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Infection, inflammation, and risk of venous thromboembolism

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SUMMARY

Venous thromboembolism (VTE), including deep vein thrombosis and pulmonary embolism, is the third most common cardiovascular disease after myocardial infarction and stroke. The incidence of VTE is increasing and VTE contributes to a substantial burden of morbidity and mortality. To be able to prevent VTE in vulnerable subjects, there is a need for more knowledge about the pathophysiology and risk factors for VTE. Acute infection and several other inflammatory conditions have been associated with VTE risk in previous studies. The aim of the present thesis was to investigate the association between both short- and long-term inflammation and VTE risk. Further, the aim was to investigate the impact of acute infection on VTE risk in hospitalized patients, and, finally, whether gut microbiome composition was associated with systemic inflammation and coagulation.

In Paper I, we used data from the fourth survey of the Tromsø Study, conducted in 1994-95, with follow-up until the end of 2012. Incident and recurrent VTE events were registered and thoroughly validated. Cox proportional hazards regression models were used to calculate hazard ratios for VTE across quartiles of the inflammatory marker neutrophil to lymphocyte ratio (NLR). We found no association between NLR measured at baseline in Tromsø 4 and future risk of incident or recurrent VTE. However, when restricting the follow-up time to the first three years after baseline, those with NLR above the 95th percentile had a 2.4-fold increased risk of VTE.

In Papers II and III, we used a case-crossover designed study with the incident VTE cases from Tromsø 4 as the study population. We found that acute infection was a frequent and strong trigger for VTE in hospitalized patients, also after adjustment for potential confounders. Moreover, we found that concomitant infection and immobilization had a synergistic effect on VTE risk. Acute inflammation, assessed by C-reactive protein (CRP), was associated with VTE risk, regardless of the cause of inflammation. The association was slightly attenuated in analyses adjusted for infection and immobilization. In stratified analyses, acute inflammation was associated with VTE risk both in cases with and without infection.

In Paper IV, we conducted a randomized, controlled trial in healthy volunteers aged 18-40 years. The intervention group received oral Vancomycin, which induced a gram-negative shift in the gut microbiome composition. Compared to the untreated control group, this change in microbiome composition was accompanied by a significant increase in coagulation factor VIII:C and high sensitivity CRP from baseline to after intervention.

SAMMENDRAG

Venøs tromboembolisme (VTE), som innbefatter dyp venetrombose og lungeemboli, er den tredje vanligste kardiovaskulære sykdommen etter hjerteinfarkt og slag. Forekomsten av VTE er økende, og sykdommen bidrar til en betydelig sykdomsbyrde og i noen tilfeller også død. Det trengs mer kunnskap om patofysiologi og risikofaktorer for VTE, for å kunne identifisere grupper med høy risiko som kan profitere på forebyggende behandling. Både akutte infeksjoner og andre tilstander preget av inflammasjon har i tidligere studier vært assosiert med økt risiko for VTE. Målet med denne avhandlingen var å undersøke sammenhengen mellom inflammasjon av både kort og lang varighet og risiko for VTE, samt å undersøke sammenhengen mellom akutt infeksjon under sykehusopphold og risiko for VTE. Videre var målet å undersøke om sammensetningen av tarmbakterier påvirker systemisk inflammasjon og koagulasjon.

I artikkel I brukte vi data fra den fjerde Tromsøundersøkelsen, som ble gjennomført i 1994-95, og deltakerne ble fulgt med registrering av validerte VTE-tilfeller til utgangen av 2012. Vi brukte overlevelsesanalyse (Cox regresjonsmodeller) for å undersøke sammenhengen mellom nøytrofil-lymfocytt ratio (NLR), som er en inflammasjonsmarkør, og risiko for VTE. Det var ingen sammenheng mellom NLR, inndelt i kvartiler, og risiko for hverken førstegangs eller gjentakende VTE. Når oppfølgingstiden ble satt til de tre første årene etter inklusjon i Tromsø 4, hadde imidlertid de med NLR over 95-percentilen 2.4 ganger økt VTE-risiko.

Ved bruk av case-crossover design med VTE-tilfellene fra Tromsø 4 som studiepopulasjon, undersøkte vi sammenhengen mellom akutt infeksjon (artikkel II) og inflammasjon (artikkel III) og risiko for VTE. Akutt infeksjon under sykehusopphold var hyppig forekommende og sterkt assosiert med VTE-risiko, også etter justering for mulige konfunderende faktorer. Samtidig infeksjon og immobilisering hadde synergistisk effekt på VTE-risiko. Akutt inflammasjon under sykehusopphold, målt ved C-reaktivt protein (CRP), var assosiert med VTE-risiko uavhengig av inflammasjonsreaksjonens årsak, og sammenhengen besto etter justering for infeksjon og immobilisering.

Artikkel IV beskriver en randomisert, kontrollert studie hvor friske frivillige i alderen 18-40 år ble invitert til å delta. Intervensjonsgruppen fikk Vancomycin kapsler, som førte til en endring i tarmfloraen til økt andel gram-negative bakterier. Sammenlignet med en ubehandlet kontrollgruppe fant vi at intervensjon med Vancomycin førte til en signifikant økning i koagulasjonsfaktor VIII:C og CRP.

LIST OF PAPERS

The thesis is based on the following papers:

I. Neutrophil to lymphocyte ratio and future risk of venous thromboembolism and mortality: the Tromsø Study
Grimnes G, Horvei LD, Tichelaar V, Brækkan SK, Hansen JB
Haematologica 2016 Oct; 101(10): e401-e404

II. Acute infection as a trigger for incident venous thromboembolism: Results from a population-based case-crossover study
Grimnes G, Isaksen T, Tichelaar YIGV, Brækkan SK, Hansen JB
Res Pract Thromb Haemost. 2017; 00:1–8

III. C-reactive protein and risk of venous thromboembolism: Results from a population-based case-crossover study
Grimnes G, Isaksen T, Tichelaar YIGV, Brox J, Brækkan SK, Hansen JB
Haematologica 2018, e-pub ahead of print April 19, 2018

IV. A Vancomycin-induced shift of the gut microbiome in gram-negative direction increases plasma factor VIII:C levels: Results from a randomized, controlled trial
Grimnes G, Hindberg K, Davids M, Nieuwdorp M, Mollnes TE, Brækkan SK, Hansen JB, Tichelaar YIGV
Manuscript

ABBREVIATIONS

APC	activated protein C
BMI	body mass index
C	control period
CI	confidence interval
CRP	C-reactive protein
CT	computer tomography
CV	coefficient of variation
DVT	deep vein thrombosis
ECOG	Eastern Cooperative Oncology Group
EV	extracellular vesicle
F	factor
FVL	factor V Leiden
HR	hazard ratio
hs	high sensitivity
HUNT	the Nord-Trøndelag Health Study
ICD	International Classification of Diseases
IL	interleukin
ln	natural logarithm
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein 1
NETs	neutrophil extracellular traps
NLR	neutrophil to lymphocyte ratio
OR	odds ratio
PAI-1	plasminogen activator inhibitor-1
PE	pulmonary embolism
r	ribosomal
RCT	randomized controlled trial
RTI	respiratory tract infection
TCC	terminal complement complex
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TNF	tumor necrosis factor
UNN	University Hospital of North Norway
UTI	urinary tract infection
VTE	venous thromboembolism
vWF	von Willebrand factor
WBC	white blood cell

1 INTRODUCTION

A rapid and efficient mechanism to stop bleeding, i.e. hemostasis, is necessary for staying alive. Likewise, the body needs to clear infection and repair tissue injury, which are taken care of by the inflammatory response. Hemostasis and inflammation are both complex processes involving activation of cascade systems, and these systems crosstalk.[1] The goal of hemostasis is to stop bleeding, and the goal of acute inflammation is to eliminate an infectious agent and to repair damaged tissue. A perfect balance of these processes and their inhibitory counterparts is not always achieved. Coagulation activity when there is no bleeding or a dysregulated inflammatory response to an infection, can be life threatening instead of life saving.

Venous thromboembolism (VTE) includes the disease entities deep vein thrombosis (DVT) and pulmonary embolism (PE). Classical symptoms and signs of DVT include redness, swelling, heat and pain in the affected extremity, most often a leg or thigh. As the name suggests, PE is traditionally thought to occur when thrombus material from a DVT breaks loose, follows the blood stream through the heart and ends up as an embolus obstructing blood flow in an artery of the lungs. However, in approximately 50% of PE cases, imaging studies have failed to identify an origin of the thrombus outside the lung.[2] Atrial fibrillation, with subsequent right sided intra-cardiac thrombus formation, accounts for some of the remaining cases[3], and de novo thrombus formation in the lungs is a possible mechanism for others.[2] Symptoms of PE include dyspnea, tachypnea, coughing, pleuritic chest pain, and in severe cases circulatory collapse and death.

The best studied acute inflammatory response is inflammation triggered by microbial infections.[4] Humans live close to microbes, and it is estimated that the number of bacteria and the number of human cells in the body are approximately the same.[5] Most of the bacteria living inside and on a body's surface- termed our microbiota- live in peace with the human host. However, when bacteria from either the body's microbiota or foreign sources invade the body, an infection results, eliciting an inflammatory response.[4] Before the era of antibiotics, infections were a major health problem associated with high mortality.[6] However, increasing use of antibiotics leads to increasing bacterial resistance to antibiotics, and infections again rise as a major health threat challenging cancer treatment and organ transplantation in modern medicine.[7,8]

Whereas chronic low-grade inflammation is an established risk factor for arterial thrombosis[9], the association between inflammation and risk of VTE has been less clear.[10] In contrast to the observed decline in incidences and mortality rates of coronary heart disease and

stroke[11,12], there has been an increase in the VTE incidence.[13] VTE is a preventable disease, but the benefit of prophylactic anticoagulant therapy has to be balanced against an increased bleeding risk. More knowledge about risk factors and mechanisms for VTE is needed in order to identify situations where preventive therapy is beneficial.

This thesis focuses on the relationship between infection, inflammation and venous thromboembolism.

1.1 Epidemiology of venous thromboembolism

VTE is the third most common cardiovascular disease after coronary heart disease and stroke[14], with an incidence in the adult population of 1 to 2 per 1000 per year in developed countries.[13,15,16] The incidence of VTE is strongly affected by age, and VTE is a rare disease prior to late adolescence.[17] After adolescence, incidence rates of VTE increase with age in both men and women[17,18], to 8 per 1000 per year after the age of 80.[17,19] Women of reproductive age (16-44 years) have a higher incidence of VTE than men at the same age, whereas men have a higher incidence than women from 45 years and above.[17] The incidence of VTE also varies between ethnic groups. Asian and Pacific Islanders have the lowest VTE incidence, followed by Hispanics, Caucasians and African-Americans.[20]

Approximately two out of three VTE cases are diagnosed as DVT alone, and the rest as PE with or without concurrent DVT.[18] Whether autopsy data is included or not affects the proportion of PEs and DVTs, as autopsy data probably overestimates the PE incidence and studies based on clinical diagnoses probably underestimate the incidence of PE.[18] An increase in the PE incidence during later years has been described.[13,21] Computed tomography pulmonary angiography was introduced in PE-diagnostics in 1998, and the increase in the PE incidence is partly attributed to better diagnostic tools.[21] However, concomitant minimal change in mortality and lower case fatality are described, and together with improved treatment, overdiagnosis (finding clinically unimportant emboli) might explain these findings.[21] During the last decades, advances have been made not only in diagnostics of VTE, but also in prophylaxis and treatment. The increased VTE rates therefore imply a need for improved identification of persons at risk as part of improved preventive strategies.

Even if a VTE can be treated and the thrombus resolves, VTE can be regarded as a chronic disease due to a high risk of recurrence. Around 30% of patients with incident VTE experience a recurrent event within the following 10 years.[22] VTEs tend to recur as the same disease entity as the first incident VTE, and patients with PE are three times more likely to suffer recurrence as PE than those presenting with DVT.[23] Overall, the recurrence risk is

greatest during the first year after an incident VTE, and declines gradually thereafter.[22] VTE events are classified as being either provoked or unprovoked. The term "provoked" refers to the presence of transient (e.g. acute medical condition, surgery, trauma, hospitalization and plaster-cast) or persistent (e.g. active cancer and wheel-chair use) environmental risk factors.[24] Cases where no such risk factors are identified are termed "unprovoked".[24] With increasing knowledge of the etiology of VTE, provoking factors might be discovered for cases we today classify as unprovoked. In population-based studies, 50-60% of incident VTEs are provoked, with some variation according to different definitions and assessment of provoking factors.[15,16,25] Unprovoked VTE is more likely to recur than VTE provoked by a transient risk factor.[26,27] Persistent risk factors, with active cancer as the most important example, yield the highest recurrence risk.[27-29]

VTE has major implications for the individual as well as for society. Short-term complications of VTE include embolization, early recurrence and death. The one-month case-fatality rate after VTE diagnosis is reported to be between 6 and 11%[15,16,30], and the one-year case-fatality rate is between 17 and 23%.[15,27,30] In cancer patients, the one-year case-fatality rate is even higher, and has been reported as high as 63-88%.[15,31] The risk of early death is 18-fold higher after PE than DVT, and it is estimated that almost 1 out of 4 PEs presents as sudden death.[32] Further, 30-day case fatality has been found to be 2.4-fold higher after PE than DVT.[30]

Chronic thromboembolic pulmonary hypertension is a rare, but serious complication, and complicates approximately 0.5-4% of acute PE events.[33] It appears when thrombi fail to resolve completely, and undergo fibrotic transformation leading to mechanical obstruction of pulmonary arteries.[33] Post-thrombotic syndrome is the most common complication after DVT, causing chronic pain, swelling and skin changes including leg ulcerations in severe cases.[34] The prevalence of post-thrombotic syndrome varies considerably between studies due to differences in study design, definitions used and follow-up time, and is reported to be 20-50%.[34-36] Risk factors for post-thrombotic syndrome include proximal DVT, previous ipsilateral DVT, older age, obesity, varicose veins and insufficient quality of the anticoagulant treatment, whereas conflicting results exist whether sex is a risk factor.[34,36]

In addition to obvious consequences for the individual experiencing complications after VTE, there is also an economic burden to society. In a Norwegian study using data from two large population-based studies (the Tromsø Study and the Nord-Trøndelag Health Study (HUNT)), participants with VTE had 62% higher risk of disability pension in age- and sex-adjusted analyses, than those without VTE.[37]

1.2 Pathophysiology of venous thromboembolism

In 1856, the German scientist Rudolph Virchow postulated a triad of factors contributing to thrombus formation, consisting of abnormal blood flow (stasis), hypercoagulability of the blood, and injury to the vessel wall (Figure 1).[38] Still, more than 150 years later, Virchow's triad remains important and relevant for our understanding of venous thrombosis, and known risk factors for VTE can be related to one or more of the factors in the triad.[39]

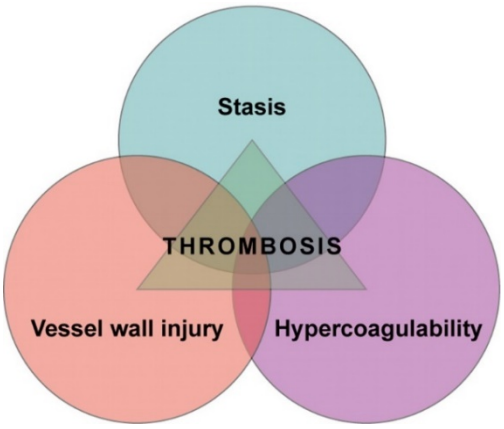

Figure 1. Virchow's triad. Three main factors contributing to thrombus formation: stasis, vessel wall injury and hypercoagulability (Adapted from Kyrle & Sabinger, Blood 2009)

Blood flows through the veins returning to the right side of the heart in a low-pressure system, and often against gravity. Skeletal muscle contractions in the legs help to squeeze blood through the veins, and venous valves prevent the blood from flowing back in the wrong direction. Autopsy and phlebography studies have demonstrated that most non-trauma-related venous thrombi originate in the sinuses behind the venous valves.[40] These sinuses are prone to thrombosis due to vortical blood flow and low oxygen tension (Figure 2).[40] These flow conditions, including a secondary slowly rotating vortex at the base of the venous valve sinus, and corresponding severe hypoxia in the deepest recesses of the sinuses, have been documented in experiments on dogs.[41,42] Localized hypoxia activates the endothelial cells lining the valve sinuses, and recruits and activates white blood cells (WBC) and platelets.[40] Even though this is not a **vessel wall injury** in a direct sense, pro-coagulant changes in the vessel wall result, via expression of adhesion molecules and release of chemo-attractants, and recruitment and binding of monocytes, platelets and extracellular vesicles (EVs) (Figure 2).[43] EVs can also be released by circulating WBC when activated, and due to their negatively charged surface and their ability to express tissue factor (TF), they are highly pro-coagulant.[44] In addition to the role of the activated endothelium, direct vessel wall damage might be of importance in some cases, e.g. following surgery or trauma, in malignancy and in thrombosis related to central venous catheter.[45,46]

Even though a pro-coagulant environment is demonstrated in venous valve sinuses, this is balanced by mechanisms preventing clot formation in most cases, and more factors are necessary to induce thrombus formation. However, the number of venous valves vary between individuals, and their role as a site for thrombus initiation is indirectly supported as those with

more valves have a higher frequency of DVT.[47] In addition to this localized **stasis** in the venous valve sinuses, more widespread stases of blood in the veins contribute to the pathophysiology of thrombus formation. Immobilization, long-haul travel, surgery, hospitalization and pregnancy are well-known risk factors for VTE sharing the feature of reduced blood flow and stasis. The observed left-sided predominance of DVT in pregnant women may be explained by compression of the left common iliac vein from the growing fetus, resulting in left-sided stasis.[48,49]

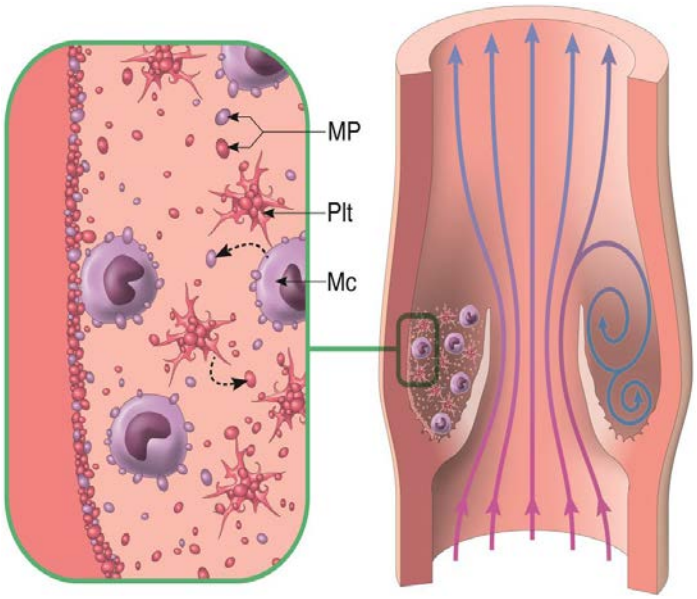

Figure 2. Venous valve sinuses as site of initial thrombus formation. A hypoxic environment exists in the venous valve sinuses, due to vortical blood flow. This activates endothelial cells, and white blood cells such as monocytes (Mc) and platelets (Plt) are recruited. When these cells are activated, tissue factor-containing extracellular vesicles, also named microparticles (MP), bud off and contribute to initiation of coagulation and thrombus formation.

Blood coagulation is essential for hemostasis and wound repair, and consists of proteins that are activated in a complex cascade reaction. The risk of bleeding has to be balanced against the risk of non-physiological clotting, to avoid arterial or venous thrombotic disease. Physiological hemostasis involves (i) platelet plug formation, (ii) activation of the coagulation cascade, which eventually leads to (iii) fibrin formation to stabilize the platelet plug, (iv) antithrombotic control mechanisms to stop clotting and (v) finally removal of the clot by fibrinolysis.

The coagulation cascade consists of two main pathways; the extrinsic and the intrinsic pathway that merge into the common pathway. Main components of these pathways are shown in Figure 3. The main trigger of the coagulation cascade is expression of TF in response to endothelial damage, from EVs or from activated monocytes.[50] The fact that TF-deficiency never has been identified in humans[50], underscores the critical role of TF in coagulation. Thrombin also plays a key role in coagulation. Thrombin causes the conversion of fibrinogen to fibrin, essential for stabilizing the platelet plug.[51] Further, thrombin activates platelets, other

coagulation factors (FVIII and FV) and induces fibrin cross-linking through activation of FXIII.[51] The intrinsic pathway serves as an amplifier of the extrinsic pathway in normal hemostasis, and seems to play an important role in formation of pathological intravascular thrombi.[52] Interestingly, the intrinsic pathway FXI is a promising target for treatment and prevention of thromboembolic disease.[53,54] Inhibition of FXI was shown to produce potent antithrombotic activity without bleeding in mouse models.[53] Further, reducing FXI levels as thromboprophylaxis was effective and safe with respect to bleeding risk in a randomized trial on patients undergoing knee arthroplasty.[54] Von Willebrand Factor (vWF) plays a role in hemostasis by facilitating binding of platelets to the vessel wall when the platelet plug is formed.[55] In the circulation, vWF binds to FVIII, thereby preventing FVIII from degradation.[55]

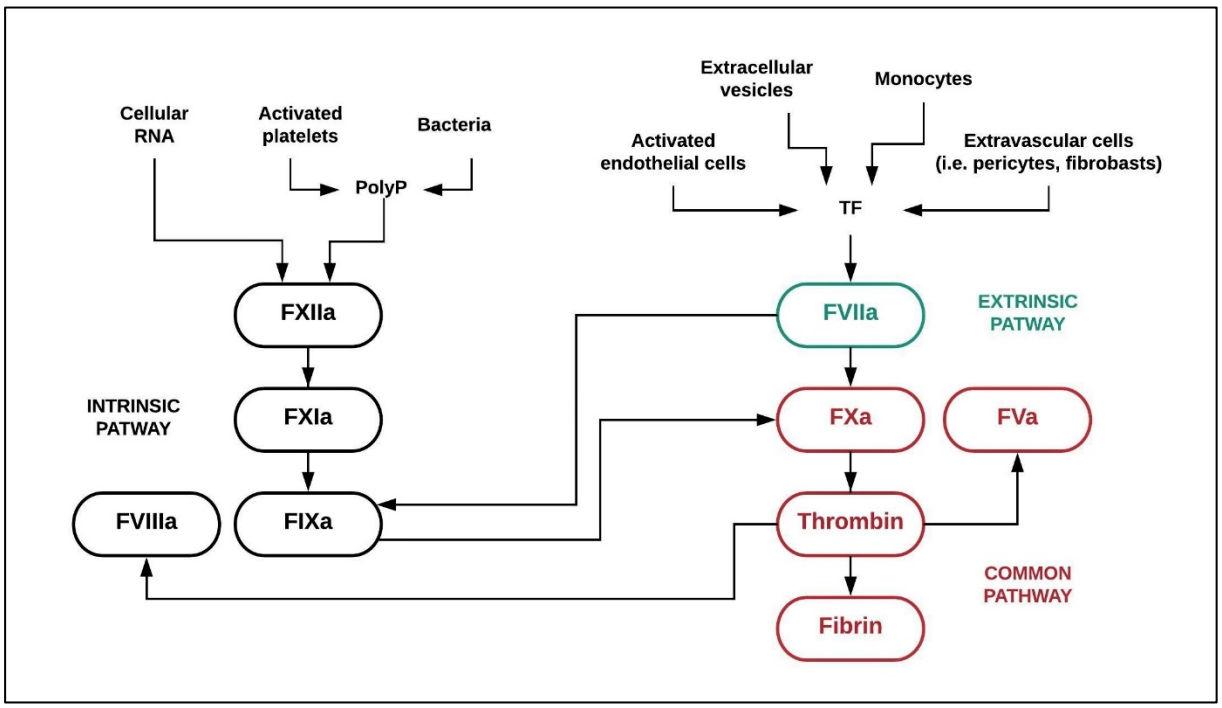

Figure 3. Overview of the coagulation cascade.
Tissue factor (TF) released from either monocytes, extracellular vesicles, activated endothelium or extravascular cells serve as a co-factor for factor (F) VII in the extrinsic pathway, while FXII in the intrinsic pathway is activated by cellular RNA or via polyphosphates (PolyP) from activated platelets or bacteria.
Activated (a) FVII interact with the intrinsic pathway, and both FVIIa and FIXa from the intrinsic pathway activate FX in the common pathway, leading to thrombin and finally fibrin formation. Thrombin also activates the co-factors FVIII and FV.
(Adapted from Mackman N, J Clin Invest 2012)

Coagulation inhibitors such as tissue factor pathway inhibitor (TFPI), antithrombin, activated protein C (APC) and protein S are important in controlling and balancing coagulation.[56] Finally, the fibrinolytic system is essential for re-establishing homeostasis after wound repair and

coagulation activation. Plasmin, formed from its inactive precursor plasminogen, cleave the cross-linked fibrin into fibrin degradation products.[57] This process is inhibited by plasminogen activator inhibitor-1 (PAI-1), adding to the complexity involved in hemostasis.[57]

Moving back to Virchow's triad, thrombophilia denotes any inherited or acquired disorders of blood coagulation or fibrinolysis, resulting in increased risk of thrombosis due to **hypercoagulability**.[56] Among the inherited thrombophilia are antithrombin-, protein C- and protein S-deficiencies, resulting in attenuated anticoagulant function, and the Factor V Leiden mutation leading to gain of procoagulant function as it leaves FV resistant to APC.[56] Having a non-O blood group according to the ABO-system implies a kind of hypercoagulable state, mainly due to higher levels of vWF and FVIII in these individuals.[58-60] Interestingly, high FVIII-levels also contribute to the hypercoagulable state in acquired thrombophilia, exemplified by pregnancy and cancer.[61,62]

1.3 Risk factors for venous thromboembolism

VTE is a multicausal disease associated with inherited and acquired risk factors.[63] A risk factor can be defined as any characteristic, attribute or exposure of an individual that increase the likelihood of developing a disease; in this case VTE. Often, more than one risk factor for VTE needs to be present for an event to occur. This has been explained by the thrombosis potential model, which shows how combinations of hereditary factors, advancing age and provoking factors may yield a thrombosis potential exceeding an individual's thrombosis threshold (Figure 4).[63]

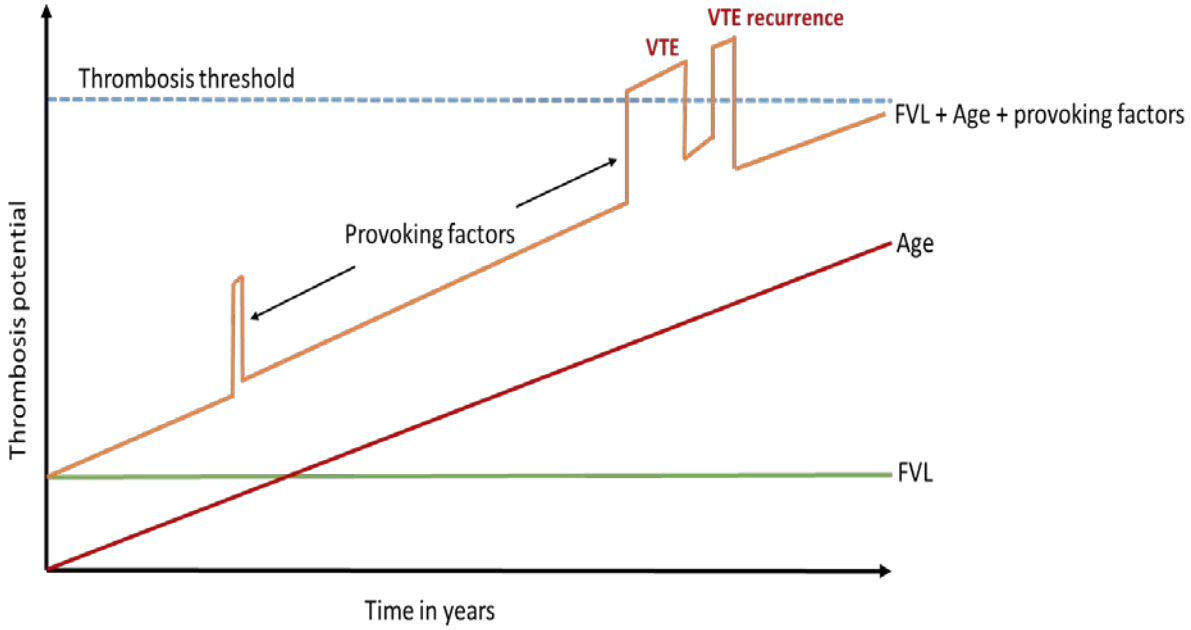

Figure 4. The thrombosis potential model. Factor V Leiden (FVL), green line, exemplifies hereditary factors. Age, red line, represents a risk factor that increases with time. The orange line demonstrates joint effects of FVL, age, and provoking factors, and shows how a provoking factor early in life may not be enough to reach the thrombosis threshold, whereas a provoking factor later in life may be enough to exceed the threshold and result in a VTE, and even in a recurrent event if a new provoking factor occurs.
(Adapted from Rosendaal F, Lancet 1999)

1.3.1 Hereditary risk factors

Family and twin studies clearly demonstrate that hereditary factors are important for the risk of VTE, and it is estimated that the heritability of the disease is around 50%.[64-66] Multiple genetic factors contribute to the VTE risk.

Non-O blood group is a highly prevalent genetic risk factor for VTE, and was present in 70% of VTE patients and 54% of healthy controls in a large meta-analysis.[67] Individuals with non-O (A_1 and B) blood group carry a 1.5 to 2-fold increased risk of VTE compared to those with blood group O.[59,60,67,68] VTE risk associated with non-O blood group is partly mediated through elevated levels of vWF and FVIII.[58,60] However, other mechanisms may also be involved, as non-O blood group remained significantly associated with VTE risk in studies where levels of vWF and FVIII were taken into account.[69,70]

FV Leiden (FVL), a mutation in the factor V gene leading to reduced ability of FV to be inactivated by APC[71,72], is present in approximately 5% of the Caucasian population[59,72], and is associated with a 3-fold increased VTE risk.[66] FVL is associated with a higher risk of DVT compared to PE, and this observation is often referred to as “the FV Leiden paradox”.[73,74] FVL

is shown to have a synergistic effect with other factors, such as oral contraceptives[74,75], pregnancy[76], obesity[77,78], cancer[79] and smoking[77] on the risk of VTE.

Prothrombin 20210A is a mutation leading to increased plasma prothrombin levels[80], and is prevalent in approximately 2% of the Caucasian population.[59,81] The prothrombin 20210A is associated with a 3-fold increase in VTE risk.[59,74,80] In some studies, the effect of this mutation has been reported to be stronger when combined with oral contraceptive use[74,75], pregnancy[76], heavy smoking[77] and obesity.[77]

Protein C and S deficiencies are caused by gene mutations present in less than 1% of the general population.[82] APC inactivates FV and FVIII and protein S serves as a co-factor for APC.[59] Several gene mutations can result in protein C and S deficiencies, and heterozygous carriers have an approximately 10-fold increased VTE risk.[82] Deficiency of the coagulation inhibitor antithrombin is rare in the general population, with prevalence varying from 0.02% to 0.2%.[83-85] Several gene mutations can result in antithrombin deficiency, which is associated with an at least 10-fold increased VTE risk.[85]

Genome-wide association studies became available in the 2000s, and have discovered additional gene variants associated with risk of VTE, although these variants display weaker associations. Up to 2015, 17 genes harboring genetic variations associated with risk of VTE had been identified.[66] In the future, novel genetic factors are expected to be identified. Potentially, these might include weak genetic factors as well as rare genetic factors associated with a high risk of VTE.

1.3.2 Acquired risk factors

Acquired risk factors for VTE include advancing age, immobilization, surgery, cancer, trauma, obesity and in women; pregnancy, puerperium and use of oral contraceptives or hormone replacement therapy.[86,87] Some acquired risk factors are classified as being provoking factors, either transient (e.g. surgery, pregnancy) or persistent (e.g. active cancer, inflammatory bowel disease), while male sex and older age are risk factors for VTE that are not considered as provoking factors.[24]

Age is an important risk factor for VTE. The incidence of VTE in the general population is 1 to 2 per 1000 per year. However, after the age of 50, the VTE incidence increases exponentially, and reaches almost 1 per 100 per year in those aged >85 years.[17] The reasons for this are not fully understood. Possible explanations include age-related increase in pro-coagulant proteins such as fibrinogen, FVIII and FIX, as well as elevated interleukin (IL)-6 and C-reactive protein (CRP) levels indicating an inflammatory state.[88] Degenerative changes of

the venous wall and valves may also contribute.[40] More comorbidities in the elderly could in theory explain some of the VTE risk. However, cancer could not explain the increased risk of VTE by advancing age in the Tromsø Study.[89] The elderly are generally less physical active[90], and immobility may contribute to increased VTE risk by age.

Obesity, defined as body mass index (BMI) >30 kg/m^2, is associated with a 2-3 fold increased risk of VTE.[91] Other anthropometric measures than BMI have also been investigated, and in the Tromsø Study, waist circumference yielded the highest risk estimates for VTE, and identified most people at risk.[92] Not only obesity itself, but also weight gain has been identified as a VTE risk factor.[93] The high and in many populations still increasing BMI constitutes a major challenge[94,95], as obesity adds to other risk factors for VTE that might not be preventable. Possible mechanisms for VTE associated with obesity include venous stasis and inflammatory properties of adipose tissue.[96] Mendelian randomization studies provide evidence for a causal relationship between high BMI and VTE risk.[97]

Height is another anthropometric measure associated with VTE risk in men[98,99] and the risk of VTE in men increased by 34% per 10 cm increase in height in the Tromsø Study.[98] Recent findings from a Mendelian randomization study confirm this estimate[100], and possible mechanisms might include a higher number of venous valves and a greater venous surface in taller people, as well as endothelial dysfunction due to a greater hydrostatic pressure and venous stasis.[100,101]

Immobilization accompanies many medical conditions, and has been associated with a 2-fold increase in the VTE risk in patients with plaster casts, neurologic paralysis, and confinement to bed for at least two to three days.[102] Also in otherwise healthy people, immobilization is associated with increased VTE risk, and long duration travel is a weak risk factor for VTE.[103] Venous stasis is the presumed mechanism for the increased VTE risk in immobilization. Hospitalization is also recognized as an important risk factor for VTE, and compared with residents in the community, hospitalized patients have >100-fold increased incidence of VTE.[104] Immobilization accounts for some of the VTE risk associated with hospitalization, as does the underlying cause of hospitalization.

Cancer is a major risk factor for VTE, associated with a 5-7 fold increased risk, and overall, cancer is responsible for 20-25% of all incident VTE cases.[15,25,105,106] The risk of cancer-associated VTE is highest the first months after cancer diagnosis, decreases gradually thereafter, but remains elevated for years.[107] The VTE risk differs across cancer sites. Hematological malignancies and cancers of the lung, gastrointestinal tract and brain are high risk sites.[107,108] Metastatic cancers yield a higher VTE risk than localized cancers.[107] The high

risk of VTE in cancer may be explained by tumor-derived pro-coagulant factors, such as TF positive EVs, inflammation and activation of neutrophils.[109] Neutrophil extracellular traps (NETs) released from these neutrophils can induce thrombosis.[110] Additionally, cancer patients are often hospitalized, undergo surgery, use central venous catheters, are prone to get infections and to be immobilized.

Major surgery is a strong risk factor for VTE, and is associated with a 4-22 fold increased VTE risk.[111,112] Major orthopedic surgery, neurosurgery, and major cancer surgery are among the high risk procedures.[113] In a study where hospitalized **trauma** patients who did not receive thrombosis prophylaxis were screened systematically for VTE, more than 50% were diagnosed with VTE.[114] One study showed that even with thromboprophylaxis, 1/3 of patients developed DVT after major trauma.[115] In this study, obesity was identified as an independent predictor of VTE.

Acute medical conditions, including congestive heart failure, respiratory disease, myocardial infarction, ischemic stroke, infections and rheumatologic disorders are also recognized as independent risk factors for VTE.[116]

1.4 Inflammation

1.4.1 Acute and chronic inflammation

The word inflammation originates from the Latin word *inflammare*- meaning "to set on fire". Inflammation occurs in response to infection and tissue injury, and might be viewed as a beneficial process combating microbe invasion and aiding tissue repair.[4] Historically, the Roman encyclopaedist Celcus, who lived in the first century AD, is credited for describing four cardinal signs of inflammation that are still valuable; *calor*- heat, *rubor*- redness, *tumor*- swelling and *dolor*- pain.[117] A fifth cardinal sign; *function laesa*- loss of function, was added by Virchow in 1871.[117] Acute inflammation featuring these five cardinal signs is easily recognizable, but inflammation can also present in more subtle ways. Inflammation is often described to be either acute or chronic, but inflammatory responses are complex, and the transition from acute to chronic inflammation is not well defined.[4]

Acute inflammation initiates within minutes or hours, gives prominent local and/or systemic signs, and is characterized by fluid and plasma exudation and a predominance of neutrophil cells.[118] When inflammation is triggered by microbial infection, it has accomplished its mission when the infectious agent is eliminated, and a switch from pro-inflammatory to anti-inflammatory mediators facilitates resolution and repair.[4] If the pathogen is not eliminated, or

other chronic inflammatory triggers, such as foreign bodies or autoimmune responses are present, features of chronic inflammation dominate.[4] Chronic inflammation has a slower onset, merit less prominent signs, but can be more severe and progressive if maintained over time. The neutrophil predominance observed in acute inflammation is replaced by monocytes/macrophages and lymphocytes.[118] Chronic inflammation does not necessarily initiate as acute inflammation, but might be induced and maintained by tissue malfunction.[4]

Cell-derived mediators, such as histamine, prostaglandins, chemokines and cytokines, are produced by WBC and activated endothelium and contribute to the inflammatory response.[118] Activated neutrophils can release NETs consisting of decondensed nuclear contents and proteins such as histones and serine proteases.[110] Microbes trapped in NETs can be digested by the remaining neutrophil cell. Additionally, the protein components of NETs contribute to coagulation and platelet activation, and risk factors for VTE such as trauma, surgery, infection and cancer are associated with NET formation.[110] Adding to theses complex responses, circulating proteins of the complement, kinin, and coagulation systems are involved in the inflammatory response, and they interact with each other. Activation of FXII is a trigger for these interrelated cascade reactions.[118]

1.4.2 Biomarkers of inflammation

A biomarker has been defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[119]

Systemic effects of inflammation are mainly due to effects from the cytokines tumor necrosis factor (TNF), IL-1 and IL-6. The response, called the acute-phase response, consists of symptoms and signs like fever, increased heart rate and malaise, leukocytosis and elevated plasma levels of acute-phase proteins.[118] These proteins are synthesized in the liver, in response to cytokines, predominately IL-6.[120] The best known acute-phase protein widely used in clinical settings is CRP. Hepatic synthesis of CRP starts rapidly after a stimulus, and serum concentrations rise above the common clinical cut-off of 5 mg/L after 5 hours, and peak 48 hours from the inflammatory stimulus.[120] IL-1β and TNF, secreted by macrophages at sites of inflammation, stimulate the expression of adhesion molecules on endothelial cells, and can enter the circulation and contribute to systemic inflammatory reactions.[118]

Different kinds of WBC may dominate in leukocytosis, even though neutrophil cells predominate in most acute inflammatory responses. In case of viral infections, lymphocytosis is observed, while eosinophil cells dominate in severe allergic responses.[118] So, total WBC

count, and differential WBC count are commonly used as markers of inflammation. Additionally, neutrophil to lymphocyte ratio (NLR), has been increasingly used in research as a biomarker for inflammation in several conditions, e.g. cancer, cardiovascular disease and infection.[121-123] Further, NLR was associated with increased risk of stroke in a dose-response pattern in a large cohort of patients with atrial fibrillation.[124] When NLR was added to the CHA_2DS_2-VASc score, a score used to predict risk of stroke in subjects with atrial fibrillation, the predictive ability was improved.[124] This finding implies that NLR might be a better inflammatory marker for prediction of thromboembolic events than CRP, as CRP has not been shown to predict stroke in atrial fibrillation.[125] The potential role of NLR as a predictor for VTE has not been investigated.

1.4.3 Inflammation, coagulation and venous thromboembolism

Extensive crosstalk exists between the cascade systems involved in inflammation and coagulation. The complement system, which is part of innate immunity, the coagulation cascade and the fibrinolytic cascade communicate through direct and bidirectional interactions.[126] The classical complement pathway can be activated by coagulation FXII.[127] Mouse models and in-vitro studies have shown that thrombin can activate the complement system through conversion of complement C5 to its active form C5a.[128] Recently, this finding has been challenged by a study using a baboon model, where thrombin and plasmin did not activate the complement system.[129] C5a in turn, amplifies coagulation by activating platelets and inducing TF and PAI-1 expression by WBC.[126] Adding to the complexity, negative feedback loops also exist. Interaction between other inflammatory pathways and coagulation includes production of TNF, IL-1β and IL-6 stimulated by thrombin, and stimulation of coagulation by various cytokines.[126] Disseminated intravascular coagulation is a serious complication to severe infections, and represents an extreme of consequences of unbalanced coagulation and fibrinolysis during inflammation. Not only is coagulation activated, its regulatory counterpart fibrinolysis is inhibited.[126] This takes place through increased levels of PAI-1 and thrombin-activatable fibrinolysis inhibitor, and through consumption of regulators such as antithrombin, protein C and TFPI.[126] Further, NETs play an important role in inflammation and thrombosis.[110]

As most inflammatory responses, the link between inflammation and coagulation has been most thoroughly studied in the context of infection. However, the link between inflammatory markers and thrombosis has also been studied in the general population using prospective studies and case-control designs. Low-grade, long-term inflammation assessed by high sensitivity (hs)-CRP[130,131] and NLR[122,132] has been consistently associated with risk of

arterial thrombosis. In contrast, most prospective studies with long follow-up time have not found an association between hs-CRP at baseline and future risk of VTE.[131,133-135] Using repeated measures from the Tromsø Study, hs-CRP was associated with a 1.8-fold increased risk of VTE in women, but not in men, after a median of 3.1 years of follow-up.[96] Other studies with a shorter follow-up time have also found an association between baseline hs-CRP and VTE risk, driven by the highest quintile of CRP in the ARIC-study[136] and only for the first year after baseline in the HUNT-study.[137] These findings might be due to underlying diseases, and the lack of a long-term association between hs-CRP and VTE risk is strengthened by the fact that genetic polymorphisms that increase CRP levels have not been associated with increased risk of VTE.[138] WBC count is not associated with increased VTE risk in the general population in long-term prospective studies[133,139], whereas pre-cancer WBC count was associated with risk of VTE in cancer patients in the Tromsø Study.[140]

While infection and injury are classic triggers of inflammatory responses, other established risk factors for VTE such as cancer, surgery and autoimmune diseases also share the feature of inflammation.[4,141-143] Active cancer and surgery are conditions associated with acute inflammation, while autoimmune diseases often involve chronic inflammation. Interestingly, VTE risk in patients with inflammatory bowel disease is especially high during disease flares (overall 3-fold increased risk compared to controls, 9-fold increased risk during flares), where acute inflammation dominates.[144] An increasing VTE risk with increasing disease-activity was also demonstrated in a population-based cohort of patients with rheumatoid arthritis and psoriasis.[145] In a case-control study of inflammatory symptoms and signs the last four weeks prior to a DVT, airway signs, gastrointestinal signs, fever and malaise were more common in cases than controls, and this result remained significant also after exclusion of cancer patients.[146]

VTE can elicit acute-phase responses and inflammation. Symptoms and signs of DVT illustrate the cardinal signs of inflammation; the affected leg is red, warm, swollen and painful, and has impaired function. In a case-control study investigating inflammatory markers, DVT-patients had higher median plasma concentrations of IL-6, IL-8 and CRP than controls at admission.[147] Lower CRP levels are found in patients with distal compared to proximal DVT.[147,148]

To summarize; low-grade inflammation assessed by hs-CRP does not predict VTE risk in studies with long-term follow-up, but inflammation seems to be of importance within a shorter time-perspective. The potential association between NLR and venous thrombosis has

not yet been investigated. Acute inflammation is a common feature of several VTE risk factors, but the impact of acute inflammation per se on VTE risk has yet to be fully established.

1.5 Acute infection

1.5.1 Definition, classification and epidemiology

As far back as 100 BC the Roman scholar and writer Marcus Terentius Varro, was quoted as noting that "small creatures, invisible to the eye, fill the atmosphere, and breathed through the nose cause dangerous diseases".[149] These dangerous diseases, i.e. infections, can be broadly classified according to the microbial agent as bacterial, viral, parasitic, or yeast infections. Based on properties of the cell membrane, bacteria can be divided into gram-positive or gram-negative, and they can be further classified to species level, e.g. gram-positive *staphylococci* and gram-negative *E. coli*. In clinical practice, the specific microbe causing an infection might remain unknown; and infections are often classified according to their clinical appearance and foci. The diseases caused by "small creatures" described by Varro, might today be classified as a viral upper airway infection or a lower respiratory tract infection (pneumonia), often caused by bacteria such as *Streptococcus pneumoniae*.

Infections can affect all parts of the body, be localized or systemic, and infection severity ranges from mild, self-limiting disease to sepsis and septic shock, based on both microbe and host properties. According to the latest definition, sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection.[150] In a recent national study from Norway, overall incidence of hospitalized sepsis was 140 per 100 000 individuals per year.[151] The sepsis incidence increases with age, and men have a higher incidence than women in all age groups.[151] Sepsis is a serious disease, with mortality ranging from 20 to 80%.[149] Immunocompromised individuals are at an increased risk of sepsis.[152] Among less severe infections, upper respiratory tract infection and cystitis are common[153], whereas for sepsis, pneumonia is the most common cause, followed by intra-abdominal and urinary tract infections.[154]

1.5.2 Acute infection and risk of venous thromboembolism

Acute infection is one of the acute medical conditions regarded as a risk factor for VTE. In a prospective Danish cohort, community-acquired bacteremia was associated with a 1.9-fold increased VTE risk the following 90 days, when compared to hospitalized controls.[155] In a case-control study based on medical databases in Northern Denmark, hospital-diagnosed systemic

respiratory tract infections (RTIs), urinary tract infections (UTIs), skin infections, intra-abdominal infections and septicemia were associated with a 3.3-fold increased VTE risk, while community antibiotic treatment was associated with a 2.6-fold increased risk.[156] In a registry-based case-control study, the risk of DVT was 2.6-fold and the risk of PE was 2.5-fold increased in the three month period following an RTI.[157] In a self-controlled case-series study from a community setting, the risk of DVT and PE were both 2.1-fold increased the first two weeks after UTI, and the risk of DVT after RTI was 1.9-fold increased.[158] Self-reported pneumonia was associated with a 5-fold increased VTE risk in a population-based case-control study (the MEGA-study), and the risk was attenuated to 3.8-fold increased after adjustment for immobilization, classical VTE risk factors and unhealthy life style.[159] In a case-crossover study, infection was the most common trigger in the three month period before hospitalization for VTE, and was associated with a 2.9-fold increased VTE risk.[160] Moreover, a reduced risk of VTE after influenza vaccination has been described.[161] However, due to the diversity of infections, differences in study designs, and the fact that possible confounding factors, such as immobilization, often co-exist, more knowledge is needed regarding the impact of acute infection on VTE risk.

1.6 Gut microbiome

1.6.1 Brief overview of the gut microbiome

The term microbiome refers to the collective genomes of our indigenous microbes or microbiota.[162] Microbiome and microbiota are, however, often used as interchangeable terms. Research interest and knowledge about the gut microbiome have increased tremendously the last decade, aided by new techniques such as 16S ribosomal (r) RNA gene sequencing and anaerobic culturing techniques.[163] More than 1000 gut bacterial species have been characterized[164], and each individual harbours at least 160 different bacterial species.[165]

Two bacterial divisions, or phyla, dominate the distal gut microbiome, namely the *Bacteroidetes*, consisiting of gram-negative bacteria, and the *Firmicutes*, consisiting of mainly gram-positive bacteria.[166] The gut microbiome changes throughout life, from a low total level of bacteria in early childhood, to higher levels and different composition of bacteria in adults.[167] The *Firmicutes/Bacteroidetes* ratio increases from birth to adulthood, while in the elderly the ratio is more similar to infants.[167] Other factors than age also affect the gut microbiome. These include diet, travel, enteric infection and use of antimicrobial agents.[168-170] So, even in healthy

individuals where most components of the gut microbiome are shared, diversity exists within individuals over time as well as between individuals.[163]

A healthy microbiome can therefore not be characterized as an ideal set of specific microbes. Rather, a core microbiome that provides all necessary functions and that is resistant to stress induced by external or internal changes, is hypothesized to characterize a healthy microbiome.[163] Why is this a topic? A possible answer is that changes to the microbiome, thought to represent absence of a healthy microbiome, are associated with disease. In inflammatory bowel disease, the gut microbiome has reduced diversity, and interactions between bacterial and host cells seem to be involved in pathogenesis.[171] Systemic antibiotic treatment affects the gut microbiome, which can result in pseudomembranous colitis caused by the bacteria *Clostridium difficile*.[171] Less obvious are the observed associations between the gut microbiome and diseases like diabetes mellitus, multiple sclerosis, obesity and atherosclerosis.[171] Although causality cannot (yet) be claimed for many of these associations, the gut microbiome deserves our attention.

1.6.2 Gut microbiome and venous thromboembolism

Is there any association between the gut microbiome and VTE risk, and if yes, how can it be explained? Changes in the gut microbiome have been found in several known risk factors for VTE. Advancing age affects the gut microbiome, and age is an important risk factor for VTE.[17] Obesity, another VTE risk factor[91], is also associated with changes in the gut microbiome. The gut microbiome of obese individuals is generally less diverse than in lean individuals, and they have more bacteria belonging to the *Firmicutes* phylum compared to the *Bacteroidetes*.[172] This ratio decreases after diet-induced weight-loss.[172] Inflammatory bowel disease is associated with both an increased risk of VTE[144] and a less diverse microbiome than in healthy individuals.[171] Infections yield a higher risk of VTE[156], and are also associated with changes in the gut microbiome, either caused by the infection itself or as a consequence of antibiotic treatment.[170] Certain cancers, mostly gastrointestinal, have also been linked to the gut microbiome.[173]

A possible link between the gut microbiome and risk of VTE is inflammation. Lipopolysaccharide (LPS) is a component of the gram-negative cell membrane, which acts as an endotoxin and causes inflammation if it enters the circulation.[172] An impaired gut barrier function- a “leaky gut”- has been observed in several conditions associated with VTE risk, such as trauma, inflammatory bowel disease and obesity.[174]

So, in several conditions associated with increased VTE risk, the gut microbiome is different than in healthy individuals. However, this is an observed association, and conclusions

regarding causality cannot be made. A hypothetical explanation for the observed association might be that thrombosis is caused by inflammation induced by translocation of LPS or other bacterial components from the gut to the circulation, but this has not yet been studied.

2 AIMS OF THE THESIS

The aims of this thesis were:

- To investigate the association between neutrophil to lymphocyte ratio and future risk of incident and recurrent venous thromboembolism, and the association between neutrophil to lymphocyte ratio and all-cause mortality after VTE in a cohort recruited from a general population (Paper I)

- To investigate the impact of acute infection, with and without concomitant immobilization, on the risk of venous thromboembolism using a case-crossover design (Paper II)

- To investigate the impact of acute inflammation, assessed by C-reactive protein, on the risk of venous thromboembolism using a case-crossover design (Paper III)

- To investigate the impact of transforming the gut microbiome in a gram-negative direction on markers of systemic inflammation and plasma FVIII:C-levels in a randomized, controlled trial (Paper IV)

3 STUDY POPULATIONS AND METHODS

3.1 Study populations and designs

The Tromsø Study is a single-center, **population-based cohort study** with repeated health surveys of the inhabitants of the municipality of Tromsø, Norway. From the first survey conducted in 1974 to the seventh survey in 2015-16, the main focus has evolved from cardiovascular disease to a broad spectrum of chronic diseases.[175] The fourth survey (Tromsø 4) was conducted in 1994-95, and is the largest survey of the Tromsø Study so far. All inhabitants aged 25 years or older living in Tromsø were invited, and 27 158 (77%) participated.[175]

In Paper I, we followed the Tromsø 4 cohort prospectively from the date of enrollment in 1994-95 until December 31, 2012. Subjects were followed until the date of a VTE, migration, death or end of follow-up, whichever came first. We investigated whether NLR, calculated from baseline measurements of neutrophils and lymphocytes, was associated with a future risk of first or recurrent VTE.

The incident VTE cases (n=707) registered from the Tromsø 4 participants until December 31, 2012, were included in the **case-crossover designed study** used in Papers II and III, where we investigated infection and inflammation as triggers for VTE. The case-crossover study was designed with four 90 day control periods and a 90 day washout period preceding the 90 day hazard period (Figure 5).

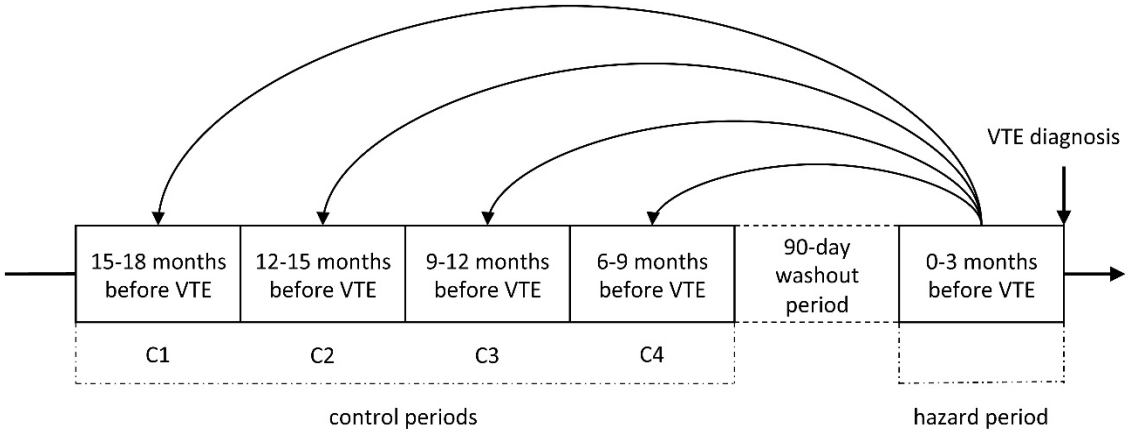

Figure 5. Case-crossover study design. Risk factors, diagnostic procedures, surgical and medical treatment, laboratory tests and diagnoses during hospital contacts were registered in four 90 day control periods and in the 90 day hazard period. A 90 day wash-out period was included between the control and hazard periods, to avoid carry-over effects.

To investigate the impact of a change in the gut microbiome in a gram-negative direction on systemic inflammation and coagulation, we performed a **randomized controlled trial** (RCT) presented in Paper IV. The study drug was Vancomycin capsules, 125 mg, in a dosage of 4 capsules three times a day for seven days. Half of the participants were randomly assigned to the intervention, and the other half served as controls. As overweight/obesity is associated with both inflammation and a different gut microbiome composition, half of the subjects in each group were normal weight (BMI <25 kg/m^2) and half were overweight or obese (BMI ≥25 kg/m^2). We invited healthy volunteers aged 18 to 40 years to participate in the trial through poster advertisement, and 43 participants, 21 in the intervention group and 22 in the control group, completed the study. Gut microbiome composition, coagulation FVIII:C, inflammatory markers and complement activation products were measured three times during the study; before intervention (T0), the day after end of intervention (T1), and three weeks later (T2), and at similar time points in the controls. Figure 6 provides an overview of the study visits.

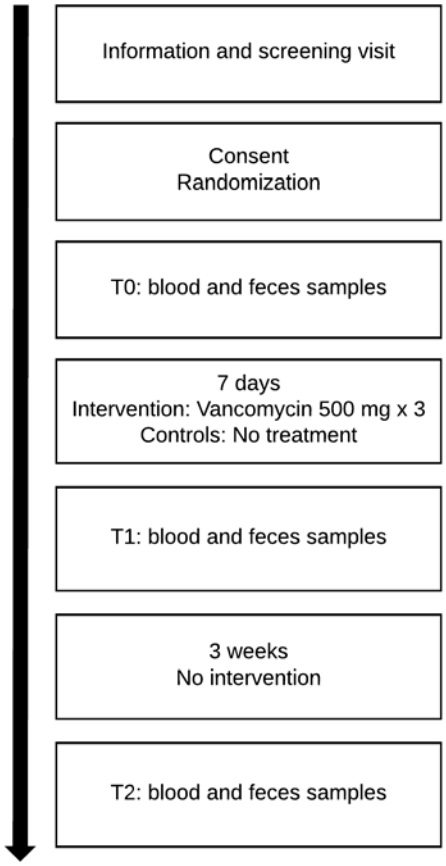

Figure 6. Overview of study visits. Blood and feces were sampled at baseline (T0), after intervention (T1) and three weeks later (T3) in the intervention group, and at similar time points in the control group.

3.2 Exposure assessment

In Tromsø 4, baseline information was collected by self-administered questionnaires, physical examinations and blood samples. Information obtained from self-administered questionnaires provided information regarding smoking habits, diabetes and use of oral contraceptives or hormone replacement therapy. Weight and height were measured in subjects wearing light clothing and no shoes. BMI was calculated by the weight in kilograms (kg) divided by height in meters (m) squared (kg/m^2). Non-fasting blood samples were collected from an antecubital

vein and analyzed in the Department of Clinical Chemistry at the University Hospital of North Norway (UNN). For measurement of WBC-, neutrophil- and lymphocyte counts, 5 ml of blood was collected into Vacutainer tubes containing EDTA as anticoagulant, and analyzed within 12 hours by an automated blood cell counter (Coulter Counter®, Coulter Electronics, Luton, UK). Neutrophil to lymphocyte ratio (NLR) was calculated by dividing neutrophil count on lymphocyte count.

For assessment of exposures in the case-crossover study, trained medical personnel searched the hospital medical records of each recorded incident VTE event for relevant risk factors, diagnostic procedures, surgical and medical treatment, laboratory tests and diagnoses during hospital admissions, day care and outpatient clinic visits in any of the control or hazard periods. A transient risk factor, or trigger, was defined by its presence during the last 90 days before each admission. If an exposure occurred over several days, it was considered to have occurred if any of the days of the exposure fell within the specified 90 day time period.

In Paper II, infection was the main exposure of interest as a VTE trigger. A bidirectional relationship exists between infection and immobilization. Immobilization is a risk factor for infection[176,177], and during an acute infection temporary immobilization is common, as patients suffering from an infection are often confined to bed.[178] Both immobilization and infection are associated with an increased VTE risk, and the impact of these factors and their interplay, possibly acting as either a confounder or an intermediate in the other factor's causal pathways, were of special interest. Infection was recorded if noted by a physician in the patient's medical record. RTI and PE may have similar symptoms, and initial misdiagnosis of PE as RTI is possible. To address this, the hospital medical records for all cases with RTI and PE recorded in the hazard period were thoroughly searched again, and the RTI diagnoses were classified as "most likely correct" (n=28), "possible" (n=37), or "most likely incorrect" (n=8) based on clinical signs and symptoms, description of radiological examinations, treatment response and information about time course. The "most likely incorrect" RTI diagnoses were recoded as "no RTI". Immobilization was defined as the presence of one of the following: bedrest for three days or more, ECOG (Eastern Cooperative Oncology Group) score of four, or other immobilizing factors specified in the patient's medical record.

In Paper III, information regarding CRP measurements was obtained from review of the hospital medical records of VTE cases. CRP were measured at request by a clinician and analyzed according to routines at the Department of Clinical Biochemistry at UNN. CRP was analyzed in serum with a particle-enhanced immunoturbidimetric assay on a Modular P (1992-2001), Hitachi 917 (2001-2008) or Cobas 8000 (2008-2012) autoanalyzer (Roche Hitachi,

Mannheim, Germany), with reagents from Roche Diagnostics (Mannheim, Germany). The analytical coefficient of variation (CV) for CRP was 3%. The lower cut-off level of the reported CRP value was 5 mg/L, and measurements of CRP lower than 5 mg/L were set to this value.

In the RCT in Paper IV, the exposure was an intended gram-negative shift in the gut microbiome composition, obtained by oral Vancomycin intake in the intervention group. Vancomycin was discovered already in 1957, and its actions and possible side effects have been thoroughly investigated and described.[179] Oral Vancomycin is not absorbed systemically, high levels are achieved in the colon and the drug is effective against gram-positive bacteria (i.e. *Staphylococci*, *Streptococci*, *Enterococci*, *Bacillus species* and *Clostridia*).[179] Oral Vancomycin is therefore a safe and suitable antibiotic choice to reach the aim of our intervention, to change the gut microbiome composition towards a gram-negative direction. All participants were asked about compliance at the study visit following the intervention. Further, to ensure that the expected effect of oral Vancomycin took place, the microbiome composition was investigated in fecal samples from T0, T1 and T2. Fecal samples were collected as close to the study visit as possible, with a maximum of 36 hours before the visit, and stored in a refrigerator until the visit. The fecal samples were then stored at -70°C until analysis of microbiome composition. Following DNA extraction (using a repeated bead beating protocol[180]) and purification (using Maxwell RSC Whole Blood DNA Kit), 16S rRNA gene amplicons were generated and purified, to allow assessment of taxonomy.

3.3 Outcome assessment

3.3.1 Identification and validation of venous thromboembolic events

Participants in Tromsø 4 were followed up from the date of enrollment in the Tromsø Study in 1994-95 to the date on which a VTE event was diagnosed, the date the participant officially moved from the municipality of Tromsø, died or to the end of the study period (December 31, 2012). Information regarding deaths was obtained from the Population Registry of Norway.
UNN is the only hospital in the municipality of Tromsø, and all hospital-based medical care in the region is provided by this hospital alone. The hospital discharge registry, the autopsy registry and the radiological procedure registry of this hospital were used to identify VTE events during follow-up. Relevant International Classification of Diseases, revision 9 (ICD-9) codes for the period 1994 to 1998 were 325, 415.1, 452, 453, 671.3, 671.4 and 671.9, and for the period 1999 to 2012 relevant ICD-10 codes were I26, I80, I82, I67.6, O22.3, O22.5, O87.1 and

O87.3.[25] Following this broad search in ICD-codes, trained personnel, who were blinded to the patient's baseline variables, reviewed the medical journals for each potential VTE case.

For a potential VTE case to be recorded, all of the four following criteria were required:

i. The presence of signs and symptoms accordant with either a DVT, PE or both.
ii. Objective confirmation by a diagnostic procedure (i.e. compression ultrasound, ventilation-perfusion scan, computed tomography (CT) scan, pulmonary angiography) or autopsy.
iii. A diagnosis of a DVT or PE noted by a physician in the patient's medical records.
iv. Initiation of therapy for the VTE (i.e. anticoagulant medication, thrombolysis, vascular surgery), or treatment was planned for, but not initiated due to a specified contraindication.

In cases where the autopsy registry was the source of the VTE diagnosis, a VTE was recorded only if the autopsy report indicated VTE as the cause of death, or as a significant factor associated with the cause of death.

All VTE events were classified as either a DVT or a PE. When DVT and PE occurred simultaneously, the event was recorded as a PE. The VTE events were further classified as provoked or unprovoked, according to the presence of provoking factors at time of diagnosis. An event was classified as provoked if any of the following were present: surgery or trauma within the previous 8 weeks, acute medical conditions (acute myocardial infarction, ischemic stroke or major infectious disease), active cancer, immobilization (bed rest >3 days, wheelchair use or long distance travel lasting ≥4 hours within the last 14 days prior to the event), or any other factor particularly described to be provoking.

3.3.2 FVIII:C and inflammatory markers

The primary outcome in the RCT described in Paper IV was difference in change from T0 to T1 in FVIII:C levels between the intervention and the control group, and secondary outcomes were differences in change from T0 to T1 in levels of inflammatory variables (hs-CRP, neutrophil count, IL-1β, IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), TNF, fibrinogen) and the complement activation products C3bc and terminal complement complex (TCC).

Non-fasting blood samples were collected at three study visits (Figure 6) at the Clinical Research Center, UNN. Blood was drawn from an antecubital vein. For analyses of FVIII:C, plasma was prepared from blood samples containing sodium citrate as anticoagulant by centrifugation in two steps, first at 2500 x *g* for 15 minutes, and then at 10 000 x *g* for 10 minutes. Plasma was stored in cryovials at -70°C, until transport (frozen, at -70°C) and analyzed at the Surgical Laboratory, University of Groningen in the Netherlands. FVIII:C was measured using an APTT assay (with Synthasil APTT reagents, Instrumentation Laboratory Werfen, New Delhi, India on a ACL top 300, Werfen CTS® Instrumentation Laboratory, MA, USA) in FVIII deficient plasma, with a CV of <5%. Neutrophil cell count, fibrinogen and hs-CRP were analyzed at the Department of Clinical Biochemistry at UNN within a few hours of sampling. Neutrophil cell count was analyzed in EDTA-blood on an automated blood cell counter by a fluorescence flow-cytometric method (Sysmex XN, Sysmex Nordic ApS), with a CV of <5%. Fibrinogen was analyzed in plasma prepared by centrifugation of sodium-citrated blood at 2500 x *g* for 15 minutes, by a clotting method (STA® -Liquid Fib, STA-R Evolution, Diagnostica Stago, France) with a CV of <5%. Hs-CRP was analyzed in serum prepared by centrifugation at 2000 x *g* for 15 minutes of blood sampled on a serum-separating tube, by an immunoturbidimetric assay on a Cobas 8000 autoanalyzer (Roche Hitachi, Mannheim, Germany). The CV for hs-CRP was 2.9% at CRP-level 1.65 mg/L, and for all CRP-levels, the CV was 4.45%.

The cytokines IL-1β, IL-6, IL-8, IL-10, MCP-1 and TNF, and the complement activation products (C3bc and TCC) were analyzed in EDTA-plasma. Whole blood was placed on crushed ice immediately after sampling, centrifuged at 2500 x *g* at 4°C for 15 minutes, and then stored at -70°C until analysis at the Research Laboratory at Nordland Hospital Trust, Bodø, Norway. The cytokines were analyzed using multiplex immunoassay technology (Bio-Plex® Multiplex System, Bio-Rad Laboratories, Inc. Hercules, CA). CVs were 6% for IL-1β, 7% for IL-6, 9% for IL-8, 5% for IL-10, 9% for MCP-1 and 8% for TNF. C3bc and TCC were analyzed by in-house enzyme-linked immunosorbent assays, with CVs of 7% and 5%, respectively.[181,182]

4 MAIN RESULTS

4.1 Paper I

Neutrophil to lymphocyte ratio and future risk of venous thromboembolism and mortality: the Tromsø Study

The aim of this study was to investigate the association between neutrophil to lymphocyte ratio (NLR) and the future risk of incident and recurrent VTE, and between NLR and all-cause mortality after VTE. NLR was measured in 25 107 participants in Tromsø 4, contributing to a total of 367 233 person-years of follow-up. After a median follow-up time of 17.7 years, 664 VTE events were registered and validated, whereof 58% DVTs and 42% PEs. Active cancer, immobilization, surgery and acute medical conditions were the most frequent provoking factors, and 273 (41%) of the cases were unprovoked. NLR was divided into quartiles based on the distribution of baseline NLR in the population (quartile 1: <1.30, quartile 2: 1.30-1.68, quartile 3: 1.68-2.19 and quartile 4: >2.19), and an extra cut-off point at the 95th percentile (NLR >3.46) was also used in the analyses.

The risk of VTE did not differ across quartiles of NLR after multivariable (age, sex, BMI, smoking and diabetes mellitus at baseline) adjustment (hazard ratio (HR) quartile 4 vs quartile 1: 1.07, 95% confidence interval (CI) 0.86-1.33, p for trend across quartiles: 0.36). NLR showed no significant association with either provoked or unprovoked VTE. To address possible regression dilution bias due to long follow-up time, analyses were conducted with follow-up time restricted to three years from inclusion in Tromsø 4, with similar results across quartiles. For participants with a NLR >3.46, however, the risk of VTE after three years of follow-up was 2.4-fold higher when compared to quartile 1 (multivariable adjusted HR 2.36, 95% CI 0.96-5.82).

Out of 664 incident VTE-cases, 107 had a recurrent VTE event and 313 died during 2669 and 3162 person-years of follow-up, respectively. There was no association between baseline NLR and risk of VTE recurrence. Both one-year (HR 1.41, 95% CI 0.91-2.20) and total (HR 1.41, 95% CI 1.03-1.94) mortality was higher in NLR quartile 4 compared to quartile 1.

In conclusion, a single measurement of NLR was not associated with risk of first or recurrent VTE, but a high NLR was associated with increased mortality among those who experienced a VTE.

4.2 Paper II

Acute infection as a trigger for incident venous thromboembolism: Results from a population-based case-crossover study

In this study, we aimed to investigate the impact of hospitalization with acute infection on the risk of VTE in patients with and without concomitant immobilization, and to explore the differential impact of respiratory tract infection (RTI) and urinary tract infection (UTI) on the risks of DVT and PE. A case-crossover study was performed, including 707 VTE patients recruited from the fourth survey of the Tromsø Study, and the occurrence of transient risk factors, or triggers, in 90 day hazard and control periods was compared using conditional logistic regression.

The median age at VTE was 71 years, and 53.6% were women. Acute infection was registered in 267 out of 707 hazard periods (37.8%), and in 107 out of 2828 control periods (3.8%), corresponding to a high risk of VTE after infection (odds ratio (OR) 24.2, 95% CI 17.2-34.0), that was attenuated to 15-fold increased after adjustment for immobilization (OR 14.6, 95% CI 10.1-21.2). After multivariable (immobilization, cancer, major surgery, trauma, red blood cell transfusion, central venous catheter) adjustment, the risk of VTE was still 11-fold increased (OR 10.8, 95% CI 7.2-16.0) after acute infection.

In stratified analyses, the risk of VTE was 20-fold increased after infection without concomitant immobilization, and 73-fold increased after immobilization without concomitant infection. The combination of acute infection and immobilization had an even greater impact on the estimated risk of VTE (OR 140.7, 95% CI 66.4-297.9), suggestive of a positive interaction on an additive scale. The risk of PE was apparently higher after RTIs (OR 48.3, 95% CI 19.4-120.0) than UTIs (OR 12.6, 95% CI 6.4-24.7), but the strength of this association diminished in sensitivity analyses where uncertain RTI diagnoses were recoded as no RTI (OR 13.9, 95% CI 6.0-32.1).

In conclusion, hospitalization with acute infection was a prevalent and strong trigger for VTE independent of immobilization. Infection and immobilization combined had a synergistic effect on the VTE risk.

4.3 Paper III

C-reactive protein and risk of venous thromboembolism: Results from a population-based case-crossover study

The purpose of this study was to investigate the impact of acute inflammation, assessed by CRP, on the short-term risk of VTE. We conducted a case-crossover study including 707 VTE patients recruited from the fourth survey of the Tromsø Study, and compared CRP measured during hospital contacts in the 90 day hazard period with the 90 day control periods.

CRP values measured during the two days prior to the date of VTE diagnosis were not included, as they could reflect an acute phase response to the VTE itself (reverse causation). The median CRP was 107 mg/L in the hazard period, and ranged from 7 to 16 mg/L in the four control periods. As CRP was not normally distributed, the natural logarithm (ln) of CRP was used in conditional logistic regression analyses. CRP levels were 58% (95% CI 39-77%) higher in the hazard period than in the control periods. A one-unit increase in lnCRP was associated with increased VTE risk (OR 1.79, 95% CI 1.48-2.16), with slightly attenuated risk estimates after adjustment for immobilization and infection.

In analyses stratified for infection, a one-unit increase in lnCRP was associated with increased VTE risk in cases with (OR 1.55, 95% CI 1.01-2.38) and without infection (OR 1.77, 95% CI 1.22-2.57). When the hazard period was compared to each control period (C) separately, we found that time between the control and the hazard period did not influence the association between CRP and risk of VTE. The estimated risk of VTE by lnCRP was 1.9-fold increased when comparing the hazard period with C1; 2.2-fold increased when compared with C2; 1.5-fold increased when compared with C3; and 1.8-fold increased when compared with C4.

In conclusion, acute inflammation assessed by CRP was a trigger for VTE in this case-crossover study, also in cases with inflammatory conditions other than infection.

4.4 Paper IV

A Vancomycin-induced shift of the gut microbiome in gram-negative direction increases plasma factor VIII:C levels: Results from a randomized, controlled trial

We hypothesized that a change in the gut microbiome composition in a gram-negative direction would lead to an increase in systemic inflammatory markers and coagulation factor VIII:C, possibly due to translocation of lipopolysaccharides from gram-negative bacteria across the gut barrier. We performed a randomized, controlled trial to investigate our hypothesis. We used oral Vancomycin 500 mg three times a day for seven days to achieve a gram-negative shift in the gut microbiome composition in the intervention group. Fecal and blood samples were collected before intervention with oral Vancomycin, the day after end of intervention, and three weeks later, and at corresponding time points in the control group, which received no intervention.

A total of 43 healthy volunteers aged 19-37 years completed the study, 21 in the intervention group and 22 in the control group. The gut microbiome composition became less diverse with a relatively higher abundance of gram-negative bacteria in the intervention group, while the microbiome composition remained stable in the control group. Three weeks after the end of the intervention, the microbiome composition became more diverse, but was not completely restored. Following intervention, the primary outcome FVIII:C increased from 104 IU/dL at baseline to 108 IU/dL, a statistically significant difference in change when compared to the control group (two-sided t-test: p=0.01). A statistically significant increase in hs-CRP (logarithmic transformed, to achieve normal distribution) were observed in the intervention group compared to the control group (two-sided t-test, p=0.04). In subgroup analyses, the change in FVIII:C and hs-CRP remained significant in those with BMI <25 kg/m^2, but not in those with BMI ≥25 kg/m^2. For the other pre-defined secondary outcomes (IL-1β, IL-6, IL-8, IL-10, MCP-1, TNF, fibrinogen, C3bc, TCC and neutrophils), there were no statistically significant differences in change from T0 to T1 between the intervention and the control group.

In conclusion, intervention with oral Vancomycin in healthy volunteers induced the expected shift in gut microbiome in a gram-negative direction. When compared to controls, there was a significant increase in FVIII:C and log transformed hs-CRP in the intervention group.

5 GENERAL DISCUSSION

5.1 Methodological considerations

5.1.1 Study design

Paper I used data from a population-based cohort study, and in Papers II and III the VTE cases were derived from the same study. Cohort studies follow a defined population with exposure status recorded at inclusion, until the outcome of interest (Paper I: VTE) or other censoring events such as withdrawal from the study, migration, death, or end of study period. The study participants are classified according to the exposure status of interest (Paper I: NLR), allowing differences in outcome to be investigated in exposed and non-exposed individuals (Paper I: NLR, quartile 1 as non-exposed). Cohort studies are well suited for investigating risk factors and the natural history of a disease, and both absolute and relative risks can be provided.[183] The clear temporal sequence between exposure and outcome is a strength of prospective cohort studies, as opposed to case-control studies, where information about exposure is collected after the outcome has occurred. The temporality is a strength when assessing causality, however, other important criteria originating from Sir Bradford Hills' work also need to be evaluated. These include strength of the association, consistency with other studies in the field, biological gradient (dose-response relationship), plausibility (plausible biological explanation exists) and experimental evidence (randomized, controlled studies).[184] As such, an association between an exposure and an outcome in one prospective cohort study is not enough to conclude on causality. A large number of participants and long follow-up time are characteristic for cohort studies, and their use might therefore be limited by the time- and resource-consuming nature.[185]

In Papers II and III, we used a **case-crossover design**. This design has some similarities with case-control studies. The main difference is that in case-crossover studies, each case serves as his or her own control, and persistent confounding factors are thereby mainly controlled for through the design.[186] An example from our study is that the presence of a genetic predisposition to VTE, such as the FVL mutation, will not differ between the control and hazard periods. This design is well suited for investigating transient risk factors, or "a more proximal cause"[187] as we did with infection in Paper II and acute inflammation in Paper III. The effect size was presented as odds ratio (OR) - the odds of having an infection in the hazard period preceding a VTE was compared to the odds of having an infection in the control periods. A case-crossover design cannot be used to obtain absolute risks or incidence rates. Exposure data are collected

retrospectively, and bias can be introduced. Different kinds of bias will be discussed later in this chapter.

In Paper IV, we conducted a **randomized, controlled trial** (RCT) to study the impact of changes in the gut microbiome on systemic inflammation and FVIII:C. RCTs are regarded as the gold standard when investigating cause and effect relationships.[184] The study participants are randomly assigned to intervention or no intervention, and the outcome of interest is compared between the two groups. The random assignment facilitates un-confounded results, as all factors other than the exposure should be randomly distributed between groups.[184] The use of a placebo intervention in the control group should always be considered when planning an RCT.[188] In the present study, we used a well-known drug, i.e. Vancomycin, to achieve the desired change in the gut microbiome composition, which was to decrease the relative proportion of gram-positive microbes and increase the proportion of gram-negative microbes. The gut microbiome composition was the direct exposure, and could be assessed by analyzing fecal samples. The outcomes of interest were objective markers of inflammation and FVIII:C measured in blood samples, and consequently, we did not expect blinding of the participants to the intervention to play a role. Therefore, the control group received no intervention instead of placebo. If we had planned an RCT to test the effect of a new painkiller, then a placebo drug would be mandatory, because in such a case, the outcome would be the participants' subjective experience of pain following the intervention, and the experience of pain can be influenced by the knowledge of getting treatment. Indirectly, a similar effect in our trial is possible. If subjects in the intervention group changed their diet based on the knowledge of the intervention with Vancomycin, this might have influenced the effect of the intervention as diet plays a role for composition of the gut microbiome.[168] To avoid this, all participants were asked to maintain their normal diet, including use of probiotics, and this was followed up by questions during the study visit after end of intervention. Importantly, the effect of the intervention on gut microbiome composition was assessed by analyses of fecal samples.

5.1.2 Generalizability

To what extent the results from a study can be directly applied to other populations is termed the generalizability, or external validity, of the study, while internal validity denotes to which extent the results are valid for the population where the study participants came from. In cohort studies, the generalizability relies on well-defined inclusion and exclusion criteria, the participation rate and loss to follow-up.[185] The participation rate has been high in all the surveys of the Tromsø Study, and 77% of the invited population participated in Tromsø 4, used in Paper

I.[175] As in most health surveys, participation rates were lower among the younger (<40 years) and the older (>80 years) population, and the participation rate was lower among men than women.[175,189] As both NLR and risk of VTE increases with age[17,190], generalization of our results to older populations should be done with caution. In the study on NLR and risk of first incident and recurrent VTE, participants with a history of VTE at baseline were excluded. Reference values for WBC count including neutrophil cell count differ in different ethnic groups, as does the risk of VTE. The Tromsø Study population is mainly Caucasian, with a Sami minority, and results are likely to be generalizable to other Caucasian populations.

The case-crossover design differs from other designs in that the study participants are chosen by the fact that they have experienced the outcome of interest, and serve as their own controls. The main question asked in case-crossover studies is not "Who gets the outcome?" but "Why did the outcome occur now?"[186] Transient risk factors, or triggers, such as acute infection in Paper II and acute inflammation in Paper III are of interest. We found infection to be a prevalent and strong VTE trigger (adjusted OR 11). ORs obtained from case-control studies can be a good approximation of relative risks. For this to be true, the study participants must be representative for the source population with respect to exposure and the outcome must be rare ("the rarity assumption").[184] A general rule is that if the prevalence of the disease is <10%, the relative risk and the odds ratio will be approximately the same, and the rarer the disease, the closer the approximation.[184] The case-crossover design resembles a case-control study except that the cases are their own controls. In our study on infection and VTE risk, infections were more common among cases than expected in a general population, and the odds ratios can therefore not be directly translated to relative risks. In other words, even though the odds of having an infection in the hazard versus the control periods is 11-fold increased, a randomly chosen patient with infection during hospitalization does not have an 11-fold increased risk of VTE.

Randomized, controlled trials offer the best protection against bias and confounding, and are therefore regarded as the gold standard when investigating causal associations.[184] However, the strict inclusion- and exclusion criteria preceding randomization do not only increase the probability that observed differences between groups can be attributed to the intervention, but also limit generalizability of the results.[191] This is especially important when testing out treatment, i.e. a new drug, as the most severe cases among the diseased population are often excluded from participation due to risk of side-effects. In the RCT described in Paper IV, we did not test out a treatment on a disease, rather we investigated the effect of a controlled change in the gut microbiome on inflammation and FVIII:C in young, healthy volunteers. The

study was designed to test out a hypothesis on pathophysiology, not a treatment. Opposite to the situation in drug trials, we investigated the population with the least chance of "positive" results, and the observed effect would probably have been at least of similar strength in subjects with conditions associated with increased VTE risk.

5.1.3 Confounding

A confounding factor denotes that an alternative explanation exists for the observed association between an exposure and the outcome. A confounder is associated with both the exposure and the outcome of interest, is not an intermediate in the causal pathway, and can either strengthen, weaken or even change the direction of the association.[184] In cohort studies, possible confounding factors are important to identify and control for in the statistical analyses. Strategies to minimize confounding include the use of multivariable statistical analysis, where possible confounders are included as covariates in multivariable regression models, and stratification, where different strata of an exposure are analyzed separately.[184] We used multivariable statistical analyses to control for confounders in Paper I. The exposure of interest, NLR, increases with age[190], and advancing age is a strong risk factor for the outcome, VTE.[17] Age as a confounder can be controlled for either by adjusting for age in the Cox regression model, or by using age as time-scale. In our study, we adjusted for age (and sex) in one model, and for additional potential confounders (BMI, smoking, diabetes mellitus) in another model. BMI is related to both NLR[190] (the exposure) and VTE[91,97] (the outcome), and adding BMI in the multivariable model seems reasonable. Smoking is related to NLR[190], but not to VTE with the exception of VTE risk attributed to smoking-related cancers.[192] Likewise, the association between diabetes mellitus and VTE seems to disappear after adjustment for BMI.[192] The debate regarding a possible link between arterial thrombosis and VTE was still ongoing at the time of our study[193], and was the background for the inclusion of smoking and diabetes in the model as these are both risk factors for arterial thrombotic disease. However, and as expected from current knowledge, risk estimates in the fully adjusted model remained essentially similar to those in the age- and sex-adjusted model.

One advantage of the case-crossover design used in Papers II and III is that persistent conditions that might confound the results of a study, are mainly controlled for by the study design. As each participant serves as his or her own control, potential confounders such as genetic risk factors for VTE or chronic medical conditions will be present in both the control and the hazard periods, and therefore not influence the association between the transient risk factor of interest and the outcome. Other transient risk factors, however, need to be taken into

account, as they might be present in either the hazard or the control periods. In Paper II, we adjusted for several confounders, with special focus on immobilization. Immobilization is a known risk factor for VTE.[102] Immobilization is also a risk factor for infections[176,177], and during an infection, immobilization is common. Immobilization can therefore be both a confounder and an intermediate in the causal pathway for the association between infection and VTE (Figure 7).

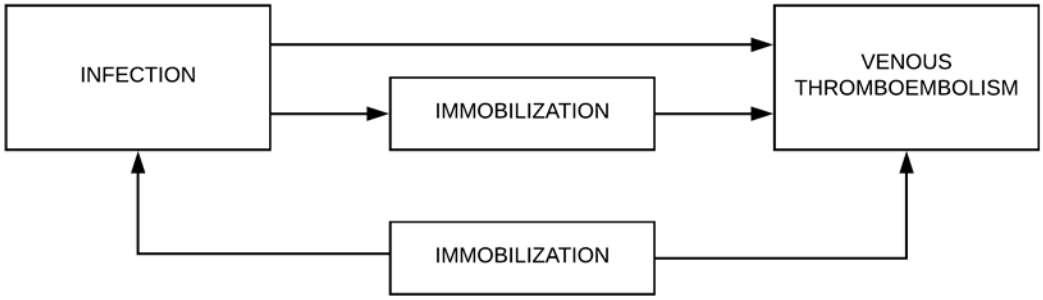

Figure 7. Possible pathways for an association between infection and venous thromboembolism, and immobilization as either an intermediate in the causal pathway (middle) or a confounding factor (bottom).

When we adjusted for immobilization in the conditional logistic regression model, the strength of the association between infection and VTE diminished from unadjusted OR 24.2 (95% CI 17.2-34.0) to adjusted OR 14.6 (95% CI 10.1-21.2), pointing towards a role for immobilization in the association between infection and immobilization. Similarly, the association between immobilization and VTE risk diminished when we adjusted for infection. Further, in stratified analyzes, we investigated the impact of different combinations of infection and immobilization on VTE risk. By including only those with infection and no immobilization, confounding by immobilization could be avoided, and in analysis of those with both infection and immobilization, we were able to discover a possible synergistic effect of the two risk factors.

5.1.4 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. The other type of error, random error, is reduced as the sample size increases. Biases may be introduced into a study at different points; during participant selection (selection bias), during data collection and/or analysis (information bias), and even in the publication process (publication bias). Bias can influence both the internal and external validity.

Selection bias denotes systematic error in the recruitment of participants in a study, so that the association between the exposure and the outcome becomes affected. This kind of bias is less likely to occur in cohort studies, since both exposed and un-exposed study participants are selected before the outcome actually occurs, and many exposures and outcomes can be investigated in one survey. However, a kind of selection bias can be introduced by different participation rates among for example age groups and sex. This kind of selection bias can be named non-response bias, and occurs when the non-responders differ from the responders.[189] In other designs, for example case-control studies, selection bias is more easily introduced, and cases and representative controls need to be recruited from the same predefined source population. In the case-crossover design, all participants are cases. In Papers II and III, the cases are derived from a well-defined cohort, and the diagnosis is thoroughly validated as described previously. In RCTs, inclusion and exclusion criteria are generally strict and well-defined, and limited generalizability is a greater concern than selection bias.[185,191]

Misclassification is a type of **information bias**. Measurement errors leading to misclassification are non-differential when they are independent of the outcome and similar across the comparison groups, or differential, when the probability of misclassification differs according to the incidence or prevalence of the outcome and differs between the comparison groups.[194] Non-differential misclassification most often leads to underestimation of the true association, while differential misclassification may introduce bias in either direction.[195] In prospective studies, of which the Tromsø Study is an example, the exposure is measured prior to the outcome, and differential misclassification is unlikely. Baseline variables such as smoking and diabetes were obtained through self-administered questionnaires, a cost-effective and efficient method. The possibility of introducing false information and misclassification when using self-administered questionnaires has to be considered, and the questions have to be prepared carefully to avoid misunderstandings. Further, questions regarding potentially sensitive information, such as smoking, alcohol consumption and sexuality might be difficult to answer correct and complete. In Paper I, we adjusted for smoking and self-reported diabetes, both potentially biased by misclassification and also regression dilution bias, which will be discussed later in this section. Later studies suggest that neither diabetes nor smoking are risk factors for VTE[192] and even if misclassification was present, results would not be biased. Adding these variables to the age- and sex-adjusted statistical models had a negligible impact.

In case-control studies, **recall bias** is more likely. Recall bias can occur when cases are more likely to recall an exposure than the controls.[194] After the occurrence of a disease or an

event, a case will often recall potential risk factors prior to the outcome differently from a healthy control.

A similar kind of **information bias** might occur in case-crossover designs. In our case-crossover study, we have retrospectively recorded risk factors for VTE present in the hazard period three months prior to the VTE, and in four preceding three month long control periods. The treating physician might be more prone to note a known VTE risk factor in the medical record if VTE was considered as a possible diagnosis, than in a control period where the patient was admitted with for instance a suspected kidney stone. This would introduce differential misclassification, where the exposure would be under-reported in the control periods, and the risk estimates for the association between the exposure and VTE would be higher than the true association.

Infection, our main exposure of interest in Paper II, was recorded if noted by a physician in the patient's medical record. For most cases of infection, the infection was also coded with an ICD-code in the discharge record. However, a patient may be diagnosed with, or treated for, several conditions during the same hospital stay, and the discharge codes are not always complete. We therefore included cases where infection was described in the medical record, even if an ICD-code for infection was not recorded. Further, the infection was registered as either certain or uncertain, based on the description in the medical records. Most typically, certain infection was recorded when a causative microbe was detected, e.g. by blood culture, and, if not, the infection was recorded as uncertain. However, infection, including sepsis, is a clinical diagnosis, and lack of microbe identification is common, e.g. when antibiotic treatment has been initiated before blood cultures and other samples for microbe detection are collected. Even if cultures are collected correctly, up to 1/3 of patients with clinical sepsis will have negative cultures.[196,197] In the analyses in Papers II and III, we included both certain and uncertain infection diagnoses, as this approach would yield the most correct number of clinically relevant infections. The recorded infections corresponded very well with cases where antibiotics were prescribed, when antibiotic use for prophylactic purposes was excluded. Acute infection can complicate hospitalization for other reasons, often with consequences for treatment and length of hospital stay. If infection was present, it is therefore very likely that it would be noted in the medical record. Altogether, the risk of information bias for the infection variable is therefore considered limited.

Immobilization might have less direct implications for patient treatment, and since it is a known risk factor for VTE, immobilization might be more likely noted in the medical record when a VTE was suspected than in the control periods. Depending on availability of data,

various definitions of immobilization have been used in different studies. In the case-crossover study on triggers of hospitalization for VTE by Rogers and co-authors[160], immobility was defined as any nonsurgical hospitalization or skilled nursing home stay, a definition probably overestimating the proportion of immobilization. In the Danish population-based case-control study investigating acute infection and VTE, immobility was defined as any inpatient diagnoses other than the infectious and other pre-defined comorbid diagnoses already included in the adjustment model.[156] Yet another definition is used in the Padua Prediction Score.[198] In this risk assessment model, reduced mobility defined as bedrest with bathroom privileges for at least three days give 3 out of maximum 20 points, and ≥4 points indicate a high risk of VTE.[198] As we used clinical data with variations in how immobilization was registered, we chose to include ECOG score of four (completely disabled, cannot carry on any self-care, totally confined to bed or chair)[199] and other specified immobilizing factors in addition to the definition used in the Padua Prediction Score. Still, immobilization might have occurred without being recorded, especially in the control periods when VTE was less likely to be expected.

In Paper III, acute inflammation assessed by CRP was the exposure of interest. In the main analyses, we included only those who had their CRP measured during the hazard period and a control period. Most likely, the CRP was lower in those where the physician did not request a CRP, and that happened more often in the control than the hazard periods. An information bias in this case would lead to underestimation of the association between CRP and VTE. To address this concern, we performed sensitivity analyses where all missing CRP values were set at the lowest reported level (CRP=5 mg/L). As expected, the OR for the association between CRP and VTE was higher in the sensitivity analysis, and probably the true risk estimate is somewhere in between the results from the two models.

In Paper I, misclassification, and thereby introduction of bias, must also be considered for the outcome, VTE. The geographical and population patterns in North Norway provides an excellent situation for developing complete outcome registries for conditions treated in hospital. UNN is the only provider of specialized health care in the region, and the nearest hospital is >200 km away. By thorough review of the hospital discharge diagnosis registry, the radiology procedure registry and the autopsy registry at UNN, all incident symptomatic VTE events during follow-up were recorded. Due to the strict validation criteria (the presence of signs and symptoms, an objectively confirmed diagnosis, a VTE was diagnosed by a physician and treatment initiated), the possibility for a false VTE diagnosis to be registered is limited. Despite all efforts made to complete the registry, there is always a possibility that some VTE events might have been missed. First, retrospective registration is dependent on complete and reliable

information in the medical records. Insufficient information is a potential source of inaccuracy in the VTE register. Second, even if VTE is normally not diagnosed and treated solely in primary care, some exceptions might exist. Home-based palliative care could provide such a case. Third, use of information from the autopsy registry increases the completeness of the VTE registry. However, autopsy rates in Norway are low[200], and cases of sudden death caused by PE might be missed. If these cases differ substantially from the recorded cases in baseline characteristics, risk profile and triggers, bias might have been introduced. However, such a difference is not likely. Fourth, a VTE event might have been diagnosed in another hospital, e.g. if the person was on holiday. In this situation, the VTE event would probably still be registered, as such patients are often referred to the outpatient clinic at UNN for follow-up.

In Paper II, a PE could, especially in the early stage, be misdiagnosed as a respiratory tract infection (RTI), due to similarities in clinical presentation. To avoid exposure misclassification and biased risk estimates, especially for the association between RTI and pulmonary embolism, the medical records of these cases were re-evaluated, and RTIs were categorized as "not likely", "possible" (but too little information in the medical record to be sure), or "likely", based on clinical signs and symptoms, laboratory and radiological examinations, treatment responses and time course. After recoding the "not likely" cases as no infection, "possible" and "likely" RTI diagnoses were included in the main analyses. To account for the possibility that some of the "possible" RTIs were actually not RTI, we performed sensitivity analyses were only the likely correct RTI diagnoses were included, and this resulted in attenuated risk estimates. Again, the true association between RTI and PE is most likely somewhere in between the results from the main and the sensitivity analyses.

Surveillance bias may occur when an exposure leads to a closer surveillance and thereby the detection of an often subclinical outcome.[184] In Paper II, patients with RTI are more likely to have a pulmonary CT scan than a patient with UTI or no infection at all, and consequently more PEs can be detected. However, in clinical practice, chest X-ray is the preferred radiology procedure when a pneumonia is suspected, and a CT scan is recommended only when there is doubt about the diagnosis, suspected complications or treatment failure. In such cases, a PE diagnosis is likely relevant, and a CT scan probably would have been performed in a patient without infection presenting with similar symptoms during a hospital stay.

In prospective cohorts with long follow-up time, another kind of bias needs to be addressed. **Regression dilution bias** might be the result when the exposure, measured at baseline, changes during follow-up without being corrected for in the analysis.[195,201] Regression

dilution bias can be introduced by true changes in the exposure variable over time, or by random measurement error in the exposure variable, and results in attenuated risk estimates (towards the null).[195] In Paper I, NLR was measured at baseline in Tromsø 4. Little knowledge exists regarding the intra-individual stability of NLR over years. In a study from the United States, diabetes, smoking and BMI were modifiable risk factors that affected NLR.[190] Further, the use of medication such as steroids could affect NLR. During a median of 17.7 years of follow-up, some of the participants would start or stop smoking, gain or lose weight, or develop diabetes, and the NLR might change. Unfortunately, neutrophil and lymphocyte counts were not included in Tromsø 5 or 6, so the exposure variable could not be updated throughout the follow-up time. To account for this, we performed sensitivity analyses where the follow-up time was restricted to three years. The result was that those with NLR above the 95th percentile had a 2.4-fold increased risk of VTE when follow-up was restricted to three years. The difference from the long-term follow-up could be partly due to regression dilution bias. This indicates that inflammation might have an effect on VTE risk in a shorter time perspective, representing a more acute than chronic inflammatory effect on VTE risk.

Publication bias implies that studies with positive findings are more often submitted and accepted for publication than studies with neutral findings. Later years, awareness of this problem and registration of planned clinical trials in public web-based registries have led to improvement. The study protocol of our RCT was registered in Netherlands Trial Registry (www.trialregister.nl) before the study was conducted. This registry is one of the officially approved registries according to the International Committee of Medical Journal Editors and the World Health Organization.

5.1.5 Missing data

Missing data are common in epidemiological research, and may introduce bias.[202] Data can be missing if 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 for other reasons. Careful planning and execution of studies are mandatory to reduce this problem. Different approaches do exist for handling missing data, but none of them are optimal. If the number of missing values for a variable is very high, the variable should be excluded.[202] If the number of missing values for the variable is relatively low, subjects with missing values can be excluded, and the variable kept for further analysis.[202] If those with missing values for a variable differ from those with complete data, deletion may introduce selection bias, in addition to reduced statistical power. A third approach, where sample size and power are maintained, is

imputation. Imputation, i.e. replacement, can be used if the missing values are missing at random (missing unrelated to the unobserved value, but related to other variables in the data set) or completely at random (missing unrelated to the unobserved value and to other observations in the data set).[202]

In Paper I, missing data for the main exposure variable, NLR, were handled by exclusion of subjects with missing data (complete case analysis). In the majority of the missing cases, the reason for the missing value would probably be pre-analytical handling of samples. As an example, the longer the bench time between blood sampling and analysis of differential WBC count, the higher the risk of failure in the analyzing process. Longer bench time is expected to occur completely at random, not related to the WBC count and not to other observations for the participant. Among 27 158 participants in Tromsø 4, 1747 (6.4%) had missing data for WBC differential count. As the Tromsø Study is large and has high participation rates, excluding subjects with missing values in this case was regarded as acceptable without substantial loss of power.

In Paper II, variables not specifically described ("missing") in the medical records were considered not present. For example, if no surgical code or description of a surgical procedure existed, we concluded that no surgery was done. Surgery represents a major intervention, and a surgical procedure should always be documented in the patient's medical record. So if no such documentation exists, this reliably means that no surgery was performed. For some variables, this could be less clear. Immobilization is one such example, and possible consequences of misclassification of this variable have already been discussed earlier in the chapter (5.1.4 Bias). Handling of missing values for CRP in Paper III has also already been discussed. In the RCT described in Paper IV, there were few missing values. Some measurements were below the lower detection limit (C3bc, IL-1β, IL-8, MCP-1, TNF), and those were set at the lower reported value. Lack of sensitive measurements in the lower range limits the possibility to identify changes within these values.

5.1.6 Sample size and study power

By increasing sample size, the power increases.[183] The power of a study describes how likely it is that an effect, if present, will be found. The probability of making a type II-error, i.e. concluding that there is no effect when there actually is an effect, is low when statistical power is high. Power calculations can be done when planning a study, and it is common to aim for 80% power. A power of 80% 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 an effect (by chance) when there is now effect in the source population, is in most cases set to 5% (or α of 0.05).

In the fourth survey of the Tromsø Study, a large cohort study with 27 158 participants followed for nearly 20 years, the study power would be sufficient in most settings. However, in subgroup analyses the number of outcomes in each group can be small, and subgroup analyses can have less power than main analyses. An example from Paper I is the analyses where follow-up time was restricted to the first three years after baseline. In those with NLR above the 95th percentile, only 8 were diagnosed with an incident VTE. A rule of thumb is that logistic and Cox models should be used with a minimum of 10 outcome events per exposure variable.[203] Therefore, the results from the sensitivity analysis in those with NLR above the 95th percentile needs to be interpreted with caution.

In Papers II and III, the number of participants was decided by the number of participants in Tromsø 4 who were diagnosed with VTE up to Dec 31, 2012. A post hoc power calculation was made for Paper II, using the observed rates of infection in the control and hazard periods, and the sample size of 707 (with 4 control periods for each case) was sufficient to find an effect of infection on VTE risk with α of 0.05 and close to 100% power.

To calculate sample size before a study is initiated, assumptions on expected effect have to be made. These assumptions are often made on the basis of previous knowledge and studies in the field. For the randomized controlled study in Paper IV, no study had been published of which results could be used for calculation of sample size. HIV-infection is associated with a 1.5-fold increased relative risk of VTE.[204] We used the difference between FVIII:C levels measured in treatment-naive HIV-patients and healthy controls to estimate sample size, and had close to 100% power to detect a similar difference between the intervention and control group in our study. However, as the difference between the groups in FVIII:C-levels was small compared to the observed difference in the HIV-study, we did a post-hoc power calculation based on the actual results in our study, and found a power of 70%. As cytokine levels have more biological variability than FVIII:C and CRP, and several participants had measurements below the lower detection limit, a larger sample size would provide more power and increase the chances of identifying a possible difference between the intervention and control group also for various cytokine levels.

5.2 Discussion of main results

5.2.1 Neutrophil to lymphocyte ratio and future risk of venous thromboembolism and mortality: the Tromsø Study (Paper I)

In Paper I, we reported that neutrophil to lymphocyte ratio (NLR) was not associated with future risk of VTE in a prospective cohort with long-term follow-up. The Saliba and co-authors'[124] study on NLR and risk of stroke in patients with atrial fibrillation suggested that NLR could be superior to hs-CRP for prediction of thromboembolic events, and inspired us to perform this study. In their large registry-based cohort study, NLR was obtained from blood samples taken the year before study start, and the participants were followed for a maximum of one year.[124]

At the time we performed our study, a possible association between NLR and VTE risk had not been investigated. Other inflammatory markers had been studied, and our results are in line with long-term follow-up studies on the association between hs-CRP and VTE risk[131,133,134]. Total WBC count, including monocytes, eosinophils and basophils in addition to neutrophils and lymphocytes, has also been investigated. In the LITE Study, no association between WBC count and VTE risk was found[133], and similar results were obtained when investigating WBC count and VTE risk in the Tromsø Study.[140] However, a high pre-cancer WBC count was associated with a 2.4-fold increased VTE risk compared to low WBC-count in patients who developed cancer during follow-up.[140]

A study from Italy investigated NLR and VTE risk in 810 cancer outpatients with primary or relapsing solid cancer at the start of a new chemotherapy regimen.[205] After a median follow-up time of 9.2 months, they found that NLR was associated with a two-fold increased risk of VTE in patients in the intermediate risk category using Khorana risk-score. These results cannot be compared to our study of a general population, as cancer is strongly associated with VTE risk, and the follow-up time in this study was less than one year.

To address the possibility of regression dilution bias due to the long follow-up time, we restricted follow-up time to three years from baseline and found that the 5% with the highest NLR values had a 2.4-fold increased risk of VTE compared to those in the lowest quartile. Statistical significance was not reached after multivariable adjustment, and there were few VTE-events (n =8) in this group. Consequently, this result should be interpreted with caution.

Anyhow, the observed association between NLR and VTE after short-term follow-up is in line with previous studies on CRP and VTE risk. In a case-cohort study using data from HUNT, CRP in the highest quintile was associated with a 1.6-fold increased risk of VTE, and this association was driven by VTE-events diagnosed within the first year after baseline.[137]

When using repeated measures of CRP in the Tromsø Study, high levels of CRP (≥ 3 mg/L versus < 1 mg/L) were associated with a 1.8-fold increased risk of VTE in women, but not in men after a median follow-up time of 3.1 years.[96]

Following our publication, NLR and VTE risk has been further investigated in a case-control study from Italy.[206] NLR was measured in 486 patients with VTE a minimum of three months after the event, and in 299 healthy controls, and in accordance with our results, they found no association between NLR and VTE risk.

We also found that baseline NLR was associated with a 1.4-fold increase in overall mortality (quartile 4 versus quartile 1). NLR as a predictor of mortality has been studied in several diseases, and NLR has been found to predict 1- and 5 year mortality in breast cancer patients[207], outcome and survival in patients with resectable cancers[208], acute exacerbations and mortality in chronic obstructive pulmonary disease[209] and short- and long-term mortality in arterial cardiovascular disease.[122] In most of these studies, NLR has been obtained after initiation of the disease, which could affect inflammation and levels of NLR, and a direct comparison to our study is therefore not possible.

Taken together, results from other studies and our study on NLR and VTE risk are quite consistent. The risk of VTE seems to be higher in more acute or short-term inflammation than in long-term, low-grade inflammation.

5.2.2 Acute infection as a trigger for incident venous thromboembolism: Results from a population-based case-crossover study (Paper II)

In Paper II, we reported that acute infection in hospitalized patients was a prevalent and strong trigger for VTE, also after adjustment for possible confounders including cancer, surgery, trauma and immobilization. Acute infection and immobilization had a more than additive effect on VTE risk. Further, our results suggested that RTI was more strongly associated with VTE in total, and PE in particular, than UTI.

Previous to our study, infection had been recognized as a risk factor for VTE in both primary care and hospital-settings.[156,158] In a population-based case-control study from Northern Denmark using information from medical databases, respiratory tract, urinary tract, skin and intraabdominal infections and septicemia diagnosed in hospital were associated with a 3.3-fold increased VTE risk after adjustment for other VTE risk factors, co-morbidities and co-medication use.[156] Similarly, they found a 2.6-fold increased VTE risk for patients with community antibiotic prescription. Even following the authors' attempts to adjust for all possible confounders, residual confounding is possible as they did not have information on

immobilization in this study. Interestingly, they found the highest risk estimates for VTE following RTIs and skin infections, and argue that misdiagnosis of PE as RTI and DVT as skin infection could not fully explain the findings. Risk of PE after RTI was highest the first two weeks after RTI, when a higher risk of misclassification might be expected, but was still 5-fold increased when time from RTI to PE was increased to 3-4 weeks. A similar pattern was seen for skin infections and DVT.[156]

In a self-controlled case-series study from a community-setting in England, UTI was associated with an age-adjusted 2.1-fold increased risk of both DVT and PE, whereas RTI was associated with an age-adjusted 1.9-fold increased risk of DVT.[158] The authors reported an 11-fold increased risk of PE the two first weeks after RTI, but chose not to include these results due to the risk of initial misclassification of PE as RTI. Other VTE risk factors were not adjusted for, but in sensitivity analyses excluding patients with a diagnosis of cancer within the last year, results remained essentially similar. The first case-crossover designed study investigating infection and VTE risk found that infection was the most prevalent trigger of hospitalization for VTE, occurring in 52.4% of the patients during the three month long hazard period before VTE.[160] Study participants were older at time of VTE-diagnosis (mean age 76.9 years) than in our study (median age 71 years), and comorbidities were common. All other risk factors registered were adjusted for in the statistical model, including immobilization. However, we cannot compare our results directly to this study as their definition of immobility was rather broad, and included all non-surgical hospitalization or skilled nursing facility stay during the 90 day risk and comparison periods.

Another case-crossover study published in 2017 found that VTE risk was 1.7-fold increased in the 30 days following hospitalization with infection compared to no hospitalization.[210] In a self-controlled case-series study, antibiotic prescription (as a proxy for infection) was associated with a 5-fold increased risk of first incident VTE, and a 2-fold increased risk of recurrent VTE.[211] A recently published case-control study found an overall 2.6-fold increased VTE risk in the 92 day period after infection, and pneumonia, UTI, oral, intra-abdominal and systemic blood-stream infections were significantly associated with VTE risk.[212] In this study, they adjusted for possible immobilizing factors such as leg paresis, hospitalizations and nursing home confinement. Again, this represents a broad definition of immobilization.

Altogether, we can conclude that acute infection is an important risk factor for VTE, especially in high risk situations. Immobilization and infection together probably contribute substantially to the increased VTE risk observed in patients during and following

hospitalization. Results from several studies, including ours, point towards a strong association between RTI and PE, but due to similarity in symptoms, and clinical and methodological challenges in distinguishing between the two, more research is needed before conclusions can be made.

5.2.3 C-reactive protein and risk of venous thromboembolism: Results from a population-based case-crossover study (Paper III)

In Paper III, we reported that acute inflammation, assessed by CRP was associated with a 1.8-fold increased risk of VTE. Adjustment for immobilization and infection slightly attenuated the association. In stratified analyses, CRP was associated with increased risk of VTE in cases with and without infection. To our knowledge, inflammation as a trigger for VTE has not been studied in a case-crossover design before, and our study adds valuable knowledge to previous findings.

A more short-term VTE risk by inflammation is supported by the nested case-control study from HUNT. They found that CRP >5 mg/L was associated with a 3-fold increased risk of VTE the first year after baseline when compared to those with CRP <0.8 mg/L.[137] In a case-control study, both IL-6, IL-8 and CRP were higher in cases presenting with DVT than in controls, and in cases, IL-6 and CRP declined after five days of follow-up.[147] The acute phase response by the DVT itself might explain these results. Another case-control study, the Leiden Thrombophilia Study, found higher CRP in cases (mean 1.49 mg/L) than controls (mean 1.12 mg/L) in samples collected a median of 18 months after the VTE-event.[213] At this time, the acute phase response should not contribute, but due to limitations in the case-control design, conclusions regarding the sequence of inflammation and VTE cannot be made based on these studies. For example, post-thrombotic complications could possibly contribute to low-grade inflammation and thereby influence the level of inflammatory markers.

Several conditions associated with an increased risk of VTE, including cancer, acute infections, autoimmune diseases and obesity, share the feature of inflammation.[22,141,204,214] In Paper II, we demonstrated that acute infection was a frequent and important trigger for VTE in the same study population. CRP is routinely used as an inflammatory marker in the diagnostic work-up and during follow-up when treating infections. To make sure that the observed association between CRP levels and risk of VTE was not entirely caused by infection, we stratified for infection, and found inflammation, assessed by CRP, to be associated with VTE risk also in cases without infection. The impact of acute inflammation might seem unexpectedly small in those with infection (adjusted OR 1.57, 95% CI 0.98-2.51). This is likely due to the

study design and statistical methods. In this analysis, CRP levels in the hazard and control periods are compared for cases diagnosed with infection in both the hazard and a control period. This implies that CRP levels are elevated in both periods, and the resulting OR probably demonstrates a more serious infection and a stronger inflammatory response in the hazard period than in the control period. This is in line with the Danish study demonstrating higher risk estimates for VTE after infection diagnosed in hospital, where more severe infections are treated, than after infections treated in the community.[156]

Similar to the associations between infection and immobilization discussed in Paper II, immobilization can also co-exist with other risk factors for VTE associated with inflammation, such as cancer, surgery and trauma. As risk estimates were only slightly attenuated in the model adjusted for immobilization, our study supports that the inflammation itself, and not only co-existing immobilization, is involved in the pathophysiology of VTE in these conditions.

5.2.4 A Vancomycin-induced shift of the gut microbiome in gram-negative direction increases plasma factor VIII:C levels: Results from a randomized, controlled trial (Paper IV)

In Paper IV, we reported findings from a randomized, controlled trial on the impact of a Vancomycin-induced change in the gut microbiome towards a gram-negative direction on inflammation and FVIII:C in young, healthy volunteers. We hypothesized that a change in the gut microbiome in a gram-negative direction would lead to an increase in systemic levels of inflammatory markers and FVIII:C, due to translocation of LPS from gram-negative bacteria across the gut wall into the circulation. As expected, intervention with oral Vancomycin led to a reduction in gut microbiome diversity and a shift towards a more gram-negative composition of the gut microbiome. In support of our hypothesis, there was a small, but significant increase in the primary outcome, FVIII:C, and hs-CRP in the intervention group when compared to the control group.

During the last decades, research interest in the gut microbiome has gone from focusing on gastrointestinal pathogens to include commensal bacteria and their role in health and disease.[215] When we planned our study, no previous study had investigated the potential impact of the gut microbiome on inflammation and coagulation. Later, results from an RCT investigating effects of gut microbiome manipulation by antibiotics on host metabolism in obese humans were published.[216] In this study they had three arms, one similar to our Vancomycin arm, one with Amoxicillin (a broad-spectrum antibiotic), and one placebo. As expected, they found that Vancomycin, but not Amoxicillin, altered the gut microbiome

composition in a gram-negative direction. They found no effect on the inflammatory variables IL-6, IL-8 and TNF. However, they did not include the downstream inflammatory marker CRP, and as discussed in our paper, CRP might be a more sensitive inflammatory marker as several cytokines together may contribute to stimulation of CRP production in the liver.[217] Further, laboratory methods may not be sensitive enough to show a difference in cytokine levels, especially in lower normal levels. Our study was small, and was underpowered for these secondary endpoints. Strong conclusions can therefore not be made, but our findings did support the hypothesis; that a targeted change in the gut microbiome composition does induce systemic inflammation and FVIII activity.

As LPS is methodologically difficult to measure, we cannot prove that this increase in inflammatory variables and FVIII:C was caused by increased LPS. Other microbial products, or even other microbes than bacteria, as for instance viruses or fungi might be involved. A small study on five patients with *Clostridium difficile*-colitis found that fecal filtrate transfer, containing bacterial debris, proteins, antimicrobial compounds, metabolic products and DNA, but no intact microorganisms, were curative and the colitis did not relapse the following six months.[218] Although limited by a small sample size and the lack of a control group, this study suggests that the impact of the microbiome is not solely dependent on live bacteria. Our hypothesis, and the possible impact of other component of the gut microbiome on systemic inflammation and coagulation should be investigated in other populations to learn more about these mechanisms.

6 CONCLUSIONS

- We found that neutrophil to lymphocyte ratio (NLR) was not associated with an increased risk of future VTE, neither incident nor recurrent. However, in those with the highest NLR (above the 95th percentile), the risk of incident VTE was 2.4-fold increased when compared to the lowest quartile when follow-up was restricted to the first three years after baseline. Those with baseline NLR in quartile 4 had a 1.4-fold increased overall mortality following the VTE event compared to those with NLR in quartile 1.

- Acute infection during hospitalization was a frequent and strong trigger for VTE. Infection and immobilization had a synergistic effect on the risk of VTE. Some of the VTE risk in immobilized patients was attributed to infection, some of the VTE risk in patients with infection was attributed to immobilization, and the presence of both infection and immobilization had an even higher impact on the risk of VTE than the sum of the two triggers alone.

- Acute inflammation, assessed by C-reactive protein, in hospitalized patients was associated with an increased risk of VTE. Concomitant immobilization did not explain the association, and inflammation was a trigger for VTE in patients with and without infection. We can conclude that acute inflammation is a trigger for VTE regardless of cause.

- Intervention with Vancomycin led to an expected reduced diversity and increased relative abundance of gram-negative bacteria in the gut microbiome composition. This change was accompanied by a small increase in FVIII:C and systemic inflammation (hs-CRP) when compared to controls. The hypothesis of an impact of the gut microbiome on systemic inflammation and coagulation was supported by our findings.

7 FINAL REMARKS AND FUTURE PERSPECTIVES

Based on the existing literature and the studies presented in this thesis, long-term, low-grade inflammation does not seem to be associated with risk of VTE. For medium-grade inflammation of shorter duration, a weak association appears, and a stronger association is demonstrated for acute and more pronounced inflammation on risk of VTE. We found that acute infection was a frequent and strong trigger for VTE in a hospital setting, and since up to 50% of incident VTE cases are related to hospitalization[22], this finding has relevance for thrombosis prophylaxis assessment. VTE is a multicausal disease, and many factors need to be taken into account to identify patients who will benefit from thromboprophylaxis. Several at-admission risk assessment models have been developed. The complex nature of VTE is however demonstrated by these risk assessment models' limited performance in external validation studies, with C-statistics of 0.58-0.64.[219] We demonstrated strong and synergistic effects on VTE risk by immobilization and infection during hospitalization, implying that these factors should be included as cornerstones when new and improved VTE risk assessment models are developed.

Due to limitations related to the use of clinical data, our findings suggesting an association between respiratory tract infections and pulmonary embolism needs to be interpreted with caution. Future studies in the field will be met with interest. PE is a common preventable cause of in-hospital death. If the suggested association between RTI and PE is confirmed, it has implications for clinical practice and outcome and for our understanding of the pathophysiology of VTE and PE in particular.

Research on the interplay between the human host and its commensal microbes is still in an early and explorative phase. The RCT presented in this thesis makes a small contribution to this field. The next decades, we will hopefully learn more about the gut microbiome and its possible impact on inflammation and coagulation.

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PAPER I

PAPER II

PAPER III

PAPER IV

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