 The Tromsø Study

The Tromsø Study is a single center, population-based cohort study with repeated health surveys of the inhabitants of the municipality of Tromsø. The first survey was conducted in 1974. To date, seven surveys have been conducted, with the last (Tromsø 7) conducted in 2015/16. The primary aim of the Tromsø Study was to determine factors related to the high cardiovascular mortality observed in the northern part of Norway.

In Tromsø 4, all inhabitants of the municipality of Tromsø aged ≥25 years were invited to the first screening visit, and 27158 participated (77% of the eligible population). The participants were followed from the date of inclusion (1994/95) until September 1, 2007, and all VTE events occurring among the participants in this cohort were registered. Participants were censored from the cohort at the date of death or migration from the Tromsø municipality. This cohort formed the basis for the case-control studies conducted in paper I and II of this thesis.

In paper I, we conducted a case-control study of patients with unprovoked VTE and healthy controls. Cases and controls were screened and included in the study between April and September 2008. We identified VTE cases from Tromsø 4 who were still alive, were between 20-80 years of age, and had experienced an unprovoked VTE 1-6 years ago. Twenty-eight cases were invited for a screening visit, and 24 of them were included in the study. For each case, one age- and sex-matched healthy control was randomly sampled from Tromsø 4. Thus, the final case-control study consisted of 24 patients with unprovoked VTE and 24 age- and sex-matched controls recruited from the general population. The study was approved by the

research ethics committee and all subjects gave informed written consent (REK Nord 114/2007).

In Paper II, we performed a nested case-control with cases and controls recruited from the fourth Tromsø Study. During follow-up (1994/95 through September 1, 2007), 462 participants experienced a VTE event. For each case, two age- and sex-matched controls (n=924) were randomly sampled from the parent cohort. The index date for the controls were the time of VTE event for the corresponding case, meaning that the controls had to be alive and without a VTE-diagnosis at the time of inclusion. In total, 47 cases and 76 controls did not have available plasma samples of sufficient quality for the TCC analyses, and the final study population consisted of 415 cases and 848 controls.

Baseline measurements from the fourth Tromsø Study was collected by self-administered questionnaires, non-fasting blood samples and physical examinations performed by trained personnel. Height and weight were measured with the participants in light clothing without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters (kg/m^2). Waist circumference was measured in centimeters at the umbilical line. Blood pressure was recorded in the seated position by the use of an automatic device (Dinamap Vital Signs Monitor). Three recordings were made at 1-minute intervals, and the mean of the two last values was used in this report. Hypertension was defined as self-reported use of antihypertensive medication or systolic pressure above 160 mmHg and/or diastolic pressure greater than 95 mmHg. Screening for factor V Leiden and prothrombin G20210A mutations was carried out on DNA isolated from blood as previously described [56].

3.1.2 Outcome measurements and validation

The University Hospital of North Norway (UNN) is the only hospital serving the region, and all diagnostic radiology and hospital care for VTE in the Tromsø region is provided by this hospital. The discharge registry covers both hospitalizations and outpatient clinic visits.

All VTE events during follow-up were identified by screening the hospital discharge diagnosis registry, the autopsy registry and the radiology procedure registry at the University Hospital of North Norway (UNN). International Classification of Diseases (ICD)-codes were used to identify VTE events. Trained personnel reviewed the patients' medical records to validate each potential VTE event. For a VTE to be recorded all the following criteria had to be fulfilled: 1) the presence of signs and symptoms of a DVT or PE or both, 2) objective confirmation by an objective diagnostic procedure (i.e. compression ultrasonography, venography, spiral computed tomography, perfusion-ventilation scan, pulmonary angiography or autopsy), 3) diagnosis of a DVT or PE noted by a physician in the medical record, 4) initiation of VTE treatment (i.e. anticoagulant treatment, thrombolysis) unless contraindications were specified. Potential VTE cases derived from the autopsy registry were adjudicated and recorded when the autopsy report indicated VTE as the cause of death or as a significant condition associated with the cause of death.

VTE events were classified as DVT or PE and if they occurred simultaneously they were classified as a PE. VTEs were also classified as provoked or unprovoked based on the presence or absence of provoking factors at the time of diagnosis. Provoking factors were recent surgery or trauma, acute medical conditions, active cancer, immobilization (bedrest > 3 days, wheelchair use or long distance travel) or any other specific factor described in the medical records.

3.2 The whole blood model

The whole blood model was initially developed by T.E. Mollnes [262], and has since then been used in a wide range of studies. The model uses whole blood anticoagulated with lepirudin, which makes it suitable to study complement and coagulation activation simultaneously, *in vitro*. Lepirudin is a hirudin analogue, which directly inhibits thrombin and thus the coagulation cascade is kept functional upstream of thrombin formation which enables us to study the initiation phase and activation of coagulation. The blockage of thrombin also enabled us to isolate the effect of polyPs on FXII activation, without the positive feedback activation from thrombin. Furthermore, lepirudin has no adverse effects on complement activation and we can study total complement activation. Anticoagulants containing calcium chelators (eg, EDTA and citrate) inhibit complement activation as well as a number of other plasma and cell inflammation markers and therefore cannot be applied in a whole blood system investigating complement. Heparin has been shown to activate or inhibit the complement system depending on the dose used [262, 263].

We conducted experimental studies using the human whole blood model. Human whole blood from healthy fasting donors was used to study cellular interactions and molecular mechanisms for the interplay between SC-polyP and complement- and coagulation-activation. The study was approved by the national ethics committee and all participants gave written informed consent before participating.

3.3 Laboratory measurements

In paper I, TF-induced thrombin generation was assessed in plasma using Calibrated Automated Thrombinoscope **(**CAT) and was performed as described by Hemker et al [264] and according to the manufacturer's instructions (Thrombinoscope BV, Maastricht, the Netherlands). Thrombin generation was measured in a Fluoroscan Ascent Fluorometer (Thermolabsystems OY, Vantaa, Finland) equipped with a dispenser. Fluorescence intensity was detected at wavelengths of 355 nm (excitation filter) and 460 nm (emission filter). Briefly, 80 µl of the plasma samples were dispensed into the wells of round bottom 96-well microtiter plates (Immulon, Lab Consult, Lillestrøm, Norway). Twenty µl of a mixture containing TF (Innovin, Bade Behring) and phospholipids (PL) (Cephaline, from rabbit brain) was added to the plasma samples to obtain a final concentration of 5 pM and 4 µM, respectively. For each experiment, a fresh mixture of 2.5 mM fluorogenic substrate (Z-Gly-Gly-Arg-AMC from Bachem, Bubendorf, Switzerland) and 0.1 M $CaCl_2$ was prepared using buffer containing 20 mM Hepes (Sigma Aldrich, St Louis, USA) and 60 mg/ml BSA (A-7030, Sigma Aldrich) with pH 7.35. The calibrator with thrombin activity of 600 nM was obtained from Thrombinoscope BV (Maastricht, The Netherlands). The thrombin calibrator corrects for donor-to-donor differences in color of plasma and inner filter effect [265]. The computer software calculated lag time (min), the time to peak (min), the peak of thrombin generation (nM) and the area under the thrombin generation curve (nM*min) or endogenous thrombin potential (ETP).

Measurements of white blood cells, platelets and hemoglobin were performed with the ABX Micros 60 cell counter (HORIBA ABX SAS, Kyoto, Japan).

In paper I, the potential activity of the classical, alternative, and lectin pathways of the complement system was assessed by a commercially available assay (Wielisa COMPL300 Total Complement Functional Screen kit from Wieslab AB, Lund, Sweden) and conducted according to the instructions provided in the manual. In brief, strips of wells for classical pathway (CP) evaluation were pre-coated with IgM, strips for alternative pathway (AP) determination were coated with LPS, and lectin pathway (LP) strips were coated with mannan. Sera were diluted in specific buffers (1/101 for the CP and LP assays, and 1/18 for the AP assay), and were incubated for 1 h at 37°C. After washing the strips, alkaline phosphatase-conjugated antihuman C5b-9 was added before incubation at room temperature for 30 min. Additional washing was performed, substrate was added, and the wells were incubated for 30 min. Finally, absorbance values were read at 405nm.

In each assay, standard positive and negative control serum provided in the kit were reconstituted with distilled water. The positive serum was a pool of five sera from healthy individuals, and the negative control consisted of heat-inactivated sera (56°C for 20 min). Complement activity was calculated using the following formula:

$$\text{Activity} = \frac{\text{mean A405 (sample)} - \text{mean A405 (negative control)}}{\text{mean A405 (standard serum)} - \text{mean A405 (negative control)}} 100\%$$

Samples as well as standard serum and negative control serum were tested in duplicates at a fixed dilution. All complement activity values are provided as % of activity in pooled normal serum. The assay is designed as a screening assay to detect deficiencies in the various complement pathways. It is not suitable to detect *in vivo* activation, but reflects the total activity potential that could be activated within each complement pathway *in vitro* [266].

In paper I, measurement of serum concentrations of MBL was performed using an MBL enzyme-linked immunosorbent assay (ELISA) kit (BIOPORTO Diagnostics A/S, Hellerup, Denmark) according to the manufacturer's instructions.

In paper II and III, plasma levels of TCC were measured using an in-house enzyme-linked immunosorbent assay (ELISA) as described previously [267]. In brief, ELISA 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 Healtcare 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.

In paper III, prothrombin fragment 1+2 (F1+2) was measured by enzyme-linked immunosorbent assay kit (Siemens, Enzygnost ® F 1+2 MONO, Marburg, Germany) according to the manufacturer's instructions. In short, thawed plasma was added to a 96 well plate and incubated for 30 minutes before addition of anti-human prothrombin monoclonal mouse antibody. After 15 minutes of incubation, the substrate Tetramethyl benzidine dihydrochloride (TMB) was added and incubated for 15 minutes. After addition of the stopping solution, the plate was read at 450nm with a reference wavelength of 650nm nm (Dynatech MR580, Dynatech Laboratories Inc., Alexandria, Va., USA). The standard curve was created by reading the wavelength of standard 1-4 with known concentrations, supplied by the kit.

3.4 Statistical analyses

All statistical analyses were conducted in SPSS for Windows, version 22.0 (SPSS Inc. Chicago, Illinois, USA), GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, 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, Spearman nonparametric correlation coefficient was used to test for correlations between variables. For complement pathway activity data and parameters of the thrombogram, between-group differences for these variables were tested with independent samples t-test. Logistic regression models were used to determine the predictive role of complement pathway activity and thrombogram parameters for VTE.

In Paper II, TCC was categorized according 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 quartiles of TCC were calculated using descriptive statistics. Logistic regression models were used to calculate odds ratios (ORs) of VTE with 95% confidence intervals (CI) according to quartiles of TCC. The lowest TCC quartile was used as the reference group. We also calculated the p-value for linear trend in OR across increasing quartiles of TCC.

In Paper III, differences between the samples were tested with Student`s t-test. For non-normally distributed data, Kruskal-Wallis test was used. P-values <0.05 were considered statistically significant.

4. Summary of papers

4.1 Paper I

IMPACT OF COMPLEMENT PATHWAYS ACTIVITY, MANNOSE-BINDING LECTIN AND TISSUE-FACTOR INDUCED THROMBIN GENERATION ON THE RISK OF UNPROVOKED VENOUS THROMBOEMBOLISM

Deep vein thrombosis (DVT) originates in the valvular sinuses of large veins in a local milieu characterized by stasis and severe hypoxia. This may induce complement- and coagulation activation, which potentially increases the risk of venous thromboembolism (VTE). We wanted to investigate whether the activity of the complement pathways, the level of mannose-binding lectin (MBL) and tissue-factor (TF) induced thrombin generation were associated with risk of unprovoked VTE. We performed a case-control study in patients with unprovoked VTE (n=24) and age- and sex-matched healthy controls (n=24). Serum complement pathway activity was measured by the total complement screen assay (Wieslab®). MBL was quantified by ELISA. Plasma TF-induced thrombin generation was measured using the CAT-assay. We found that activity in the highest quintile of the classical pathway was associated with increased odds of unprovoked VTE (OR 4.5, 95% CI; 0.8-24.7). Moreover, MBL deficiency (≤100 ng/ml) was associated with unprovoked VTE (OR 3.5, 95% Cl; 0.8-15.3). VTE patients had shortened TF-induced lag-time (4.8±0.6 min vs. 5.8±2.1 min, $p<0.001$) and a higher endogenous thrombin potential (ETP) (1383±267 nM*h vs. 1265±247 nM*h, $p=0.07$) than controls. No association between the classical complement pathway activity or MBL deficiency, and parameters of TF-induced thrombin generation was observed. These results suggest that high activity of the classical complement pathway, and MBL deficiency, might be associated with an increased odds of unprovoked VTE.

4.2 Paper II

COMPLEMENT ACTIVATION ASSESSED BY TERMINAL COMPLEMENT COMPLEX AND FUTURE RISK OF VENOUS THROMBOEMBOLISM

Growing evidence support a link between key components of the complement system and risk of venous thromboembolism (VTE). However, it remains uncertain whether activation of the complement system, assessed by soluble terminal complement complex (plasma TCC), is associated with future risk of incident VTE. The aim was to investigate the association between plasma levels TCC and future risk of incident VTE in a nested case-control study and to explore genetic variants associated with plasma levels TCC using protein quantitative trait loci (pQTL) analysis of exome sequencing data. The study population consisted of 415 subjects with VTE events and 848 age- and sex-matched controls who were selected from a population-based cohort, the Tromsø Study. Logistic regression models were used to calculate odds ratios (ORs) with 95% confidence intervals (CIs) for VTE across quartiles of plasma levels of TCC. Whole exome sequencing was conducted in 709 subjects (355 VTE patients and 354 control subjects) using the Agilent SureSelect 50Mb capture kit. The risk of VTE increased across quartiles of plasma TCC, particularly for unprovoked VTE (p for trend 0.02). Participants with plasma TCC in the highest quartile (> 1.40 CAU/mL) had an OR for unprovoked VTE of 1.74 (95% CI: 1.10-2.78) compared to those with plasma TCC in the lowest quartile (≤ 0.80 CAU/mL) in analyses adjusted for age, sex, body mass index. The OR for VTE by high plasma TCC was substantially higher in individuals with short time between blood sampling and VTE event. We found no significant association between genome-wide and complement-related gene variants and plasma TCC. Our findings showed that plasma levels TCC are independent of gene regulation and that high levels of plasma TCC are associated with VTE risk and unprovoked events in particular.

4.3 Paper III

THE EFFECT OF SHORT- CHAINED POLYPHOSPHATES ON COAGULATION AND COMPLEMENT ACTIVATION IN A HUMAN WHOLE BLOOD SYSTEM

Polyphosphates (polyPs) are naturally occurring, highly anionic linear polymers of monophosphate units. Short-Chained (SC)-PolyPs are secreted from platelets upon stimulation and are reported to inhibit complement activation under experimental conditions and to facilitate propagation of coagulation activation. We aimed to investigate the impact of SC-polyPs on coagulation- and complement activation in a whole blood model. Blood was collected from healthy fasting individuals and anticoagulated with the thrombin inhibitor lepirudin. Blood was incubated with SC-polyPs (0-100 µM) and *E.coli* (10^7 CFU/ml). Complement activation was monitored by plasma terminal C5b-9 complement complex (TCC) and coagulation activation by prothrombin fragment 1+2 (F1+2) using ELISA assays. Infestin was used to block the coagulation activation product FXIIa, and the impact of tissue factor (TF) on coagulation activation was monitored by TF mRNA expression and use of blocking anti-TF antibodies. We found that SC-polyP in concentrations 40 and 100 µM promoted coagulation activation ($p<0.01$ compared to time-matched controls), but to a lesser extent than long-chained polyp in whole blood. *E.coli* induced a time-dependent (0-30 min) complement activation in whole blood ($p<0.001$), but SC-polyP did not modify this *E.coli*-induced complement activation. Preincubation with the FXIIa-inhibitor infestin, completely blocked SC-polyP-induced coagulation activation (plasma F1+2: 1939±619pmol/l with 100µM polyP vs. 578±285pmol/l with infestin and 100µM polyP; $p<0.001$) without affecting complement activation. In conclusion, SC-polyP triggered FXII-dependent coagulation activation without affecting complement activation in a whole blood model.

5. General discussion

5.1 Methodological considerations

5.1.1 Study design

Paper I and II in this thesis utilize data from a large population-based cohort, the Tromsø Study. The Tromsø Study has a high attendance rate (77%) and all inhabitants ≥25 years were invited, which makes it a suitable cohort for studying the general population. Paper I has a case-control design, whereas paper II has a nested case-control study.

Case-control studies are more efficient and less resource demanding than cohorts. A case-control study is an observational study that compares attributes of sampled cases and controls. However, most case-control studies are retrospective, and prone to reverse causation and recall bias, and can therefore not be used to assess cause-and-effect relationships. Paper I of this thesis is a case-control study where cases were derived from the fourth Tromsø Study and the controls were derived from the sixth Tromsø Study. The blood samples were drawn 1-6 years after the VTE event, and thus it is not possible to assess if the increase in potential complement activation is a cause or a consequence of the VTE event. However, we only recruited cases with a VTE event >1 year before blood sampling to minimize the risk of reverse causation.

Paper II in this thesis is a nested case-control study where all participants were sampled from the same parent cohort (i.e. the fourth Tromsø Study), and matched on age and sex. A nested-case-control study enables us to limit the use of expensive analysis without sacrificing the temporal advantage of cohort studies. The blood samples were taken at inclusion in Tromsø 4 (1994/95), frozen after blood collection, and thawed and analyzed several years later. In this study, we know that the increase in TCC in VTE cases is not due to the VTE since the blood

samples were taken before the event. This enables us to speculate in cause-and-effect specific relationships between TCC levels and VTE.

5.1.2 Bias

Bias is the term for systematic errors in epidemiological research that results in incorrect estimates of the true effect of an exposure on the outcome. Biases may be introduced into a study at different points and are divided into different types:

Selection bias occurs when there is a systematic error in recruitment of study participants so that the association between exposure and outcome gets affected. This is less likely to occur in cohort studies, since both cases and controls are selected before the outcome occurs and many exposures and outcomes can be studied in one survey. There is, however a chance for a non-response bias in cohorts, where the responders are different from the non-responders [268]. In other designs, such as a case-control study, selection bias is more easily introduced. The controls have to be representative for the population from which the cases are derived with respect to the distribution of exposures and confounders. The likelihood of achieving this is higher if the cases and controls are recruited from the same predefined source population. The selection of controls may be biased since there is a risk of including controls that do not represent the source population of the cases. In paper I, we randomly sampled participants from the fourth Tromsø study and invited them to participate as controls in our study. In paper II, the controls were randomly sampled from Tromsø 4. In both studies, the controls were matched on age and sex to minimize the risk of selection bias. Moreover, in both papers I and II, the cases were derived from a well-defined cohort, and the diagnosis was thoroughly validated, hence reducing the possibility of selection bias in our cases.

Misclassification bias can be non-differential or differential. A misclassification is non-differential when it is independent of the outcome and similar across comparison groups. Non-differential misclassification most often leads to underestimation of the true association. Studies including baseline information obtained through self-administered questionnaires are vulnerable to misclassification as participants may report their exposure levels incorrectly. Moreover, random errors in biological measurements may lead to misclassification.

Regression dilution bias is an example of non-differential misclassification. The regression model assumes that variables like smoking habits, cholesterol levels, and dietary habits stay the same throughout the study period. If the variable changes during the follow-up, and this is not corrected for in the analysis, it might lead to a dilution of the true effect [269, 270]. Regression dilution bias can be introduced by true changes in the exposure variable, or by random measurement errors in the exposure variable, and leads to attenuated risk estimates [269]. In a cohort, where blood samples are taken before the outcome occurs and the exposure levels do not affect the outcome assessments, the misclassification of exposure is likely non-differential. Plasma levels of modifiable biomarkers are expected to change over time, [269] when there is a long follow up time. Accordingly, in Paper II, we found that the risk of VTE by plasma levels of sTCC varied substantially over time between blood sampling and the VTE event, and this phenomenon was likely explained by regression dilution.

Misclassifications are differential if the misclassification is dependent on the value of an exposure. Differential misclassifications can introduce biases in either direction [269]. In case-control studies, with measurements collected after the cases and controls are chosen, there is a potential for differential misclassification. This might be due to **recall-bias**, which implies that the cases and the controls might recall their medical history differently, and describes the tendency for cases to recall an exposure more often that controls [271].

Differential misclassification can also occur in prospective studies if the outcome assessment/adjudication is influenced by the known exposure levels (e.g. if a participant is more likely to be referred to screening for an event due to his/her risk factor level). In paper I the VTE events were symptomatic, objectively confirmed by radiological procedures and adjudicated by persons who were blinded for the measurements of potential complement activation, which diminishes the possibility of differential misclassification.

5.1.3 Missing data

Missing data is common in medical research, and it is not possible to eliminate the possibility of missing data completely. Missing data can be introduced when participants do not respond to questions in a questionnaire, if equipment fails, procedures are not followed, by loss or errors in laboratory handling of samples, or any other reason that leads to loss of data [272]. Careful handling and execution of studies are important to reduce this problem. However, there is no optimal approach to ensure avoidance of missing data. If the number of missing data of one variable is very high, the variable should be excluded from the study. If the number of missing data of one variable is low, the subjects with missing values can be excluded [272]. If those with missing data differ from those with complete data, exclusion might lead to selection bias. In addition, exclusion of subjects will reduce the statistical power. To maintain sample size, and thus the statistical power, one can replace missing data. Imputation (i.e. replacement), can be used if the missing values are missing at random, if the data is not missing at random one can use multiple imputations [272]. In paper II, we excluded 47 (10%) cases and 76 (8%) controls due unavailable plasma samples of sufficient quality for the analyses. Since the proportion of missing was low, and likely non-differential (i.e. the chance of having an insufficient blood sample would not depend on the outcome), exclusion of these cases did probably not influence the results of our study.

A study has high external validity if the results are applicable to the whole source population, and to similar populations (i.e. the study has high generalizability). The age and sex distribution of the Tromsø population is comparable to other Western populations [273]. The incidence and prevalence of cardiovascular diseases, traditional cardiovascular risk factors and the incidence of VTE and cancer is comparable to the incidence found in other studies of similar populations [273, 274]. The high attendance rate (77% of those invited) suggest that the Tromsø Study has high external validity.

5.1.4 Sample size and statistical power

The power of a study describes how likely it is to find an effect if present. Statistical power is affected by the size of the effect (effect size) and the size of the sample used to detect it. Bigger effects are easier to detect than smaller effects, while large samples offer greater test sensitivity than small samples. The statistical power can be increased by increasing the sample size [275]. The probability of making a type II error i.e. concluding that there is no effect when there is one is low when the statistical power is high. Power calculations should be done when planning a study and it is common to aim towards 80% power, which means that there is an 80% probability that a type II error will not occur. The acceptable probability of making a type I error i.e. to find and effect when there is none, is in most cases set to 5%.

In a case-control the statistical power in mainly determined by the number of cases you have, but can be increased by including more than one control per case [276]. However, in subgroup analysis, the number of outcomes in each group can be small, and subgroup analysis can have less power than the main analysis. To calculate the sample size before a study is conducted,

assumptions on expected effect have to be made. These assumptions are often made based on previous knowledge and studies in the field.

It is a challenge to calculate sample size in experimental studies, as we in most cases do not know the outcome of the study. In paper III, we could not estimate the effect on polyP on coagulation- and complement activation in our whole blood model since it had never been done before.

5.1.5 The whole blood model

Using the human whole blood model in paper III makes the experimental system more physiological relevant than using buffer, plasma, or serum models. We can model the result of the influence of known agents such as polyP, but also take in to account the influence of other factors residing in the blood. Although the complexity of whole blood reduces our ability to provide exact mechanisms for the effect, it allows us to evaluate the effect of interventions when this complexity is preserved, making it closer to a clinical situation.

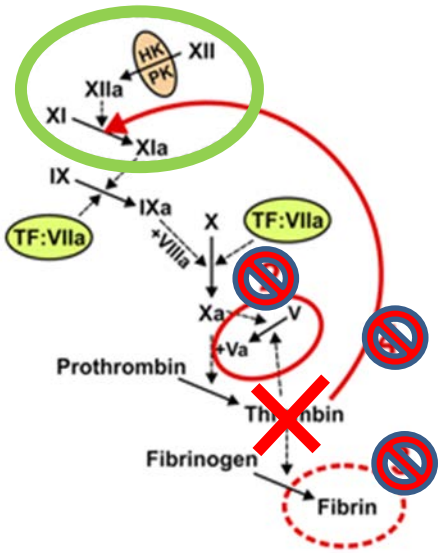

Figure 8. The effect of SC-polyP in our whole blood model where thrombin is blocked. The potentiation effect on coagulation activation of SC-polyP through thrombin are blocked, leaving us with the isolated effect of SC-polyP on FXII activation (adapted from Morrissey, Blood 2012).

There are important limitations to consider when using the whole blood model. The model represents an intermediate between *in vitro* and *in vivo* experiments, and although the complexity of the blood is preserved there are still processes and reactions, which are lost when removing it from the endothelial lining of the vessels. The fact that whole blood has to be anticoagulated is also a limitation as it interferes with the natural processes in the blood. Lepirudin was the most suitable anticoagulant in our experiment since it does not interfere with complement activation or Ca^{2+} levels and enables us to study the coagulation system upstream of thrombin. However, as mentioned previously, thrombin might be an important player in the crosstalk between complement- and coagulation activation, and we lose this possible interaction between the systems. On the other hand, the use of lepirudin is advantageous when studying polyP's effect on coagulation activation, since it makes it possible to isolate the possible effect of polyPs on FXII activation, without the positive feedback for thrombin (Figure 8).

5.2 Discussion of main results

5.2.1 High potential activity in the classical pathway of the complement system and MBL deficiency is associated with increased odds for unprovoked VTE (paper I)

In paper I, we found that subjects with potential activity levels within the highest quintile of the classical pathway had a higher risk of unprovoked VTE. Moreover, MBL deficiency and low lectin pathway activity, was associated with increased risk of VTE.

We recruited VTE patients from a population-based cohort and age- and sex-matched apparently healthy controls from the same source population, making it a representative study population for the general population. To study complement activity we used an assay designed as a screening assay to detect deficiencies in the complement pathways. It is not suitable to detect *in vivo* activation, but reflects the total activity potential within each complement pathway by *in vitro* activation [266]. The pathway activity was measured as a percentage of a standard pathway activity derived from normal human serum, defined to contain 100% activity in all three pathways. The percentage of normal pathway activity was used to assess if the potential activity of the complement pathways showed different risk estimates for VTE. The use of this method is a novel approach to study the association between complement pathways and diseases. However, the results should be considered as exploratory since these measurements represent the potential for activation and not activation *in vivo*.

The classical pathway of the complement system and risk of VTE has been studied in systemic lupus erythematosus (SLE) patients. In a Swedish study, SLE patients with a history of VTE had increased deposition of C1q, C3, and C4d on platelets compared to SLE patients without VTE [245], indicating classical pathway activation. Accordingly, we found an OR of 4.5 for the association between high potential serum activity of the classical pathway and unprovoked VTE in a study with participants from the general population. As thrombin is known to activate

complement component C3 [219], one possible explanation for the association between high potential complement activity and VTE could be hypercoagulability. We investigated this possibility by measuring thrombin generation in plasma by Calibrated Automated Thrombogram (CAT). We found that high potential activity of the classical complement pathway was not associated with parameters of TF-induced thrombin generation. This indicated that the association between high potential activity of the classical pathway of the complement cascade and VTE risk was independent of coagulation parameters. One possible mechanism for the association between high activity of the classical pathway of the complement system and VTE risk might be the hypoxic state found in the veins, particularly in the deepest recess of the valvular sinus, where the thrombus forms [1, 38]. Hypoxia has been found to activate the complement system [277] and re-oxygenation of hypoxic HUVEC cells incubated with 30% serum leads to activation of the classical pathway [278]. It is reasonable to think that the hypoxic state in the valvular sinuses leads to activation of the complement system, which again can initiate thrombus formation.

Between 5-20% of the population is estimated to be MBL-deficient [279, 280]. Samples that were low in the lectin pathway activity assay, were therefore quantified for MBL concentration in a separate ELISA to ensure that subjects did not have a MASP deficiency. Even though >99% of the lack of lectin pathway activity is due to MBL deficiency, occasionally MASP-2 deficiency has been found, of which one case is reported in Norway [281]. MBL levels have been found to remain stable throughout life, making it a good candidate as biomarker for various diseases. Age, gender, and diurnal variation do not affect MBL levels of an individual, and there is only a mild increase during acute phase reactions [282]. Individuals with MBL levels below 100ng/ml have high prevalence of associated diseases [279, 283]. MBL-deficient individuals have increased susceptibility to various types

of infectious disease, as well as several noninfectious disorders, including autoimmune disorders [284-286]. In addition, the lectin pathway MBL associated serine protease-1 (MASP-1) has been shown to be a significant contributor to coagulation in a mouse model with occlusive thrombosis [286], and have thrombin-like properties [287, 288]. Based on this we suggested that MBL deficiency would be protective against VTE. Prior to our study, two smaller studies had investigated the association between genotypes associated with MBL deficiency and risk of VTE in patients with SLE. One study found that VTE in SLE patients were unrelated to the MBL genotype [278]. However, in a cross-sectional study including 114 SLE patients, the prevalence of VTE was higher in subjects with MBL-deficient genotypes [279]. In our case-control study, VTE patients had 3.5-fold higher odds of MBL deficiency (≤100 ng/ml) than controls. The MBL levels were strongly correlated with potential lectin pathway activity, and similarly to MBL levels, low lectin pathway activity showed a trend towards an increased risk of VTE. The underlying mechanisms for the possible association between MBL deficiency and VTE risk is not obvious, but may be a consequence of the predisposition to autoimmune- [280, 281] and infectious disease [282, 283], in individuals with MBL deficiencies, both of which are associated with the risk of VTE [284, 285].

Traditional case-control studies are retrospective in nature, meaning that blood samples are often drawn after the event of interest. In our study, blood samples were drawn 1-6 years after the VTE event. Thus, it is not possible to determine whether increased complement pathway activity was present before the event or was a consequence of the VTE event (reverse causation). However, the rather long time between the VTE event and blood sampling strengthened the probability of that the increased complement activities were

present prior to the event and not an acute reaction of the VTE event. Due to the low number of participants included in this study the risk of statistical type 1 and 2 errors are considerably increased. Statistical analysis of such a small sample size has limitations, and the results should be interpreted with caution. Thus, a larger, prospective study should be conducted to validate these findings.

5.2.2 Complement activation assessed by TCC is associated with future risk of VTE (paper II)

In Paper II, 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 all VTE, and especially unprovoked VTE, increased across quartiles of plasma TCC. Subjects with plasma TCC in the highest quartile had 74% higher risk of unprovoked VTE compared to those with plasma TCC in the lowest quartile. Furthermore, we found no significant association between genome-wide and complement-related gene variants and plasma levels of TCC.

Prior to our study, no study had investigated complement activation assessed by plasma TCC and risk of VTE. The studies done on complement and VTE risk were restricted to animal models, with the exception of one large population-based cohort (the Copenhagen General Population Study). In the latter study, subjects with plasma C3 levels in the highest tertile had 31% higher risk of VTE after adjustment for CRP and BMI [243]. This study investigated the association between plasma levels of C3 and future risk of VTE and not the activation of the complement system. This led to the question of whether C3 was a mediator of VTE risk or merely an innocent bystander. The impact of C3 on VTE risk has also been investigated in an animal model. C3-deficient mice had lower incidence of venous thrombosis and reduced

thrombus size compared to wild-type mice [287]. The pivotal role of C3 in complement activation [243] may suggest that complement activation drives the observed link between C3 and VTE risk. To investigate this we measured the end stage of complement activation, namely the formation of the terminal TCC complex C5b-9, also called the membrane attack complex (MAC) when assembled on a membrane. If the terminal pathway is activated in the fluid phase, the TCC complex will build up in a soluble form (plasma TCC), that can be measured in plasma as an indicator of complement activation. We found that plasma TCC was associated with increased risk of VTE, and especially unprovoked VTE. This indicates that it is activation of the complement system that is associated with VTE risk, and the observed association between C3 and VTE is due to complement activation.

Several studies has found that the dysregulation of the complement system is associated with different arterial and cardiac diseases [289-291]. Depositions of the TCC [292, 293] and other complement components have been found in human infarcted myocardium [294]. It was previously shown that re-oxygenation of hypoxic human endothelial cells leads to activation of the complement system via the classical pathway [278]. As discussed for the classical pathway and VTE risk, the hypoxic state found in the deepest recess of the valvular sinus, where the thrombus forms [1, 38] may also activate the complement system [277, 295] and potentiate thrombus formation. This may be part of the explanation of why complement activation is associated with VTE risk. Moreover, in our study, genome-wide and complement-related gene variants showed no significant association with levels of plasma TCC. This strongly suggests that plasma TCC is determined by environmental rather than genetic factors. Subjects susceptible to future VTE may exhibit a certain pathophysiological milieu in the valvular sinuses, which makes them prone to complement activation and thereby future risk of VTE, or have dysregulation of the complement system in these locations. This

further supports the results from paper I where we found that high potential classical pathway activity of complement was associated with higher risk of VTE, independent of TF-induced thrombin generation.

It is not only the location of thrombus formation which may explain the effect of complement activation on VTE risk. Evidence supports that complement activation is a mediator of VTE risk through platelet- and coagulation activation. If the TCC is incorporated in the cell membrane of platelets, it results in the exposure of negatively charged phosphatidylserine (PS) on the activated platelet surface [290] that may assemble and amplify coagulation reactions [291]. It can also lead to the formation of procoagulant microvesicles (MVs) [211], which is associated with risk of VTE [145], and secretion of procoagulant granules from the cytoplasm of platelets [212]. In addition, the complement system has direct procoagulant activities, including the ability to cleave and activate coagulation factors [214] and increase TF expression in various cell types [214, 215].

This study was a nested-case-control study and the blood samples were obtained at inclusion in Tromsø 4 (1994/95), and analyzed several years later. The time sequence between exposure (high plasma TCC) and outcome (VTE) makes it possible to speculate about a potential causal relationships between plasma TCC levels and VTE risk. As plasma levels of TCC are modifiable and will fluctuate over time, the association to VTE risk is likely to be underestimated in our study with long follow-up time (regression dilution bias). To address the possibility of regression dilution bias we performed statistical analysis where we restricted the maximum time from blood sampling to the VTE events. Consequently, we found that the risk of VTE by plasma TCC was higher with shorter time between blood sampling and VTE.

This indicates that the true association between plasma levels of TCC and VTE risk is stronger than found in our study with long follow-up.

Similar to the results from paper I, the risk of VTE is higher in subjects with high activation of the complement system. We can assume that the complement system is involved in the pathogenesis of VTE based on the results from previous studies and our results on complement activation and VTE risk. Functional studies are needed to investigate the molecular mechanisms behind the association between plasma TCC and VTE risk.

5.2.3 The role of polyphosphates in coagulation and complement activation (paper III)

In paper III, we investigated the effect of short chained (SC) polyphosphates (polyP) on coagulation- and complement activation in a human whole blood model where thrombin was blocked. We found that SC-polyP significantly increased prothrombin fragment 1+2 (F1+2) levels in whole blood after 60 minutes, indicating that it activates the coagulation system. Inhibition of FXIIa abolished the SC-PolyP induced coagulation activation, showing that SC-polyP plays a role in initiating activation of the contact pathway of coagulation. We found that SC-polyP did not influence *E.coli*-induced TCC formation in whole blood.

No studies had investigated the direct effect of platelet-sized polyPs on coagulation activation in whole blood where the Ca^{2+} concentrations are kept physiological. Our whole blood model takes advantage of the fact that thrombin is blocked. This enables us to investigate the isolated effect of SC-polyPs on FXII activation. To the best of our knowledge, no such investigations of SC-polyPs had previously been performed in a whole blood model with physiological Ca^{2+} concentrations.

For many years the true activators of the contact pathway were unknown and most of the studies done on contact activation used artificial surfaces. Platelet-sized polyPs where found to be an activator of the intrinsic pathway of coagulation through activation of factor FXII [7, 255], and to bind tightly to the proteins responsible for contact activation [255, 256]. Clinical studies have shown that elevated levels of plasma FXII is associated with coronary heart disease and atherosclerosis [296, 297], and studies in mice showed that FXII deficiency protected against thrombosis [150]. In addition, cationic inhibitors of polyP reduced venous and arterial thrombosis in animal models [298] . This made platelet polyP an interesting molecule to study in the contact activation and in the pathogenesis of VTE. However, later studies showed that the ability to activate FXII in the absence of blood cells was dependent on polymer length [256, 258, 299], and now bacterial long-chained polyPs (>200 units) are recognized as a surface for assembly of the coagulation proteins necessary for activation of the contact pathway of coagulation [43]. Nowadays, SC-polyP is thought to be involved in acceleration of coagulation activation and not in the initiation [300]. Contrary to this, we found that SC-polyP significantly increased plasma levels of F1+2 in human whole blood after 60 minutes, and that this effect was abolished when pre-incubating blood with the FXIIa specific inhibitor infestin. This indicates that SC-polyPs activate FXII in human whole blood. This was further proved by the fact that tissue factor (TF)-mRNA levels in blood incubated with SC-polyPs were not elevated and we did not observe any effect of anti-TF antibody on SC- polyP induced coagulation activation.

Over the past decades, several studies have reported that activated platelets promote coagulation in a FXII-dependent manner [301-304]. Indeed, the presence of platelets in our whole blood model might explain why SC-polyP could activate FXII, but not by others that

used systems without platelets. Platelet-derived polyPs have been found to bind to and aggregate on the platelet surface and form nanoparticles that promote FXII activation [259]. The aggregation of polyPs on the platelets surface leads to a high local concentration of polyPs. Moreover, it is possible that there is a presence of longer chained polyPs on the platelet surface after activation [259]. In addition, polyPs have been found to bind to the platelet surface together with FXII [305]. These findings together with our results, which show that SC-polyPs activate FXII in whole blood, makes it reasonable to speculate that the ability of SC-polyPs to activate FXII is dependent of the presence of platelets.

When we started this work, only two studies had investigated the effect of polyPs on activation of the complement system. Wijeyewickrema and colleagues found that SC-polyP acted as a cofactor for C1 inhibitor (C1-INH), which is the major negative regulator of the classical- and lectin pathway. [261]. They found that polyP potentiated the inhibitory function of C1-INH, dampening complement activation [261]. Moreover, Wat and colleagues found that polyP suppressed the terminal pathway of complement by destabilizing the assembly of the TCC in a size and concentration dependent manner. They found that P_1 and $polyP_{>1000}$ suppressed hemolysis in a concentration-dependent manner [260], and that 100µM SC-polyP inhibited the terminal pathway of complement with the same magnitude as LC-polyP in serum. We found no such effect in whole blood when *E.coli* was used as a complement activator and blood was preincubated with 100 µM SC-polyP. These results seem conflicting, but it might be explained by the differences in study methods. Wijeyewickrema et al. used a buffer system with purified proteins, and Wat et al. used a system with 2% serum, which makes these systems suitable to study molecular mechanisms but they do not necessarily reflect what happens *in vivo*. Our whole blood system is more suitable for studying an *in vivo* situation as it represents an interface between an *in vivo* model

and an *in vitro* system. Our results indicate that the presence of blood cells and plasma in physiological concentrations interferes with the possible inhibitory function of SC-polyPs on complement activation and that SC-polyPs found in human platelets do not exert an inhibitory function on the complement system.

The whole blood model used in this study gives us the opportunity to study the effects of polyPs in the presence of blood cells and plasma factors. Using lepirudin as an anticoagulant gives us a unique opportunity to analyze the initiation phase of thrombin generation. The fact that thrombin is inhibited in our model has to be kept in mind when interpreting the results, as it might influence the observations. On the other hand, inhibition of thrombin makes it possible to investigate the isolated effect of SC-polyPs on FXII activation without the influence of thrombin induced feedback activation of FXI and FV.

6. Conclusions

1. Low activity of the lectin pathway, reflected by MBL deficiency, and high activity of the classical pathway might be associated with risk of unprovoked VTE. Parameters of thrombin generation (lag-time and ETP) were associated with VTE risk, but displayed no associations with the activity of the lectin and classical complement pathways. These findings are hypothesis-generating and exploratory, and a larger prospective study is warranted to validate our findings.

2. Complement activation *in vivo*, assessed by plasma levels of TCC, was 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 plasma TCC, suggesting that local or systemic environmental factors are the dominating determinants of plasma TCC. Functional studies are warranted to investigate the molecular mechanisms behind the association between plasma TCC and VTE risk.

3. SC-polyphosphates activate the intrinsic pathway of coagulation through FXII in a human whole blood system where thrombin is blocked, but do not suppress *E.coli*-induced complement activation.

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