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Combined effects of cancer and prothrombotic genotypes on the risk of venous thromboembolism

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Summary

Venous thromboembolism (VTE), a collective term for deep vein thrombosis and pulmonary embolism, is a severe and multifactorial disease. Heritability has been found to explain up to 60% of VTE events, however, the role of genetics on VTE in cancer is scarcely studied. VTE occurs frequently in cancer patients and is a common cause of morbidity and mortality in this patient group. The aim of the present thesis was to investigate the impact of individual prothrombotic genotypes and the combination of genotypes in a genetic risk score (GRS) on the risk of VTE in cancer patients. Further, VTE may be the first sign of an underlying malignancy, and therefore we also aimed to investigate the effect of prothrombotic genotypes on VTE risk in subjects with an occult (i.e. undetected) cancer.

All four papers in the present thesis utilize data from the fourth survey of the Tromsø Study (Tromsø 4), conducted in 1994-1995. The study populations in Paper I and III are also recruited from the second survey of the Nord-Trøndelag Health Study (HUNT 2), conducted in 1995-1997. Paper IV is based on the Scandinavian Thrombosis and Cancer (STAC) Cohort, which consists of merged data from the Tromsø 4 Study, the HUNT 2 Study and the Danish Diet, Cancer and Health (DCH) Study. Participants were followed from date of enrollment (1993-1997) in the different surveys to the date of an incident VTE event, the date of death or migration, or until end of follow-up (2007-2012). All potential cases of incident VTE events and cancer diagnoses during this time-period were recorded.

We reported the effect of several single nucleotide polymorphisms (SNPs) on VTE risk in subjects with and without cancer. A SNP of the GP6 gene (rs1613662), affecting platelet adhesion and activation, displayed a decreased risk of VTE in cancer-free subjects, while an increased risk was observed in cancer patients homozygous for GP6 SNP. The genotype was also found to be associated with prothrombotic and metastatic cancers. These findings support a role of platelet reactivity in the pathogenesis of VTE, which may differ according to cancer status.

The risk of VTE was also found to increase by the presence of ABO (rs8176719), and risk alleles in F5 (rs6025), F2 (rs1799963), FGG (rs2066865), and F11 (rs2036914) in both cancer-free subjects and in cancer patients. Moreover, a synergistic effect was discovered for the genetic variants of FGG, FVL and ABO in combination with cancer on the VTE risk. We found a dose-response relationship between number of risk alleles in the 5-SNP score (genetic risk score, GRS) and VTE risk in subjects with and without cancer, and the combined effect of cancer and high-number of risk alleles (≥4 risk alleles) yielded a supra-additive effect for the risk of VTE. However, the five prothrombotic genotypes, alone or combined, did not increase the risk of VTE in occult cancer.

Our findings suggest that the genetic risk score and prothrombotic genotypes may be useful for identifying cancer patients at increased risk of VTE.

Sammendrag

Venøs tromboembolisme (VTE), en fellesbetegnelse for dyp venetrombose og lungeemboli, er en alvorlig og multifaktoriell sykdom. Det er vist at opp mot 60% av VTE hendelser kan forklares av arvelighet, likevel er genetikkens rolle lite undersøkt hos personer med VTE og kreft. VTE forekommer hyppig blant kreftpasienter og er en vanlig årsak til sykelighet og dødelighet i denne pasientgruppen. Formålet med denne avhandlingen har vært å undersøke hvordan individuelle protrombotiske genotyper og kombinasjonen av genotyper i en genetisk risikoskår (GRS) påvirker risikoen for VTE hos kreftpasienter. VTE kan være det første tegnet på en underliggende malignitet, og vi hadde derfor også som mål å undersøke effekten av protrombotiske genotyper på risikoen for VTE blant personer med okkult (dvs. ikke påvist) kreft.

Alle fire artiklene i avhandlingen bruker data fra den fjerde Tromsøundersøkelsen (Tromsø 4), gjennomført i 1995-1997. Studiepopulasjonene i artikkel I og III er i tillegg rekruttert fra den andre Helseundersøkelsen i Nord-Trøndelag (HUNT 2), gjennomført i 1995-1997. Artikkel IV er basert på «the Scandinavian Thrombosis and Cancer (STAC) Cohort», som består av sammenslåtte data fra Tromsø 4 studien, HUNT 2 og den danske «Diet, Cancer and Health» (DCH) studien. Deltakere ble fulgt fra registrering (1993-1997) i de ulike studiene til datoen for en førstegangshendelse av VTE, datoen for død eller flytting, eller til studieslutt (2007-2012). Alle potensielle tilfeller av førstegangs VTE og kreftdiagnoser i denne tidsperioden ble registrert.

Vi rapporterte effekten av flere ulike «single nucleotide polymorphisms» (SNPs) på risikoen for VTE blant personer med og uten kreft. En SNP i GP6 genet (rs1613662), som påvirker blodplateadhesjon og blodplateaktivering, viste seg å redusere risikoen for VTE hos kreftfrie, mens en økt risiko for VTE ble observert hos kreftpasienter homozygote for GP6 SNP'en. Genotypen var også assosiert med protrombotiske og metastaserende krefttyper. Disse funnene støtter at blodplater spiller en rolle for patogenesen av VTE, hvilket kan variere i henhold til kreftstatus.

Risikoen for VTE økte for både kreftfrie personer og kreftpasienter med ABO rs8176719 og risikoalleler for F5 (rs6025), F2 (rs1799963), FGG (rs2066865), and F11 (rs2036914. Videre oppdaget vi en synergistisk effekt mellom kreft og genetiske varianter av FGG, FVL og ABO på risikoen for VTE. Vi fant også et dose-respons forhold mellom antall risikoalleler i 5-SNP skåren (GRS) og VTE risikoen blant personer med og uten kreft. Kombinasjonen av kreft og et høyt antall risikoalleler (≥4) utøvde en supra-additiv effekt på risikoen for VTE. Dog var der ingen sammenheng mellom de fem protrombotiske genotypene, alene eller i kombinasjon, og risikoen for VTE hos personer med okkult kreft.

Våre funn tyder på at den genetiske risikoskåren og protrombotiske genotyper kan være nyttige for å identifisere kreftpasienter med økt risiko for VTE.

List of papers

The thesis is based on the following papers:

I. Genetic variation of platelet glycoprotein VI and the risk of venous thromboembolism

Skille H, Paulsen B, Hveem K, Gabrielsen ME, Brumpton B, Hindberg K, Gran OV, Rosendaal FR, Brækkan SK, Hansen JB

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II. Fibrinogen gamma gene rs2066865 and risk of cancer-related venous thromboembolism

Paulsen B, Skille H, Smith EN, Hveem K, Gabrielsen ME, Brækkan SK, Rosendaal FR, Frazer KA, Gran OV, Hansen JB

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III. Combined effects of five prothrombotic genotypes and cancer on the risk of a first venous thromboembolic event

Skille H, Paulsen B, Hveem K, Gabrielsen ME, Brumpton B, Hindberg K, Gran OV, Rosendaal FR, Brækkan SK, Hansen JB

Journal of Thrombosis and Haemostasis. 2020 July; 10.1111/jth.15011

IV. Prothrombotic genotypes and the risk of venous thromboembolism in occult cancer

Skille H, Paulsen B, Hveem K, Severinsen MT, Gabrielsen ME, Kristensen SR, Næss IA, Hindberg K, Tjønneland A, Brækkan SK, Hansen JB

Manuscript

Abbreviations

ANC	Awareness of Neutropenia in Chemotherapy
AP	attributable proportion due to interaction
APC	activated protein C
AUC	area under the curve
BMI	body mass index
CATS	Vienna Cancer and Thrombosis Study
CCR	the California Cancer Registry
CI	confidence interval
CRN	Cancer Registry of Norway
CT	computed tomography
CVC	central venous catheter
DCH	Diet, Cancer and Health Study
DOAC	direct oral anticoagulant
DVT	deep vein thrombosis
F	factor
FGG	fibrinogen gamma gene
FVL	factor V Leiden
GATE	the Genetic Attributes and Thrombosis Epidemiology Study
GP6	glycoprotein VI
GRS	genetic risk score
GWAS	genome-wide association study
HR	hazard ratio
HUNT	Health Survey in Nord-Trøndelag (Helseundersøkelsen i Nord-Trøndelag)
IR	incidence rate
ITAC	the International Initiative on Thrombosis and Cancer
LMWH	low-molecular-weight heparin
MARTHA	the Marseille Thrombosis Association Study
MEGA	Multiple Environmental and Genetic Assessment
MV	microvesicle
NETs	neutrophil extracellular traps
NPV	negative predictive value
OR	odds ratio
PE	pulmonary embolism

PPV	positive predictive value
PTS	post-thrombotic syndrome
RCT	randomized controlled trial
RERI	relative excess risk due to interaction
RIETE	Registro Informatizado de Pacientes con Enfermedad TromboEmbólica
SI	synergy index
SIR	standardized incidence ratio
SNP	single nucleotide polymorphism
SOME	Screening for Occult Malignancy in Patients with Idiopathic Venous Thromboembolism
STAC	Scandinavian Thrombosis and Cancer
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TWAS	transcriptome-wide association study
UNN	University Hospital of North Norway
VEGF	vascular endothelial growth factor
VKA	vitamin K antagonist
VTE	venous thromboembolism
vWF	von Willebrand factor
WHO	World Health Organization

1. Introduction

Venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common multifactorial disease with a potentially fatal outcome.[1] It occurs in 1-2 per 1000 persons per year in the general population, [2-5] affecting all age groups, ethnicities and both genders.[4,6] A DVT is the formation of a thrombus in the deep veins, arising most frequently in large veins of the lower extremities, but can also occur in deep veins of the upper extremities, abdominal veins and cerebral veins and sinuses.[7] Prevalent signs and symptoms of DVT are pain, swelling and erythema of the affected extremity.[8] Since autopsy studies by Virchow in the 19th century, PE has until recently mainly been considered as a complication of DVT. A PE often occurs by embolization of the original DVT, i.e. parts or all of the blood clot dislodges, travels via the blood stream to the pulmonary circulation where it lodges and, subsequently, obstructs blood flow. However, imaging studies have reported that concurrent DVT can be found in less than 50% of PE patients, indicating other etiologies for some PE cases.[9,10] Other possible origins for PE are de novo thrombus formation in pulmonary arteries,[9,10] or embolization from a right sided cardiac thrombus caused by atrial fibrillation.[11] The clinical course of a PE ranges from asymptomatic to fatal circulatory collapse.[8,12] Nevertheless, frequent signs and symptoms seen in PE patients include dyspnea, tachypnea, coughing and pleuritic chest pain.[8]

The association between cancer and venous thrombosis has been known for more than a century and is well established.[13-15] As the French physician Armand Trousseau is credited for describing the relationship between VTE and cancer in 1865, the spontaneous formation of a venous thrombus in relation to an underlying malignancy has been termed the Trousseau's syndrome. The term is particularly known in the field of medicine not only because Trousseau described the association, he also diagnosed himself with a VTE secondary to gastric cancer only two years later, and then died shortly after.[16] Not many people know that Jean Baptiste Bouillaud had already reported the association nearly half a century earlier, in 1823.[14,17]

Since the time of Bouillaud and Trousseau, several studies have convincingly demonstrated the link between cancer and VTE, [18,19] and today cancer is acknowledged as one of the most important risk factors for VTE in the population.[15] Cancer patients who develop VTE have a shortened life expectancy compared to cancer patients without VTE.[20,21] Furthermore, the clinical consequences of VTE, such as post-thrombotic syndrome (PTS), recurrent VTE and treatment-related bleeding, are more prevalent in cancer patients than in cancer-free subjects.[20,22] The two-way relationship between venous thrombosis and malignancy has also been confirmed in studies describing increased risk of cancer after a VTE event,[23-28] and approximately 5% of patients with unprovoked VTE are diagnosed with cancer

within one year.[29-33] This finding has raised the question to what extent we should screen for cancer in subjects with VTE, which has been heavily debated.[30,31,34]

Despite the quantity of knowledge on cancer-related VTE assembled over the last decades, the incidence of VTE in cancer is high and increasing.[35] Cancer is a heterogeneous disease, and the risk of VTE depends on the interaction between tumor cells, treatment regime, the hemostatic system and individual patient characteristics. A strong hereditary component is found in VTE patients, and several genotypes are established as patient-related risk factors for VTE.[36] However, the role of prothrombotic genotypes in the complex interplay with malignancy is not yet well established, as only a few of these genotypes have been studied in cancer patients.

Even though VTE is a potentially preventable disease by the use of antithrombotic treatment, current guidelines do not recommend routine anticoagulant prophylaxis to all cancer patients due to the high risk of bleeding and uncertain benefit-to-harm ratio in these patients.[37-39] The severe complications and potentially fatal outcome of VTE in cancer stresses the need and importance of identifying high-risk subjects, to determine who would benefit from targeted prevention. Thus, even 150 years after the death of Trousseau, there are still knowledge gaps to fill. Hence why I chose genetic risk factors for cancer-related VTE as the topic of my thesis.

1.1. Epidemiology

1.1.1. Venous thromboembolism in cancer patients

Cancer is a strong and independent risk factor for VTE,[35,40] and studies have consistently demonstrated that 20-30% of all first venous thromboembolic events are cancer-related.[2,19,41,42] The relative risk (RR) of VTE in subjects with active cancer ranges from 4 to 7, compared to the general population or subjects without cancer.[35,40,43,44] Studies have reported an absolute risk (cumulative incidence) of VTE in cancer, which varies widely from 1% to 12%.[45-47] The wide range reported might be attributable to methodological variations between studies, such as study population, follow-up duration, definition of active cancer and assessment of outcome (i.e. cancer and VTE). Further, cancer is a heterogeneous disease and risk estimates of VTE are highly dependent on different risk factors, which can be broadly stratified into cancer-related, treatment-related and patient-related risk factors. A meta-analysis from 2012 of 38 individual studies demonstrated this heterogeneity as the overall risk of VTE in average risk patients with cancer (those representative of all patients with cancer type under investigation or overall cancer) and high risk patients with cancer (those with high-grade or metastatic

disease or treated with therapeutic strategies that increase VTE risk), were 13 per 1000 person-years (95% CI 7-12) and 68 per 1000 person-years (95% CI 48-96), respectively.[48]

The VTE incidence varies between ethnic groups, with Asian/Pacific Islanders having the lowest risk of both first-time VTE and cancer-related VTE.[49] Interestingly, there appears to be little difference in the incidence of cancer-related VTE between African-Americans, Hispanics and Caucasians.[49] The incidence of VTE in cancer is high and several studies have noted an increasing incidence of cancer-related VTE.[35,50,51] A large cohort study of more than 83000 cancer patients derived from four different United Kingdom databases presented an increase in overall incidence of VTE among cancer patients from 10 per 1000 person-years (95% CI 8-14) in 1997 to 19 per 1000 person-years (95% CI 18-21) in 2006, while no similar trend was seen in the 577000 cancer-free controls.[35] The rise in VTE incidence is reported for different cancer types.[35] Several factors could be the explanation for the increasing incidence, including greater awareness of cancer-related VTE and improvements in diagnostic imaging for detection and staging of cancer, which may incidentally identify VTE events. Additionally, more aggressive cancer treatments (e.g. chemotherapy and surgery) and increasing survival, leading to elderly patients undergoing more cancer treatments, also increase VTE risk.

Incidental (i.e. asymptomatic) VTE is a relative common finding in cancer patients.[52] Extended use of computed tomography (CT) scan for evaluation of cancer treatment effect and improved CT scan technology may have affected the increase in incidence of VTE over time, especially incidental VTE. In a recent retrospective study by Di Nisio *et al.*, 3.2% of cancer patients had asymptomatic VTE, while 2% presented with symptomatic VTEs.[53] Similar rates of recurrence and mortality have been noted in patients with symptomatic and asymptomatic cancer-related VTE, and guidelines suggest that incidental PE should be managed like symptomatic events.[54-57]

There are a limited number of studies investigating the differences in clinical presentation of VTE between cancer patients and cancer-free subjects. Bilateral DVT seems to be more prevalent in cancer patients than non-cancer.[58-60] In a recent study, the rates of PE and symptomatic proximal DVT were similar, however, rates of symptomatic thrombosis in inferior vena cava and iliac veins, upper limb DVT and bilateral lower limb DVT were higher in cancer patients compared to cancer-free subjects.[61] It is to be noted that the relative high prevalence of upper limb DVT in cancer, could partly be explained by the frequent use of central venous catheters (CVC) in these patients.[62] Lastly, cancer is shown to be common in rare forms of VTE such as extrahepatic portal vein obstruction, Budd-Chiari syndrome and thrombosis of mesenteric veins.[63]

VTE is a serious disease in cancer patients. In general, clinical consequences such as recurrent VTE, PTS and bleeding complications are typically more common and more severe in cancer patients

suffering a VTE event than cancer-free VTE patients.[64-66] The International Initiative on Thrombosis and Cancer (ITAC) developed the first international evidence-based clinical practice guidelines in 2013 to provide clinicians with practical and accessible recommendations for treatment and prevention of cancer-related VTE.[67] The ITAC guidelines were updated in 2019 and recommend patients with cancer-related VTE initial treatment with low-molecular-weight heparin (LMWH) for a minimum of 6 months, or DOACs (direct oral anticoagulants, i.e. Rivaroxaban or Endoxaban) for the same duration of time in cancer patients who are not at high risk of gastrointestinal or genitourinary bleeding.[67] Thus, cancer patients receive an extended duration of anticoagulant therapy. However, despite adequate anticoagulation, treatment failure occurs frequently in cancer patients with VTE and subsequently, cancer patients have a two to nine-fold higher risk of VTE recurrence compared to cancer-free subjects.[66,68-70] In the Tromsø Study, the incidence rate of recurrent VTE per 100 person-years, were 8.5 following a cancer-related VTE versus 3.6 following a non-cancer unprovoked VTE.[71] In a cohort study of 477 subjects from Olmsted County diagnosed with cancer-related VTE, the cumulative incidence of VTE recurrence was 18% at three months and 27% at one year.[72] Furthermore, this study also reported an increased risk of treatment-related bleeding in cancer, with the cumulative incidence of major bleeding being 2.5% and 4.7% at three months and at one year, respectively.

Cancer-related VTE is associated with poor survival. A retrospective study by Khorana and colleagues found that in-hospital mortality was two to five fold increased in neutropenic cancer patients hospitalized with VTE compared to those without VTE.[73] Similarly, a study of the California Cancer Registry (CCR) from 1993 to 1995, determined that VTE diagnosis was a significant predictor of increased mortality the first year at all 12 cancer sites investigated, with hazard ratios (HRs) of 1.6-4.2 after adjustment for race, age and cancer stage.[45] VTE was associated with increased mortality in localized, regional and metastatic-stage cancers, with metastatic cancers, not surprisingly being the strongest predictor of decreased survival. The strong association between metastatic-stage disease and development of VTE suggests that the biological aggressiveness of the cancer itself may be the main cause of death in cancer patients with VTE. However, it is possible that the cause of death could be related to the VTE event itself (i.e. massive PE), VTE treatment (bleeding due to anticoagulants), cancer treatment (i.e. major surgery, chemotherapy or radiation treatment) or presence of comorbid conditions.

Altogether, cancer-related VTE leads to substantial increase in consumption of healthcare resources and healthcare costs. As the incidence is increasing and VTE treatment is resource-intensive and costly, the disease burden of VTE in cancer is not expected to decrease. More effective agents and less costly management strategies are needed. In a retrospective study of 529 cancer patients, VTE and VTE-related complications occupied 6% of the bed-capacity at the oncology department.[74] Further,

the mean hospitalization cost for VTE in cancer patients was more than double the cost per episode of VTE in the general medical population.[74,75]

1.1.2. Venous thromboembolism as a first sign of cancer

As described by Armand Trousseau, VTE can be the first manifestation of an occult cancer. Several studies have shown that VTE-patients have an increased risk of subsequent cancer compared to the general population.[23-28] The reported rates of occult cancer detection in patients with unprovoked VTE seem to have been decreasing significantly over time.[76] A systematic review published in 2008 by Carrier and colleagues based on 34 studies, reported that the prevalence of occult cancer detection following an unprovoked VTE event was up to 10%,[25] and that the risk of occult cancer detection was 7-fold increased in VTE patients compared to the general population.[25] More recent clinical studies have reported that approximately 3.7 to 5.0% of patients with unprovoked VTE are diagnosed with cancer within the first year following a VTE event.[33,77] Similarly, a large prospective study reported a 5% rate of occult cancer detection over a follow-up period of 30 months.[78] A systematic review and individual patient-level meta-analysis from 2017 reported a prevalence of occult cancer detection of 5.2% (95% CI 4.1-6.5%) during 12 months in patients with unprovoked VTE.[31] A large case-control study suggested that even though the risk of occult cancer was strongest within the first year following a VTE event, the risk remained elevated for up to six years for multiple myeloma, colon and pancreatic cancer.[79]

The incidence of occult cancer detection are thought to vary widely according to the presence or absence of provoking factors for VTE (i.e. surgery, immobilization, infection etc.). In a study by Prandoni and colleagues, only 1.9% of patients with provoked VTE develop cancer during follow-up compared to 7.6% in patients with unprovoked events.[80] Similar findings was reported by Carrier and coworkers in a systematic review where the one-year incidence of cancer following a VTE event was 10% (95% CI 8.6-11.3%) and 2.6% (95% CI, 1.6-3.6%), for unprovoked and provoked VTE, respectively. However, using the Scandinavian Thrombosis and Cancer (STAC) cohort, the risk of cancer did not vary as greatly in provoked and unprovoked VTE events.[81] The risk of cancer was 4.5-fold (95% CI 3.4-5.8) increased for unprovoked VTE and 3.5-fold (95% CI 2.4-5.2) increased for provoked events the first 12 months following a VTE event. The difference was even smaller after the initial 12 months, suggesting that VTE may be the first sign of malignancy regardless of the presence of other provoking factors.

The risk of cancer after VTE does not differ according to the origin of the VTE event (i.e. lower limb, upper extremities, abdominal veins etc.). A large Danish registry study presented essentially similar risks of cancer during the first year of follow-up in subjects with superficial VTE (2.2%), DVT

(2.7%) and PE (2.9%).[27] The corresponding standardized incidence ratios (SIRs) were 2.5 (95% CI 2.1-2.9) for superficial VTE, 2.8 (95% CI 2.6-2.9) for DVT and 3.3 (95% CI 3.0-3.5) for PE. Comparable results was seen in the STAC cohort, with the risk of cancer after 12 months yielding HRs of 4.1 (95% CI 3.1-5.4) for DVT and 4.0 (95% CI 2.8-5.6) for PE.[81] Further, a large multicenter, prospective observational study reported an overall incidence of cancer of 1.4% (95% CI 0.9-2.1) for distal DVT and 1.5% (95% CI 0.8-2.4) for proximal DVT.[82]

The cancer sites found following a VTE event constitutes a large and heterogeneous group.[18,23,24,26] In a meta-analysis of four large cohorts, the highest relative risk of cancer after VTE was found for ovarian, pancreatic, liver, hematological, brain, lung and kidney cancer, while the lowest risks was reported for cancers of breast and bladder.[28] Jensvoll and colleagues found the highest HRs following a VTE event in the STAC cohort, for kidney, ovarian, lymphatic, pancreatic, stomach and lung cancer.[81] Subjects diagnosed with cancer subsequent to a venous thrombotic event have a higher prevalence of malignant disease and advanced stages,[20,24,83] and consequently poorer prognosis compared to cancer patients without VTE.[20,83]

Several studies have investigated predictors of cancer in patients with unprovoked VTE. Analysis of a randomized controlled trial of patients with unprovoked VTE, reported that age, previous provoked VTE and current smoking were associated with higher HRs of cancer the following year after a VTE event.[84] Using the RIETE registry (the Registro Informatizado de Pacientes con Enfermedad TromboEmbólica), several biomarkers for cancer were found to be independent predictors such as patients aged 60 to 75 years, unprovoked (i.e. idiopathic) VTE, bilateral deep vein thrombosis and anemia.[83] No significant association was detected for measured platelet count, D-dimer levels, surgery or anticoagulant treatment. However, other studies have shown a correlation between D-dimer levels measured at the time of the VTE event and risk of subsequent cancer,[85-87] suggesting that high plasma D-dimer levels at incident VTE diagnosis should be taken into consideration when the decision to screen for underlying cancer is made.

Sørensen and co-workers were the first to demonstrate that patients with VTE and a following cancer diagnosis have poor prognosis compared to cancer patients without a prior or concurrent VTE event at the time of cancer diagnosis.[20] In this Danish population-based study, the one-year survival rate was found to be 12% in patients with a cancer diagnosed at the same time as the VTE event, compared to 36% in cancer patients with no previous or concurrent VTE event. Further, the one-year survival rate was only 38% for cancer patients diagnosed with VTE within one year after the cancer diagnosis, compared to 47% in the cancer controls who never had a VTE, matched for cancer type, age, sex and year of diagnosis.[20] They also found that VTE was associated with advanced stages of cancer, with higher prevalence of distant metastasis among VTE-patients compared to those without VTE. In

the prospective RIETE registry, VTE patients with occult cancer had a mortality rate of 20% in 80 days, compared to 5.4% in 80 days for VTE patients without occult cancer.[83]

During the last decade, there has been an ongoing debate on to what extent patients with VTE should be further examined for an occult cancer (i.e. limited vs extensive screening). Several studies have evaluated a more extensive cancer screening strategy, which may include CT or fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT scanning.[33,78,88] As the clinical benefit may increase by applying extensive screening only to patients at high risk of occult cancer, the RIETE score and the Screening for Occult Malignancy in Patients with Idiopathic Venous Thromboembolism (SOME) score have been introduced.[84,89] In a recently published individual patient data meta-analysis of prospective studies, Mulder and colleagues evaluated the predictive performance of the RIETE and SOME score.[90] Both scores had a poor predictive discriminatory performance between low- and high-risk patients, which does not support the use of these scores in daily clinical practice. In a Canadian randomized controlled trial, limited screening versus limited screening combined with abdominal and pelvic CT scan, did not differ in average time to cancer diagnosis, nor mortality.[33] A study comparing FDG PET/CT with limited screening found that extensive screening did not result in higher rates of cancer after unprovoked VTE.[88] One could speculate that extensive screening might result in earlier cancer detection, however, it does not seem to increase the rate of occult cancer detection, decrease morbidity, or increase survival or cost-effectiveness.[33,76] Thus, the mortality rates of cancer remains the same by the use of extensive and limited screening.

Currently, the limited screening approach is recommended for patients with unprovoked VTE. Limited screening usually consists of medical history, physical examination, laboratory investigations (complete blood count, calcium, urinalysis, and liver function tests), and chest X-ray, as well as age- and gender specific cancer screening (colon, breast, cervix and prostate) according to national guidelines.[32] The limited screening should also be used for patients with VTE at unusual sites. Further, patients with splanchnic vein thrombosis or cerebral vein thrombosis should be tested for an underlying myeloproliferative disorder. Additionally, for those with splanchnic vein thrombosis and aplasia or hemolytic anemia, and patients with Budd-Chiari syndrome, they suggest testing for paroxysmal nocturnal hemoglobinuria. Routine cancer screening in patients with provoked VTE is not recommended.

Few studies have evaluated the incidence of occult cancer detection in patients with recurrent unprovoked VTE. However, the incidence of occult cancer detection seems to vary according to whether the VTE is an initial or recurrent event. In the cohort study by Prandoni *et al*, it was reported that patients with recurrent VTE had a particular higher risk of cancer (OR 2.3, 95% CI 1.0-5.2) compared to patients with a first lifetime unprovoked VTE.[80] In the Tromsø Study, the majority of those

with an incident VTE during the occult cancer period (54%) who also experienced a recurrent VTE, had a VTE recurrence before the date of cancer diagnosis, and 69% experienced a recurrent event either before or within 5 days of the cancer diagnosis.[91] These findings indicate that the recurrent VTE often occurs prior to cancer detection, while the patients receive anticoagulant treatment. As there is limited knowledge on recurrent VTE and occult cancer detection, there has been some uncertainties regarding cancer screening in these patients. Today, the limited screening is recommended for this patient group, nonetheless, clinicians are recommended to maintain a lower threshold for cancer investigations and extensive screening procedures than for those used for patients with an incident unprovoked VTE.[32]

1.2. Pathophysiology of venous thromboembolism in cancer

Hemostasis is the physiological process that prevents bleeding after a vascular injury while maintaining blood flow of the general circulation, holding vital importance and delicately balancing pro- and antithrombotic mechanisms. Simplified, VTE occurs as a result of changes in blood flow (stasis), changes in blood composition (hypercoagulability), and/or damage of the vessel wall which may overcome the protective anticoagulant pathways and trigger thrombosis. The triad of pathophysiological alterations is referred to as Virchow's triad (Figure 1, Figure 2).[92-94] Figure 2 is an overview of risk factors in the pathogenesis of VTE in the general population.

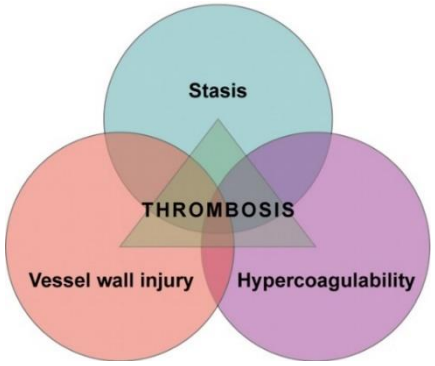

Figure 1. Virchow's triad. Three main factors contributing to thrombus formation.

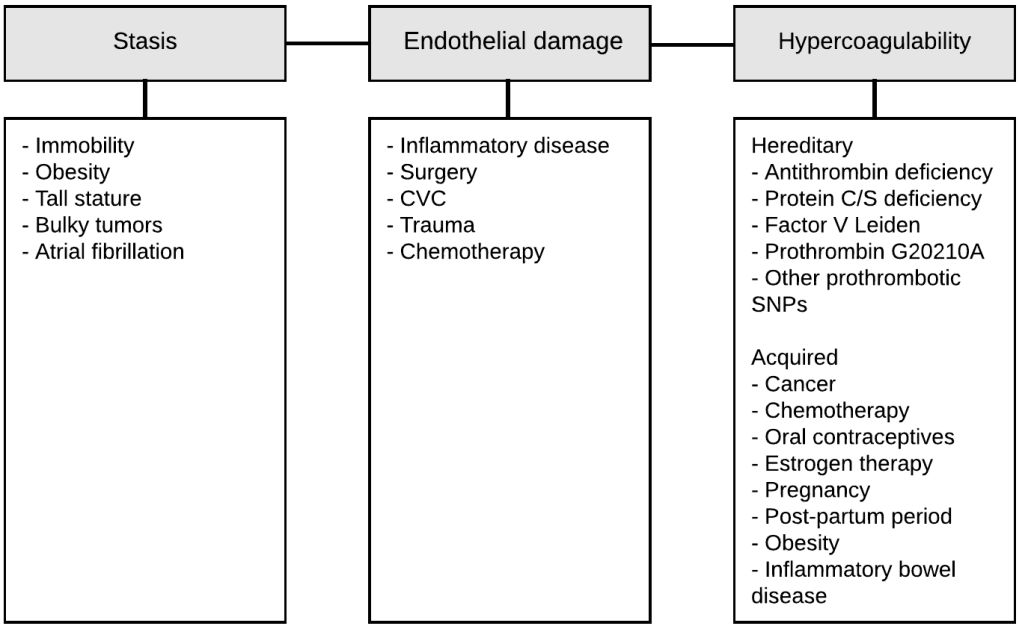

Figure 2. Categorization of some risk factors for venous thromboembolism.

Primary hemostasis is the process of platelet activation and adhesion, and secondary hemostasis refers to the initiation of the coagulation cascade. The coagulation cascade is a sequential process of different pathways (i.e. the extrinsic, intrinsic and common pathway), culminating in fibrin formation, which is the central stabilizing component of a blood clot (Figure 3).[95]

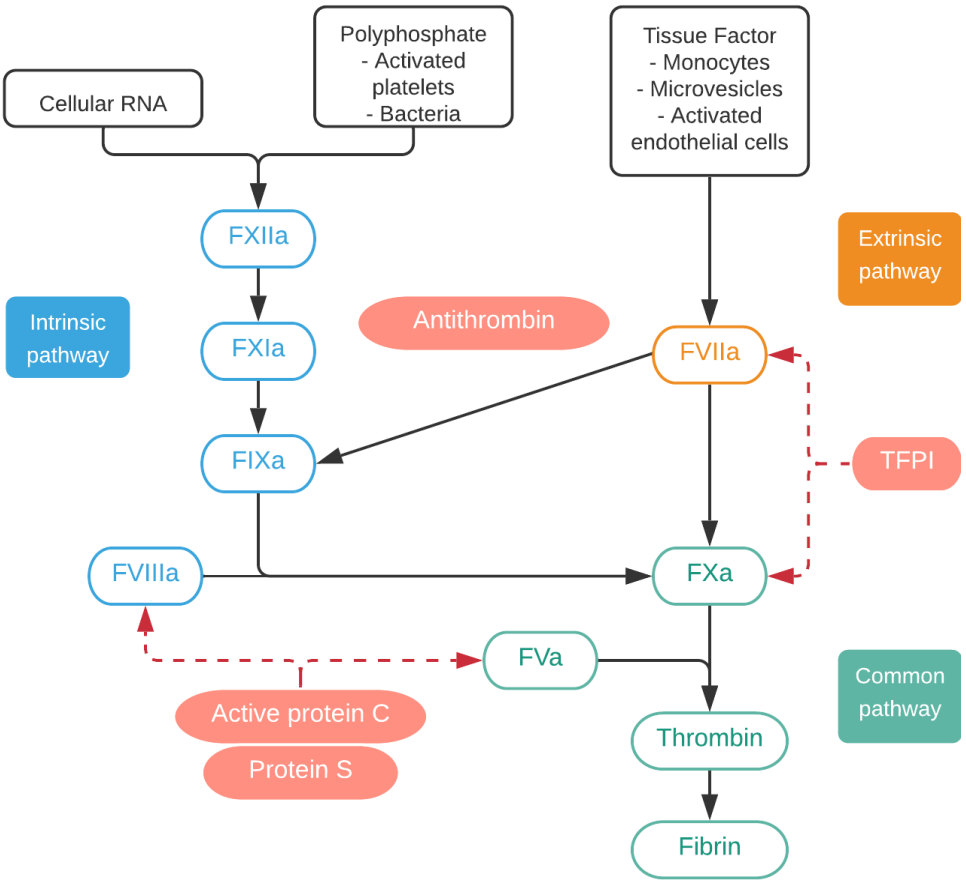

Figure 3. A simplified figure of the coagulation cascade. Pathological activation of the extrinsic pathway (FVIIa and TF) occurs via expression in monocytes, microvesicles, and activated endothelial cells. Cellular RNA and polyphosphates released by activated platelets and bacteria activate the intrinsic pathway (FXIIa, FXIa, FIXa and FVIIIa). (Adapted from Mackman, Journal of Clinical Investigation, 2012)

Tissue factor (TF) is the main trigger for the extrinsic pathway of the coagulation cascade, and has a key role in hemostasis.[95] Under pathological conditions, TF is expressed on circulating monocytes, microvesicles (MVs) and activated endothelial cells.[94] FXII and FXI provide an alternate route of clotting initiation through activation of FIX, in the intrinsic pathway. This pathway may also be triggered by cellular RNA and polyphosphates (PolyP) released from activated platelets or bacteria, resulting in formation of a venous thrombus.[94] The common pathway consists of FXa, FVa and thrombin (FIIa), which converts fibrinogen to fibrin.[94] Tissue factor pathway inhibitor (TFPI) inhibits FXa and the TF/FVIIa complex, antithrombin inhibits all coagulation factors, and activated protein C (APC) inactivates FVa and FVIIa. The cascade is thoroughly regulated, and disorders of the coagulation proteins can lead to excessive bleeding or thrombus formation.

Although endothelial damage with exposure of TF is a main trigger of the coagulation cascade, the majority of VTEs develop in the presence of intact endothelium.[94,96] Autopsy and phlebography studies have shown that most non-trauma-related VTEs originate in sinuses behind venous valves.[97] This is indirectly supported by the increased risk of DVT in subjects with more valves.[98] As blood travels against gravity in veins, some is caught in a secondary vortex of the valve sinuses, leading to hypoxia (Figure 4). Immobility can result in prolonged blood stasis and further potentiate hypoxia in these regions.[97] Localized hypoxia activates endothelial cells, and recruits and activates white blood cells and platelets.[97] Endothelial cells mobilize P-selectin and von Willebrand factor (vWF) on their surface, which recruit leukocytes and platelets expressing TF, which then activates the coagulation cascade. In addition, activated endothelial cells can downregulate endothelial expression of protein C receptor and thrombomodulin, and upregulate TF expression.[99]

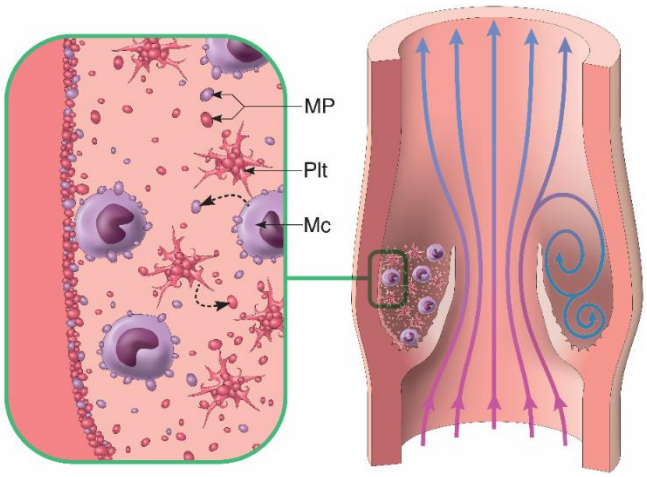

Figure 4. The venous valvular sinus as a predilection site for DVT initiation. Blood is trapped in a vortex of the valve pockets, and the resultant hypoxia activates the venous endothelium, leading to the recruitment and binding of leukocytes, especially monocytes (Mc), platelets (Plt) and TF-positive microparticles (MP). Consequently, TF from activated monocytes and microparticles may activate the coagulation cascade and initiate thrombosis formation.

Changes in blood composition are essential in the pathogenesis of VTE. The term thrombophilia describes the tendency of VTE development on the basis of a hypercoagulable state, which can be a result of both inherited and acquired disorders of blood coagulation or fibrinolysis. Inherited disorders may induce thrombus formation by decreasing levels of anticoagulant factors, such as antithrombin, protein C and protein S, or increase procoagulant factors and the thrombus formation tendency (i.e. gene mutations such as Factor V Leiden (FVL), prothrombin G20210A and non-O blood group).[36,100] Several known risk factors for VTE may cause acquired thrombophilia, such as obesity, pregnancy, oral contraceptives, and importantly, cancer.

Cancer itself represents a ***hypercoagulable state***, and the pathophysiological mechanisms of endothelial damage, hypercoagulability and stasis of Virchow's tirad, are important features of cancer in development of VTE. Coagulation activation and tumor growth and progression are closely related. Cancer cells can activate the coagulation system through several mechanisms, (i) by production of procoagulant, fibrinolytic, and proaggregating activities, (ii) release of proinflammatory and proangiogenetic cytokines, and lastly, (iii) through direct interaction with vascular and blood cells (e.g.

endothelial cells, leukocytes and platelets) by adhesion molecyles.[101] Studies have shown that cancer patients have increased levels of coagulation factors V, VIII, IX, and XI as well as increased markers of coagulation activation such as elevated plasma D-dimer levels.[102] Further, cancer-induced deficiency of the vWF cleavage protein ADAMTS-13 has been described, resulting in unusually large vWF multimeres, which increases thrombosis risk.[103] In normal vascular cells, TF is not expressed, except when induced. Malignant cells however, have an abnormal expression of TF. In a study by Kakkar and colleagues, cancer patients had significantly higher levels of both TF and FVIIa compared to cancer-free subjects, suggesting a strong activation of the extrinsic pathway.[104] Interestingly, the intrinsic pathway is found not to be as important in cancer-related VTE pathophysiology, as levels of FXIIa are only slightly elevated in cancer patients.[105] Cancer cells are also found to induce hypercoagulability through inflammatory responses with increased level of circulating proinflammatory cytokines,[106-108] and inhibition of fibrinolytic activity through expression of plasminogen activator inhibitor-1 (PAI-1).[109-111] Moreover cancer tumors release cell-free DNA and growth factors that promote release of neutrophil extracellular traps (NETs) from neutrophils, which are suggested to play a key role in inflammatory-mediated thrombosis.[112]

Activated platelets play an important role in the hypercoagulable state of malignancies, where they promote angiogenesis, tumor progression and metastasis.[113,114] Previous studies have shown that elevated platelet counts in cancer patients are frequently observed and associated with decreased survival.[113,115] Platelet activation by thrombin leads to release of vascular endothelial growth factor (VEGF) and other growth factors, thereby stimulating angiogenesis and inhibiting apoptosis.[17,116] Cancer cells express P-selectin ligands and adhere to various cells including platelets, in addition to enhance P-selectin expression in these cells. This results in enhanced adhesion to the endothelium and a “cloak” of platelets surrounding the tumor cells, protecting them from circulatory immune cells or natural killing cells.[17,116]

Venous stasis may be a result of tall stature, obesity, pregnancy, conditions preventing normal function of the skeletal muscles and normal blood flow or atrial fibrillation. It could also occur in patients with malignancy as a result of tumor expansion leading to compression of blood vessels nearby. Furthermore stasis is also seen as a consequence of immobilization secondary to surgery, cancer treatment, complications, and advanced cancer stage.[117]

Vascular trauma and ***endothelial damage*** may be a result of tumor invasion by solid tumors. Consequently, the endothelium is activated and may increase VTE risk. Chemotherapy, radiotherapy and surgery are all cancer treatment modalities that could result in vessel wall injury and through the same pathway as solid tumor invasion, induce coagulation.[118] The vessel wall may also be damaged by insertion of CVCs, or cancer treatment (i.e. surgery, chemotherapy and radiation).[119,120]

1.3. Venous thromboembolism – a multicausal disease

VTE is a multicausal disease. The thrombosis potential model proposed by Frits Rosendaal (Figure 5), illustrates the key concepts of VTE where several factors must be present for a VTE event to occur.[121] A risk factor can be any attribute, characteristic, or exposure of an individual that increases the likelihood of disease development. The model by Rosendaal illustrates the complex interplay between genetic and acquired risk factors, and how VTE occurs once a set of sufficient risk factors have accumulated in a patient and the thrombosis threshold is exceeded. Further, the model demonstrates the dynamics of interactions between risk factors and how risk factors can result in either additive or synergistic effects. As the red line in Figure 5 represent, VTE has a strong age-dependency, indicating that more risk factors have to be present for VTE to occur in children than in adults or elderly.[121] The green line represents intrinsic factors that are stable over time such as the genetic variation FVL. The orange line demonstrates the combined effect of FVL, age and other provoking factor (e.g. surgery, cancer or immobilization), with sufficient pathophysiological changes to exceed the thrombosis threshold. The thrombosis potential remains increased following a VTE event, and a provoking factor may exceed the threshold again, resulting in a recurrent event. After the recurrent VTE, the risk of another recurrent VTE event is even higher than following the incident VTE.

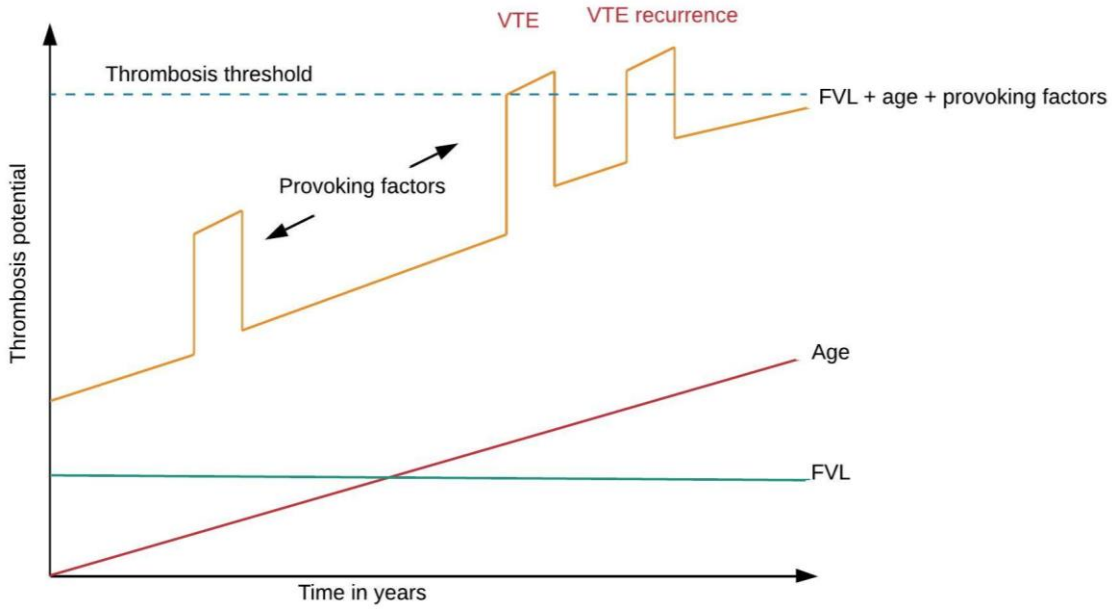

Figure 5. The thrombosis potential model. (Adapted from Rosendaal, Lancet, 1999)

A fundamental challenge in the management of patients suffering a VTE event and prevention of VTE occurrence, is that no obvious preceding cause or risk factor is identified in 30-50% of VTE cases (i.e. an unprovoked event). This underlines the disease complexity and that further research is necessary to unravel the causes of VTE and to improve strategies for VTE prevention, both in the general population and in cancer patients.

Risk factors for venous thromboembolism can be categorized into ***acquired*** and ***genetic*** factors. Several ***acquired risk factors*** for VTE have been established for the general population. The risk of VTE increases exponentially with age,[2,3,122,123] and thus, VTE is mainly a disease of the elderly.[4,122] The incidence of VTE is 0.6 per 100 000 per year in children, whereas 1 per 100 in elderly.[124,125] Numerous medical illnesses and autoimmune diseases are known acquired risk factors for VTE, such as congestive heart failure,[126-129] acute infections,[127-130] myocardial infarction,[128,129,131] ischemic stroke,[126-129] inflammatory bowel disease,[128,132-135] systemic lupus erythematosus,[133,134] and chronic kidney disease.[136-138] Hospitalization for an acute medical condition is associated with an 8-fold increased VTE risk.[37] Further, anthropometric measures including obesity (i.e. body mass index (BMI) ≥30 kg/m^2) and tall stature, are found to have a 2- to 3-fold and 2- to 4-fold increased VTE risk, respectively.[139-144] Other important and well-known risk factors include surgery,[40,128,145] previous VTE,[68,71] immobilization,[121,126,146] trauma,[40] pregnancy and puerperium,[147,148] oral contraceptives,[128,145] hormone replacement therapy,[128,149,150] and lastly, use of CVCs.[145,151] Cancer is one of the strongest acquired risk factors for VTE and will later be described in detail by the stratification of different features of the disease.

VTE is to a large extent a genetic disease. It is estimated that ***genetic risk factors*** account for as much as 50 to 60% of VTE risk.[152-155] Inherited thrombophilia can be the result of either gain-of-function mutations of procoagulant factors or loss-of-function mutations of anticoagulants, with the loss-of-function mutations being less frequent, however, tend to be associated with higher risk estimates for VTE.[156] The first discovery of inherited thrombophilia was antithrombin deficiency in a family from Skjervøy, Norway, in 1965, where a 12 year old boy and his mother were found to have significantly lower activity in antithrombin and heparin co-factor.[157] The following decades, genetic variations of ABO-blood group were found to affect VTE risk,[158] and deficiencies of protein C and its co-factor protein S were discovered.[159,160] Non-O blood type is present in approximately 65% of the population and is associated with a 1.5- to 2.0-fold higher risk of VTE,[161,162] partly mediated by higher levels of vWF and FVIII.[163] Heterozygous protein C and protein S deficiencies occur in less than 1% of the general population, but when present, the VTE risk is 10-fold increased.[163] Since these discoveries, a number of relatively common prothrombotic genetic variations and single nucleotide polymorphisms (SNPs) have been elucidated (Table 1). Factor V Leiden (FVL), also known as rs6025, is a missense mutation (arginine to glutamine) discovered in 1994 that results in APC-resistance with abnormal decomposition of FVIII.[156,164] Heterozygous carriers of FVL have a 2- to 5-fold increased VTE risk, and the risk is further dramatically increased in homozygous carriers.[165] The prothrombin mutation, G2021A or rs1799963, was identified only two years after FVL and is a gain-of-function mutation,[166] associated with higher levels of prothrombin (FII) and thus, a 1.5- to 3.0-fold higher VTE risk.[161,167]

About ten years then passed before the discovery of a novel genetic risk factor for VTE.[36] In 2005, the rs2066865 of the fibrinogen gamma gene (FGG) was discovered and found to be associated with reduced levels of one of the three subunits of fibrinogen.[168] The T-allele of the FGG SNP has a frequency of 0.25, and has been found to increase VTE risk by an OR of 1.61.[168,169]

Genome-wide association studies (GWAS) became available in the 2000s, resulting in major advances in understanding the role of genetic variations on VTE risk. GWAS approaches allowed identification of genotypes associated with VTE (Table 1). In 2019, GWAS and transcriptome-wide association studies (TWAS) of larger study populations revealed several novel susceptibility loci for VTE, whit some loci located outside the known coagulation pathways.[170] However, the majority of VTE-associated SNPs encode proteins that are involved in the coagulation cascade, altering the function and/or levels of proteins.[163,171] Bezemer and coworkers were the first to conduct a large-scale association genetic study on VTE.[172] Although the novel SNPs identified from GWAS display weaker associations with VTE, with OR ranging from 1.11 to 1.35, the SNPs may be of clinical significance if they interact with other risk factors (e.g. cancer) for VTE, giving supra-additive risk estimates. Supra-additive risk estimates are seen when two exposures have a synergistic effect (i.e. biological interaction) on an outcome, resulting in a joint effect greater than the expected sum of the individual exposures.[173] Biological interaction can be approached in several ways, e.g. by calculating the relative excess risk due to interaction (RERI) or the proportion attributable to interaction (AP) or assessing the synergy index (SI). Biological interaction will be further discussed in later sections of the thesis.

Table 1. Known prothrombotic genotypes associated with VTE.[36,171]

Gene	Site	Associated phenotype	Frequency	VTE OR
Genes associated with VTE identified before GWAS				
F2	rs1799963	↑ F11	0.02	2.50
F5	rs6025	APC resistancy	0.05	3.00
FGG	rs2066865	↓ Fibrinogen γγ'	0.25	1.47
ABO	rs8176719	↑ VWF, ↑ VIII	0.3	1.50
PROCR	rs867186	↑ sEPCR, ↑ PC	0.07	1.22
PROS1	Multiple	VTE	Rare	~ 10
SERPINC1	Multiple	VTE	Rare	~ 10
Novel SNPs associated with VTE identified by GWAS				
VWF	rs1063856	↑ vWF	0.37	1.15
STXBP5	rs1039084	↑ vWF	0.46	1.11
GP6	rs1613662	↑ platelet function	0.82	1.15
F11	rs2289252	↑ FXI	0.41	1.35
F11	rs2036914	↑ FXI	0.52	1.35
C4BPB/C4BPA	rs3813948	↑ C4BP	0.08	1.18
KNG1	rs710446	↓ aPTT	0.45	1.2
SERPINC1	rs2227589	↓antithrombin	0.10	1.29
TSPAN15	rs78707713	Unknown	0.88	1.28

Several of the SNPs discovered through the GWAS approach got our attention. Glycoprotein VI (GP6) rs1613662, is an A/G single nucleotide variation in amino acid 219, resulting in a serine to proline substitution, affecting the GPVI receptor for collagen.[174] Carriers of the G-allele of the GP6 SNP have been found to express fewer GPVI receptors on platelets,[175] causing less platelet adhesion and platelet activation.[176] Observational studies have over the last two decades demonstrated a 20% decreased risk of VTE in carriers of the GP6 G-allele, and inversely, that A-allele carriers have a 15% higher risk of VTE than those not carrying the A-allele.[177,178] The allele frequency of the A-allele at GP6 rs1613662 is found to be 0.82.[36] Another SNP identified in recent years, is rs2036914 of F11 encoding for coagulation factor XI. The presence of the C-allele at the F11 SNP is found to correlate to higher plasma levels of F11 and to increase VTE risk by an OR of around 1.35.[36,172,179,180]

Furthermore, the combination of prothrombotic genotypes may improve risk prediction models for VTE.[171,181] Emerging studies have attempted to create genetic risk scores (GRS) based on several VTE-associated SNPs to improve prediction of VTE. Using a large case-control study of cancer-free subjects, de Haan and colleagues created a GRS based on 31 VTE associated SNPs.[181] SNPs with the highest odds ratios of VTE were added one by one to construct a more parsimonious GRS with fewer SNPs. This resulted in a score of five VTE-associated SNPs (ABO rs8176719, F5 rs6025, F2 rs1799963, FGG rs2066865, and F11 rs2036914), performing just as good as the score of all 31 SNPs for VTE risk assessment (Figure 6). The 5-SNP score has been evaluated in several studies, in both incident and recurrent VTE, with a predictive capacity ranging from 0.59 to 0.69.[181-183] Nonetheless, the authors concluded that in order for the GRS' to become useful in a clinical setting, high-risk subjects need to be identified in whom genetic profiling will be cost effective.[181]

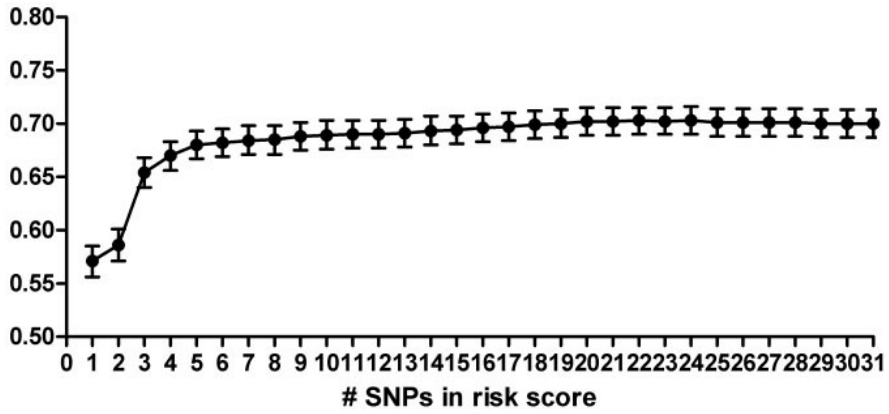

Figure 6. Area under the ROC of genetic risk scores based on increasing numbers of SNPs. SNPs were added in order of the OR as found in the literature. The figure presents how a GRS of the five SNPs with the highest OR for VTE perform similarly to a GRS of VTE associated 31 SNPs. (Adapted from de Haan, Blood, 2012).

1.4. Risk factors for venous thromboembolism in cancer

Risk factors for VTE in cancer can be grouped into *cancer-*, *treatment-* and *patient-related* risk factors (Figure 7). Further, several biomarkers for VTE risk are detected in cancer patients. A better understanding of clinical risk factors and biomarkers in this patient group, could improve prediction of VTE risk in individual cancer patients, and thus, identify high-risk patients that would benefit from thrombosis-prophylaxis.

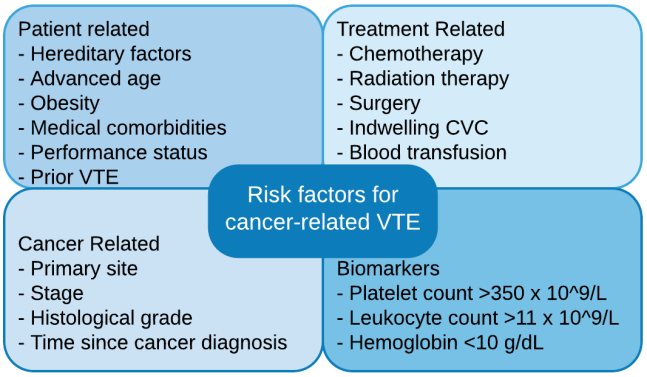

Figure 7. Categorization of risk factors for cancer-related VTE. (Adapted from Gran, Thrombosis Research, 2018)

1.4.1. Cancer-related risk factors for venous thromboembolism in cancer

Extensive work has been published on cancer ***type*** and subsequent risk of VTE.[15] Even though the VTE incidence vary between studies, and studies might be difficult to compare due to differences in study population, design and duration of follow-up, they have consistently reported pancreatic, brain, lung and ovarian cancers to induce the highest VTE risks.[35,44,48,184] Further, the literature has also reported a moderate increase in VTE risk for myeloma, lymphomas, gastrointestinal, and kidney cancer, and a rather low VTE risk is induced by breast and prostate cancers.[44,45,50] However, it needs to be addressed that although breast, prostate and colorectal cancers are associated with lower risks of VTE, they still contribute to the overall disease burden of VTE due to the high prevalence of these cancer types. In the STAC cohort, the incidence rates of VTE per 1000 person-years were 7.5 (95% CI 3.7-14.9), 9.0 (95% CI 4.8-16.7) and 33.2 (23.4-47.3) 0-6 months following a cancer diagnosis date for breast, prostate and colorectal cancer, respectively.[185]

Cancer ***stage*** highly correlates with VTE risk, and metastatic disease is one of the strongest risk factors for VTE in cancer.[43-45,48] In a large Danish follow-up study of more than 55000 cancer patients, VTE risk was strongly dependent on cancer stage, with an adjusted RR of 2.9 for subjects with stage I and II, and RRs of 7.5 and 17.1 for those with stage III and IV, respectively.[44] Similar trends were seen for 12 different cancer types in the CCR, with increased RR for VTE in metastatic cancer patients compared to those with localized disease.[45] Additionally, the Multiple Environmental and Genetic Assessment (MEGA) Study found that patients with metastatic cancer had a 20-fold (95% CI 3-149) increased VTE risk compared to patients with localized cancer.[43] It appears that fast growing cancers, i.e. cancer that are biologically aggressive, correlates with high VTE risk. This is evidenced by short survival time, and in the CCR study of 13000 women with ovarian cancer, 15% of those with metastatic

disease died within three months after the cancer diagnosis date and an additional 15% died in the four to 12 months following the cancer diagnosis.[186] Furthermore, slow-growing cancers such as breast and prostate cancer, have significantly lower thromboembolic tendencies.[45,187]

The ***histological subtype*** and ***tumor grade*** of cancers are also found to influence VTE risk.[186,188,189] In the CCR, different subtypes of lung cancer affect the risk of VTE to different degrees, with the risk of VTE being 2-fold higher for subjects with adenocarcinomas compared to squamous cell lung cancer, with incidence rates of 9.9 and 4.4 per 100 person-years, respectively.[188] Not surprisingly, high grade tumors induce higher risk of VTE than low grade tumors. In an observational cohort study of 747 cancer patients, the Vienna Cancer and Thrombosis Study (CATS), the risk of VTE was 2-fold higher for high-grade tumors (grade 3 and 4) compared to low-grade tumors (grade 1 and 2), after adjustment for sex, age, cancer type, cancer stage and histological type.[190]

The incidence of VTE is clearly highest the first few months following a cancer diagnosis, and ***time since cancer diagnosis*** is therefore established as a risk factor for VTE. Using the MEGA Study, Blom and coworkers found that the risk of VTE was highest the first three months after a cancer diagnosis (OR 53.3, 95% CI 8.6-334.3).[43] In the same study, the risk decreased to 14-fold (OR 14.3, 95% CI 5.8-35.2) in the four to 12 months following the cancer diagnosis date, and a 2.4-fold increased risk was found even up to 10 years after cancer diagnosis. In a recent report from the STAC cohort, VTE risk was apparently highest in the 6 months after cancer diagnosis, but when mortality was taken into account, the risk estimates were substantially lowered and became equally high in the period 6 months before and 6 months after cancer diagnosis.[185] This could indicate that the cancer itself, rather than treatment-related factors (e.g. surgery, chemotherapy, and radiotherapy), or complications of cancer (e.g. immobilization and infections) cause the increased VTE risk.

1.4.2. Treatment-related risk factors for venous thromboembolism in cancer

VTE is a frequent complication of ***surgery*** in both cancer and cancer-free subjects. The 30-day cumulative risk of VTE after cancer surgery is found to be 1.6%.[191] White and colleagues found that cancer patients undergoing major surgery had a 2- to 4-fold higher risk of VTE compared to cancer-free patients after major surgery.[192] In a cohort study of surgical patients with cancer and benign neoplasms, higher rates of VTE were found in cancer patients, despite the similar treatments. Further, in a study by Gould and colleagues, the risk of cancer-related VTE was particularly high after surgery of the abdomen and pelvis.[193] Contrary, another study found no association between surgery and VTE in cancer patients,[194] and some studies have even found a protective effect of major surgery on VTE risk in patients with breast, colon and ovarian cancer.[186,195,196] The proposed protective effect of

surgery might be explained by the selection of patients most eligible for surgery, such as cancer patients with better performance status and less advanced cancers. Further, surgical removal of tumors may increase survival, improve the disease burden and thus, reduce VTE risk in these patients.

Chemotherapy is associated with a 2- to 6-fold increased risk of VTE compared to the general population, and several studies have convincingly demonstrated the association between chemotherapy and VTE.[40,194] In a case-control study of Olmsted County, the risk of VTE was 4.1 (OR 4.1, 95% CI 1.9-8.5) for cancer patients not receiving chemotherapy, while the risk increased to 6.5-fold (OR 6.5, 95% CI 2.1-20.2) when chemotherapy was used.[40] In a recent systematic review and meta-analysis, the pooled incidence of VTE was 7% during neoadjuvant chemotherapy.[197] The highest VTE rates were found for patients with bladder and esophageal cancer. The incidence of VTE in cancer patients receiving chemotherapy is increasing, from 3.9% per hospital admission in 1995, to 5.7% per hospitalization in 2003.[50] Further, some chemotherapy agents are associated with higher rates of VTE.[198] Immunomodulatory drugs (e.g. thalidomide and lenalidomide) increase VTE risk, especially when combined with dexamethasone or chemotherapy in treatment of multiple myeloma.[199]

Conversely to chemotherapy, data on the epidemiology and clinical features of VTE during ***radiotherapy*** are scarce. Radiotherapy may be initiated in early stages of cancer, when the risk of VTE is high, or as a part of radical cancer treatments of localized tumor, in addition to or without chemotherapy. Further, radiotherapy and chemotherapy are also used in palliative settings for pain relief, to reduce tumor compression of surrounding tissue etc. In a prospective study using the CATS data, 47% of cancer patients were treated with radiotherapy.[200] In this study, radiotherapy yielded a 2.3-fold (95% CI 1.2-4.4) increased VTE risk. Using the RIETE database, Guy and coworkers were the first to use a large cohort study to investigate the association between radiotherapy and VTE.[201] They found that 13% of cancer patients who had experienced a VTE event, received radiation therapy. Cancer patients with VTE receiving radiotherapy in this cohort had a higher rate of cerebral bleeding, PE recurrence and DVT recurrence during the course of anticoagulant treatment, compared with cancer patients who had suffered a VTE but did not receive radiotherapy.

Central venous catheters (CVC) are vital components of cancer therapy in several cancer types and those undergoing hemodialysis, parenteral feeding, and in administration of blood products, hydration and different drugs. CVCs are associated with higher rates of DVT in the upper extremities, and the incidence of CVC-related VTE in cancer patients varies from 0.3% to 28.3% in different populations.[202] In a study on 400 cancer patients with a newly implanted port that were followed for a median of 12 months not receiving thromboprophylaxis, 8.5% (95% CI, 6.0 -11.7) had symptomatic VTE.[203] Other treatment-related factors such as ***blood transfusion*** and erythropoiesis-stimulating agents are also found to increase VTE risk in cancer.[204,205]

1.4.3. Patient-related risk factors for venous thromboembolism in cancer

Acquired risk factors for venous thromboembolism in cancer

Several of the acquired risk factors for VTE in the general population apply to cancer patients. However, while the effect of ***increasing age*** on VTE risk in the general population is clear, the impact of high age in cancer patients is inconclusive. In a Danish population-based cohort of 57791 cancer patients, the crude incidence rates of VTE increased with increasing age, but the adjusted relative risk showed the opposite tendency with the VTE risk declining by increasing age.[44] In an Italian prospective observational study of cancer patients undergoing surgery, patients above the age of 60 had a 2.6-fold higher risk of VTE compared to cancer patients under the age of 60 years.[206] Most studies have however, found no association between age and VTE risk in cancer. In a large registry-based study of 40787000 hospitalized cancer patients, the incidence rates were essentially similar in cancer patients aged 40-59 years and 60-79 years.[51] In a study by Chew and colleagues based on the CCR, no association was found between advancing age and overall cancer-related VTE. However, the effect of high age differed among cancer sites, displaying a modest effect on VTE for patients suffering from non-Hodgkin's lymphoma, breast- and ovarian cancer.[45] Finally, age is not included in the Khorana risk score for assessment of VTE risk in ambulatory cancer patients, as they have not found an association between age and cancer-related VTE.[207]

A history of ***previous VTE*** is a risk factor for recurrent VTE in the general population.[68,71] The risk of VTE due to previous VTE is even stronger in cancer patients, and the recurrence rate is significantly higher compared to subjects without cancer.[208] In the Tromsø study, the cumulative incidence of VTE recurrence in cancer patients was 2.7% (95% CI 1.0-7.0) at 30 days, 8.2% (95% CI 4.3-15.7) at 6 months, 16.3% (95% CI 9.9-25.9) at 12 months and 22.0% (95% CI 16.2-41.0) at two years following an incident VTE event. In a study by Prandoni and colleagues, the 12-month cumulative incidence of VTE recurrence was 20.7% in cancer patients on anticoagulants, while as low as 6.8% in cancer-free subjects receiving anticoagulant treatment.[66] Active cancer is associated with a 2- to 9-fold higher risk of VTE recurrence compared to cancer patients without VTE,[1,66,209] and similar to cancer-free subjects, the risk of recurrence in cancer is higher after a DVT event compared to PE events.[210]

The presence of ***medical comorbid conditions*** may increase VTE risk in cancer, especially when several comorbidities are present at the same time. In a study of hospitalized cancer patients, Khorana and coworkers found an increased VTE risk by the presence of renal disease (OR 1.5), pulmonary disease (OR 1.4), arterial thromboembolism (OR 1.5) and anemia (OR 1.4).[50] Further, acute infection has been proposed as an important risk factor for cancer-related VTE (OR 1.8),[50] with a particularly increased risk in neutropenic patients.[51] Several studies have demonstrated that increasing number of

comorbidities enhance VTE risk in cancer.[186,188,195,196,211] Using the CCR study of 108255 patients with breast cancer, three or more medical comorbidities present were found to be a predictor for VTE (HR 2.9, 95% CI 2.4-3.5) when compared to patients with no comobidities.[195]

Obesity, defined as a BMI ≥35 kg/m^2, is one of the variables in the Khorana risk model for cancer-related VTE, displaying an OR of 2.5 (95% CI 1.3-4.7).[207] Overweight and obesity are major risk factors for a number of chronic diseases, and are considered a rising problem in both high- and low-income countries. A meta-analysis reported a 2.3-fold increased risk of VTE in obese patients in the general population.[212] However, the association between high BMI and VTE in cancer is less established. Contradictory to the findings of Khorana *et al.*, BMI ≥35 kg/m^2 was not associated with cancer-related VTE in European populations included in the Vienna Cancer and Thrombosis Study (CATS).[213] Further, obesity was associated with increased VTE risk in some studies on ovarian cancer,[214,215] while no association was seen in other studies on ovarian and prostate cancer.[216,217]

Immobilization is common in cancer patients as immobilization is related to surgery, cancer treatment with chemotherapy or radiation, and end-stages of cancer. Although it is a strong predictor for VTE in the general population, immobilization has been scarcely investigated in cancer. Immobilization was present in 23% of cancer patients in the Tromsø Study and were found to be the most frequent provoking factor for both cancer-related VTE and VTE in cancer-free subjects.[218] In a prospective observational study by Agnelli and coworkers, immobilization after surgery, defined as bedrest more than three days, displayed a 4.5-fold (95% CI 2.5-7.8) increased VTE risk in cancer patients.[206]

Biomarkers for venous thromboembolism in cancer

The Food and Drug Administration (FDA) defines a biological marker, shortened biomarker, as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or response to an exposure or intervention, including therapeutic interventions".[219] Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers.[219] A biomarker is not an assessment of how an individual feels, functions or survives.[219] A number of subtypes of biomarkers have been identified according to their presumed applications. One single biomarker may meet multiple criteria for different uses, and some definitions may overlap. Categories of biomarkers include biomarkers of susceptibility/risk, diagnostic biomarkers, monitoring biomarkers, prognostic biomarkers, predictive biomarkers, response biomarkers, and safety biomarkers.[219] Further, a biomarker that indicates the potential for developing a disease or medical condition in an individual who does not currently have clinically apparent disease or the medical condition is classified

as a susceptibility/risk biomarker.[219] These biomarkers are fundamental for the conduct of epidemiological studies about risk of disease.[220] The most ideal biomarkers have high sensitivity and specificity for the outcome of interest, is safe to measure and does not vary across comparable groups (e.g. sex, ethnicity). In recent years, several biomarkers have been identified for VTE in cancer, of which several different biomarkers have been included in prediction models for cancer-related VTE.

Studies have consistently demonstrated no association between white blood cell count[221,222] and platelet count[139,223,224] with the risk of VTE in the general population. However, both high levels of white blood cells and platelets have been found to be associated with VTE risk in ambulatory cancer patients.[207] The Awareness of Neutropenia in Chemotherapy (ANC) Study group registry includes roughly 4000 cancer patients which are followed from prior to chemotherapy initiation, to a maximum of four rounds of chemotheraphy.[207] In this cohort, ***leukocytosis*** (>11 x 10^9/L), ***thrombocytosis*** (≥350 x 10^9/L) and ***low hemoglobin*** levels (<10 g/dL) were identified as biomarkers for chemotherapy-associated VTE, with a 2.2-fold, 1.8-fold and 2.4-fold increased risk of VTE, respectively. Similar findings for leukocytosis were found for cancer patients in the RIETE registry, with a 60% increased risk of recurrent VTE in cancer patients with leukocytosis at the VTE event.[225] Further, pre-cancer diagnosis platelet count and leukocyte values were associated with incident cancer-related VTE in the Tromsø Study, whereas no association was found for cancer free subjects. In this study, leukocytosis (defined as leukocytes ≥8.6 x 10^9/L) was associated with a 2.4-fold increased VTE risk,[221] and thrombocytosis (defined as platelet count ≥295x 10^9/L) with a 2-fold increased VTE risk.[226] Moreover, in the Tromsø Study, the combination of elevated platelet counts and leukocyte counts displayed a synergistic effect with an age- and sex-adjusted three-fold (HR 3.0, 95% CI 1.8-5.0) increased risk of VTE in cancer, compared to cancer patients with low values of both platelets and leukocytes.[226] These findings suggest that platelets and leukocytes play a role in the pathogenesis of VTE in the presence of a malignant environment.[226] Both activated platelets and platelet-derived microparticles promote a procoagulant membrane surface for thrombin activation,[227] which again enhances platelet-tumor interaction and further tumor progression.[228] Thus, elevated platelet counts may result in a larger membrane surface facilitating an interaction between tumor cells and coagulation factors, and thereby promoting coagulation activation.

Markers of hemostasis activation, especially ***D-dimer***, have been found to be elevated in cancer patients.[229-232] D-dimer is a product of degradation of cross-linked fibrin, and positive measures in blood indicate activation of both coagulation and fibrinolysis. Elevated levels of D-dimer are found to be associated with increased risk of VTE in the general population,[233-235] and today, D-dimer is used in the clinical algorithm for VTE diagnostics.[236] The sensitivity and negative predicting value are high, i.e. it is unlikely that a VTE event is present if the D-dimer levels are below cutoff.[237,238] The specificity

however, is low, as other conditions also can result in elevated D-dimer, such as infections, trauma, cancer, and inflammation. In a cohort study of 108 lung cancer patients, Ferroni and colleagues found that the risk of VTE was 11-fold increased for patients with lung cancer and elevated D-dimer (>1.5 µg/ml), compared to lung cancer patients with D-dimer levels below 1.5 µg/ml.[232] In the prospective CATS cohort of 821 cancer patients, elevated D-dimer levels (>75th percentile) were associated with a 2.4-fold (95% CI 1.4-4.0) increased risk of VTE in cancer.[239]

P-selectin is a marker of activation of platelets and endothelial cells.[240,241] It is an adhesion molecule stored in α-granules of platelets and Weibel-Palade bodies of endothelial cells that is expressed on the cell surface when these cells are activated,[240,241] and mediates adhesion and migration of leukocytes, platelets and cancer cells in inflammation, thrombosis and cancer growth and metastasis.[242] Again, in the CATS Study, plasma P-selection levels above the 75th percentile was found to be an independent risk factor for VTE with a HR of 2.6 (95% CI 1.4-4.9) after adjustment for age, sex, surgery, radiotherapy and chemotherapy.[243]

Finally, other biomarkers suggested for VTE in cancer patients are tissue factor-positive microparticles (TF+ MPs), factor VIII and C-reactive protein.[244-247] Additionally, elevated levels (≥358 pmol/L) of ***prothrombin fragments 1+2*** (F1+2), i.e. products of prothrombin cleavage, have been found to be associated with a 2.0-fold (95% CI 1.2-3.6) increased risk of VTE in cancer, compared to cancer patients with F1+2 levels below 358 pmol/L.[200] In the same study, a combined effect was observed for the presence of both elevated prothrombin fragments and D-dimer levels, indicating an even higher risk of VTE when two biomarkers of a prothrombotic state are present.

Today, a few risk assessment scores that aim to stratify cancer patients by their VTE risk exist. However, none of these risk models are recommended in current guidelines. Risk reduction for VTE is mainly seen in high-risk patients compared to low-risk patients. Furthermore, the risk of bleeding on anticoagulant treatment is high and the benefit-to-harm ratio should be taken into consideration. Thus, the decision to provide anticoagulation for prevention of VTE in cancer should ideally be provided by a valid risk stratification strategy based on the presence of risk factors and biomarkers for cancer-related VTE.[248] With a concrete and individual approach, only high-risk cancer patients of VTE would receive thromboprophylaxis, avoiding the burden and risks of anticoagulants in low-risk cancer patients. The Khorana Risk Score is the most recognized risk prediction score for VTE in cancer.[207] The Khorana risk model was derived and validated in an independent cohort of 1365 patients receiving chemotherapy in the observational Awareness of Neutropenia in Chemotherapy (ANC) Study Group Registry.[207] This score includes different clinical and laboratory parameters, with points given for specific cancer sites (i.e. stomach, pancreatic, lung, lymphoma, gynecological, bladder and testicular cancer), elevated platelet and leukocyte count, low hemoglobin or use of erythropoietin-stimulating

agents (ESAs), and high BMI. The Khorana prediction score has been externally validated in the Vienna CATS cohort and several other studies.[249-251] However, the reproducibility in some cancer patients and populations has been questioned, and several variations of the prediction model has been proposed to improve the Khorana risk score. Ay and coworkers found an improvement of accuracy to identify high-risk patients when D-dimer levels and P-selectin were added to the Khorana risk prediction model (the extended Vienna CATS Score).[252] However, P-selectin is not measured routinely in cancer patients, which limits its clinical use. Verso and colleagues included type of chemotherapy to the Khorana risk assessment score (the PROTECHT score), resulting in an improved ability to identify patients at high VTE risk.[250] Further, Pelzer and coworkers replaced BMI with WHO performance status in the Khorona score (the CONKO score) for patients with pancreatic cancer.[251] Comparison of risk prediction scores has been made, and in a cohort study of 876 cancer patients the Vienna CATS and PROTECHT scores appeared to discriminate better between low- and high-risk patients of VTE than the CONKO score and the Khorana score.[253] Even though today's biomarkers and prediction models seem to be able to differentiate between cancer patients at high and low risk of VTE, the risk assessment models are not adequate to be used in clinical practice. There is still a need for research in this field.

Genetic risk factors for venous thromboembolism in cancer

The impact of genetic risk factors on cancer-related VTE is not well-established, as cancer patients often are excluded from studies investigating the association between genotypes and VTE. As discussed in the thrombosis potential model, inherited risk factors alone may only mildly increase VTE risk, but in the presence of other provoking factors such as cancer, genetic variants may push the patient's thrombosis potential above threshold and thus, result in thrombus formation (Figure 5). Prothrombotic genotypes are potential biomarkers for risk stratification of VTE in cancer, being of particular interest as they are fixed, only need to be measures once, and are not influenced by malignant disease, interventions or complications of cancer.

Factor V plays an important role in the coagulation cascade, functioning as a cofactor for conversion of prothrombin to thrombin and promoting activation and degradation of factor VIII.[254,255] Factor V Leiden (FVL) is a gain-of-function missense mutation that results in APC resistance by debilitated down-regulation of activated factor V by APC, and in an abnormal degradation of factor VIII by APC.[256-258] A total of 3-7% of the European population are carriers of FVL, and the genetic variation is very rare in Asian and African populations.[165] Several studies have reported no association between ***FVL*** and VTE risk in different cancers such as gynecological,[259] breast,[260,261] pediatric,[262] gastrointestinal[260,263] and unselected cancers.[264,265] The lack of associations in these studies may be

explained by small population sizes (67-281 subjects) and the prevalence of the genetic variation. However, later population-studies with larger study populations have found an increased VTE risk by the presence of FVL. In a large case-control study using data from the Multiple Environmental and Genetic Assessment (MEGA) Study, the VTE risk was 3-fold increased in subjects with FVL, 5-fold increased in subjects with cancer, and notably, 12-fold (OR 12.1, 95% CI 1.6-88.1) increased in subjects with the presence of both FVL and cancer.[43] Similar results were found in an Austrian cohort of nearly 1000 cancer patients, with a HR of 2.0 (95% CI 1.0-4.0) for VTE risk in cancer patients with FVL compared to cancer patients without FVL.[266] Further, in a case-cohort derived from the Tromsø Study, FVL was associated with a 2-fold increased VTE risk in cancer and displayed a synergistic effect on VTE risk when combined with cancer.[267] A meta-analysis of 10 studies showed that FVL was associated with CVC-related VTE in cancer patients, with a pooled OR of 4.6 (95% CI 2.6-8.1).[268]

The prevalence of ***prothrombin G20210A*** mutation is found to be 0.7-4% in the general population and varies from 0.6 to 2.6% in cancer patients.[167,264,269-272] The genotype is a gain-of-function mutation resulting in high plasma levels of factor II (prothrombin).[273] Most studies have demonstrated a positive association between G20210A and VTE with risk estimates alternating between 1.5 and 6.7,[43,263,274-276] while some smaller studies have found no association.[260,264,277] In the MEGA Study, the prothrombin genotype displayed an OR of 2.3 (95% CI 1.6-3.3) for VTE risk in carriers of the risk allele compared to non-carriers. In the same study, no analysis was done for cancer patients with the prothrombin mutation, as there were zero control participants with the genotype in this group. Further, the pooled OR for CVC-related VTE in cancer patients with G20210A was found to be 4.9 (95% CI 1.7-14.3) in a meta-analysis of seven studies.[268] The F2 G20210A mutation is also associated with slightly increased plasma levels of F11 leading to increased thrombin generation, which results in a hypercoagulable state.[171]

Non-O blood group is thought to be the most common genetic risk factor for VTE.[162] Recent studies have established that A1 (about 90% of A blood) and B blood groups are associated with a 1.5 to 2-fold increased risk of VTE, while A2 (about 10% of A blood) and O blood groups have a protective effect against VTE. [161,177,278,279] The increased risk of A1 and B blood groups is thought to be mediated by elevated levels of vWF and FVIII, however, the association between these blood groups and VTE remains even after adjustments for vWF and FVIII.[163] Several SNPs, such as rs8176719, rs8176750, rs505922 and rs817646, have been used to differentiate between O and non-O blood groups. Non-O blood has in most studies been identified by the presence of these SNPs, and the rs8176719 has been most widely studied with regards to VTE. Non-O blood group has been found to be an independent risk factor for cancer-related VTE in pancreatic cancer,[280] in children with acute lymphoblastic leukemia[281] and adults with malignant gliomas.[282] In the Tromsø Study, non-O blood was found to be

associated with a 30% increased risk of cancer-related VTE (HR 1.32, 95% CI 0.95-1.83), compared to cancer patients with O-blood group.[283] In the same study, the combined effect of non-O blood group and cancer on VTE risk was 12-fold (HR 12.29, 95% CI 9.63-15.68) higher than in non-cancer subjects with blood group O.[283] In a study of 219 patients with myeloproliferative disorders (essential thrombocytosis, polycythemia vera and primary myelofibrosis), no association between the ABO SNP rs8176719 and VTE risk was found.[284]

Other SNPs have also been investigated in relation to VTE risk in cancer patients, however, most of these studies were small and had limited statistical power.[283] In a study of 78 cancer patients, none of the nine genetic variations investigated (i.e. FVL, FV H1299R, FII G20210A, MTHFR C677T, MTHFR A1298C, PAI-1 4G/5G, β-fibrinogen–455 G→A, FXIII Val34Leu and GpIIIa HPA-1a) were found to be associated with VTE risk.[285] Further, in 1079 tumor patients derived from the Vienna CATS Study, the polymorphisms of fibrinogen (-455G>A) and FXIIIa (Val34Leu) were not found to be associated with VTE risk.[286] Finally, a study of 60 cancer patients found no association between three different genetic variations of the FVII gene (−323ins10-bp, −401GT, and −402GA) and VTE risk.[287]

Genetic risk scores may have the potential to predict VTE risk in cancer. Preliminary data based on 251 cancer patients undergoing chemotherapy treatment, showed that a GRS of 11 SNPs performed better in detection of high risk patients of VTE, compared to the Khorana Risk Score (AUC 0.70 vs. 0.55).[288] Further, a Swedish population-based study of around 4000 women with breast cancer investigated the risk of VTE based on the presence of nine established VTE loci, with the highest risk found for subjects above 59 years in the top 5% of the polygenic risk score.[289] As described in section 1.3 of this thesis, de Haan and coworkers created a GRS of 5 prothrombotic SNPs, which has been evaluated in cancer-free populations.[181] Further, in recent years the 5-SNP score has also been tested in combination with other conditions such as ischemic stroke, body height and family history of myocardial infarction.[290-292] However, the predictive capacity of the 5-SNP score has not been evaluated in cancer.

During the last decades, major advances have been made in our understanding of genetic risk factors in VTE development. However, the role of prothrombotic genotypes on VTE in cancer is scarcely studied in larger studies, and several established prothrombotic genetic variants such as GP6 rs1613662 and FGG rs2066865 have never been investigated in cancer patients. Moreover, no previous study have investigated any of the known prothrombotic SNPs or combination of SNPs in a GRS on VTE risk in occult cancer. A deeper understanding of prothrombotic genotypes in cancer-related VTE might improve risk prediction, thromboprophylaxis guidelines and thus, improve prognosis for these patients. There is a need to adequately evaluate established and novel genetic risk factor for VTE in cancer, and further, to develop and validate genetic risk assessment models for VTE in cancer patients.

2. Aims of the thesis

The aims of the thesis were:

- To investigate the association between GP6 rs1613662 and VTE risk in the general population and stratified by cancer status to explore the combined effects of GP6 rs1613662 and active cancer on the risk of VTE (Paper I)

- To investigate the joint effect of FGG rs2066865 and active cancer on the absolute and relative risks of VTE in a population-based case-cohort (Paper II)

- To investigate the impact of increasing number of risk alleles in the 5-SNP score on the risk of VTE in patients with and without cancer using a population-based case-cohort (Paper III)

- To investigate the effect of five individual prothrombotic genotypes and a genetic risk score (GRS) on VTE risk in occult cancer, using a large case-cohort recruited from the general population (Paper IV)

3. Methods

3.1. Study populations

3.1.1. The Tromsø Study

The Tromsø Study is a single center prospective population-based study with repeated health surveys of the inhabitants of Tromsø municipality, North Norway.[293] Overall, seven surveys (Tromsø 1-7) of the Tromsø Study have been conducted, ranging from 1974 to 2016. The study was first initiated to investigate causes of the high cardiovascular mortality in northern Norway, and to develop methods to prevent myocardial infarctions (MIs) and strokes. However, the study has evolved over four decades and now provides information on a wide range of examinations and diseases.[293] Further, the study offers several favorable features, such as a long-term follow-up, a longitudinal design, repeated measurements, high attendance rates and a single center follow-up.

The fourth survey of the Tromsø Study (Tromsø 4), conducted in 1994-1995, was the Tromsø survey used for this thesis. In Tromsø 4, all inhabitants aged 25 or older were invited, and 27158 individuals participated, yielding an attendance rate of 77%. All participants aged 55 to 74 years and a random 5-10% sample in the other age groups were invited to a second, more extensive examination. VTE registration started on January 1, 1994 and all incident and recurrent VTE events were registered until December 31, 2012. Tromsø 4 was included in all four papers (I-VI) of this thesis. However, in Paper I and III, the Tromsø 4 survey was combined with the second survey of the Nord-Trøndelag Health Study (HUNT 2).[294] Finally, in Paper IV, Tromsø 4 was included as one of three cohort studies merged into one large population-based study, the Scandinavian Thrombosis and Cancer (STAC) cohort.[295]

3.1.2. The Nord-Trøndelag Health Study

The HUNT Study encompass health information and biologic material of the inhabitants of Nord-Trøndelag County, Norway. The study was primarily designed to determine the prevalence of diabetes, hypertension and undiagnosed tuberculosis, and to assess the quality of health care provided to patients with these conditions. Data in the HUNT Study has been collected through the four following population surveys: HUNT 1 (1984-1986), HUNT 2 (1995-1997), HUNT 3 (2006-2008) and HUNT 4 (2017-2019). Overall, approximately 150 000 individuals have participated, and more than 100 000 subjects have provided blood samples. The main objective of the second survey of the HUNT Study (HUNT 2) was public health issues such as cardiovascular disease and other chronic conditions in accordance with national health priorities. In HUNT 2, all individuals aged 20 years and older living

in Nord-Trøndelag were invited and 66 140 participated, giving an attendance rate of 71%.[294] Participants of HUNT 2 were followed from the inclusion in 1995-1997, until December 31, 2008. HUNT 2 was used for Paper I and III, and were also included in the STAC cohort, used for Paper IV.

3.1.3. The Scandinavian Thrombosis and Cancer Cohort

In Paper IV, participants were included from the STAC cohort, which is a large population-based cohort comprising of data from three Scandinavian cohorts: The Tromsø 4 Study, the HUNT 2 Study and the Danish Diet, Cancer and Health (DCH) Study. The Tromsø 4 Study and HUNT 2 Study have already been described in the sections above. The DCH Study was conducted in 1993-1997 and aimed to assess the effect of diet and lifestyle factors on the development of cancer and several other chronic diseases. All inhabitants aged 50 to 64 years and living in the urban areas of Copenhagen and Aarhus, without a previous cancer diagnosis were invited to take part in the DCH Study. In total, 57 053 individuals participated, being 35% of those invited.[296] The STAC cohort was created to investigate the epidemiology and risk factors for VTE in cancer. Study participants were followed from date of inclusion (1993-1997) until end of follow-up (2007-2012) in the individual cohorts. Registration of VTE events was done until December 31, 2007 in HUNT 2, April 30, 2008 in the DCH Study and December 31, 2012 in Tromsø 4. Cancer diagnoses were registered until December 31, 2008 in HUNT 2 and December 31, 2012 in Tromsø 4 and the DCH Study. In total, 144 952 individuals aged 19 to 101 years, without previous VTE or cancer were included in the STAC cohort.[295]

A case-cohort design was used for all the four papers in this thesis. This was done to limit the costs and time required for genotyping in our studies. All incident VTE events during follow-up were included as cases in the different studies, and a sub-cohort of age-weighted subjects randomly selected from the original cohort(s) was sampled. The case-cohort design is further explained and discussed in the section of methodological considerations.

3.2. Baseline measurements and prothrombotic genotypes

Baseline data at study inclusion in Tromsø 4, HUNT 2 or the STAC cohort, was obtained by physical examination, non-fasting blood samples and self-administered questionnaires. Trained personnel recorded blood pressure using an automatic device. Three measurements were performed after two minutes at rest in Tromsø 4 and HUNT 2, and the average of the two last measurements were chosen. In the DCH Study, blood pressure was measured twice after five minutes at rest, and the lowest measurement was used. Body height and weight were measured in participants wearing light clothing

and no shoes, and was used to estimate the body mass index (BMI, kg/m^2) as body weight (kg) divided by height squared (m^2). Information on diabetes mellitus, smoking status, physical activity, education level and history of cardiovascular disease (MI, angina or stroke) was obtained from standardized, validated self-reported questionnaires.

For participants in Tromsø 4 and HUNT 2, DNA was isolated from whole blood and stored at minus 70^{o}C at the national CONOR (Cohort Norway) biobank, located in Levanger, Norway. All blood samples drawn from participants in the DCH Study were processed and frozen within two hours at minus 20^{o}C, and at the end of the day of collection, all samples were stored in liquid nitrogen vapor (max minus 150^{o}C). The following SNPs were genotyped: rs1613662 in the glycoprotein 6 gene (GP6), rs2066865 in the fibrinogen gamma gene (FGG), rs8176719 in ABO (non-O blood type), rs6025 in F5 (FVL), rs1799963 in F2 (prothrombin G20210A), and rs2036914 in F11. In the Tromsø Study, rs1613662 (GP6), rs8176719 (ABO), rs6025 (F5), rs1799963 (F2) and rs2036914 (F11) were genotyped using the Sequenom platform, and rs2066865 (FGG) by the TaqMan platform, as previously described.[291] The HUNT Study performed genotyping using the Illumina HumanCore Exome array. In the DCH Study, genotypes were determined using predesigned TaqMan SNP genotyping array, as described elsewhere.[179]

Participants were considered carriers of the prothrombotic risk gene when one or two risk alleles were present. In Paper I, homozygosity for the major allele at GP6 rs1613662 was used as reference group and risk according to carriership of the minor allele was investigated. In Paper II, the FGG SNP was genotyped and the presence of one or two risk alleles (minor alleles) was defined as hetero- and homozygosity, respectively. For the last two papers, Paper III and IV, ABO rs8176719 (non-O blood type), F5 rs6025 (Factor V Leiden), F2 rs1799963 (prothrombin G20210A), FGG rs2066865 and F11 rs2036914 were genotyped. The major allele of the different SNPs was used as reference group, however, for the F11 SNP, the minor allele was used as reference as the minor allele of the F11 SNP has been found to decrease VTE risk.[180] For the ABO SNP, zero risk alleles were classified as O blood type, and thus one or two risk alleles present were considered non-O blood type. In Paper III and IV, we did not differentiate between hetero- and homozygotes. A genetic risk score conceived by de Haan and colleagues was created by summarizing the number of risk alleles from the five sequenced SNPs in Paper III and IV.[181]

3.3. Cancer assessment

3.3.1. Identification and validation of cancer diagnoses

Incident cancer diagnoses during follow-up were identified by linkage to the Cancer Registry of Norway (CRN) (Paper I to IV) and the Danish Cancer Registry (Paper IV) by the use of participants' individual unique national civil registration number, which is assigned to all residing in the Nordic countries. The CRN and the Danish Cancer Registry are similarly organized, receiving information from several medical sources such as general practitioners, hospital doctors, death certificates and pathological laboratories. The registries are also linked to the Norwegian National Cause of death Registry and the Danish Register of Cause of Death in the respective countries. Cancer registration and reporting cancer cases has been mandatory by law since 1953 in Norway and 1987 in Denmark.[297,298] Reports have found both cancer registries complete and valid, reporting a completeness of 98.8% in Norway and 95-98% in Denmark.[297,298] The percentage of microscopically confirmed diagnoses in the registries were 94% in Norway and 93% in Denmark, respectively.[297,298] The two cancer registries provide information regarding the cancer diagnosis date, cancer location (ICD10 codes C00-96 and ICD7 codes 140-205), histological grade (ICO-3) and cancer stage (localized, regional, distant or unknown). Subjects with non-melanoma skin cancers (ICD10 C44) and no other cancer diagnosis were regarded as cancer-free, due to the non-metastatic potential of this disease.

3.3.2. Definition of occult cancer and active cancer

Temporal proximity to cancer is shown to be a strong predictor for VTE risk.[24,35,185] Studies have found an increased VTE risk already one year preceding the cancer diagnosis date, with a seven-fold increased risk six months prior to the cancer diagnosis date.[24,25,185] Further, previously undiagnosed cancer is frequent in patients with unprovoked VTE, with a period prevalence of undiagnosed cancer increasing from 6.1% at baseline to 10.0% from the time of VTE diagnosis to 12 months after.[25] Hence, we defined *occult cancer* (the presence of an undiagnosed cancer) as the 12 months prior to a cancer diagnosis date. In Paper IV, we investigated the effect of individual and combination of prothrombotic genotypes in a GRS on the risk of VTE in occult cancer, compared to subjects that were cancer-free. Two sensitivity analyses were additionally performed to test the robustness of the occult cancer variable, where the occult cancer period was defined as six months and two years preceding the cancer diagnosis. VTE events occurring after the occult cancer period were censored from the analysis as the main objective was to investigate VTE's related to occult cancer, and not overt cancer.

In Paper I, II and III, we investigated different prothrombotic genotypes and a GRS on the risk of VTE in *active cancer*. Several studies have found that nearly 50% of cancer-related VTE events occur in a two and a half year period around the cancer diagnosis (i.e. from six months before cancer diagnosis to two years after),[24,35,185] an observation that supports evidence suggesting that VTE risk is closely related to the rate of cancer growth, rather than the extent of cancer.[196] We therefore chose this timeframe to define the active cancer period. Thus, a VTE event was classified as related to active cancer if it occurred within six months preceding a cancer diagnosis until two years following the cancer diagnosis date. Patients who were still alive and VTE-free at the end of the active cancer period were censored at this time, as information regarding cancer progression and remission was not available and extending the active cancer period could result in dilution of the results by including VTE events that were not related to cancer.

3.4. Assessment of venous thromboembolism

Only first lifetime, objectively confirmed symptomatic VTE events were included in the Tromsø Study, the HUNT Study and the STAC cohort. Trained personal reviewed and validated the medical records for each potential case of VTE in both in- and outpatients. A VTE event were classified as DVT or PE, and if both conditions occurred concurrently the VTE were recorded as a PE. The VTE events were further classified as provoked or unprovoked, depending on the presence of provoking factors at the time of VTE diagnosis. The definitions of provoking factors were slightly different between the three cohorts in the STAC cohort. However, in the STAC cohort, all three cohorts defined surgery, trauma, active cancer, CVC's and marked immobilization as provoking factors.

In Tromsø 4, all VTE events in the study period from January 1, 1994 until December 31, 2012 were identified by searching the hospital discharge diagnosis registry, the radiology procedure registry and the autopsy registry at the University Hospital of North Norway (UNN).[143] UNN is the sole provider for all VTE-related health care and diagnostic radiology procedures for VTE events in the area. The discharge diagnosis codes of interest were the International Classification of Diseases, revision 9 (ICD-9) codes 325, 415.1, 452, 453, 671.3, 671.4 and 671.9 for the period 1994 to 1998, and ICD-10 codes I26, I80, I81, I82, I67.6, O22.3, O22.5, O87.1 and O87.3 for the period 1999 to 2012.[124] A diagnosis of VTE was verified and recorded when the presence of clinical symptoms of DVT of PE was combined with objective confirmation by radiologic procedures (i.e. compression ultrasonography, venography, pulmonary angiography, spiral CT, perfusion-ventilation scan, or autopsy), and resulted in a VTE diagnosis and treatment initiation (i.e. LMWH, vitamin K antagonist or similar anticoagulant medications, thrombolytic therapy, vascular surgery). For cases derived from the autopsy registry, a

VTE was recorded only if the autopsy report indicated the VTE event as the cause of death or as a significant contributing cause of death.

In HUNT 2, VTE events during follow-up from January 1, 1995 until December 31, 2007 were identified by searching the hospital discharge registry and the radiology procedure registry at the two local hospitals, Levanger Hospital and Namsos Hospital, and at the tertiary-care center of the region, St Olav's Hospital.[2] The discharge diagnosis codes used were ICD-9 codes 415, 451, 452, 453, 325, 362.3, 433, 557.0, 634–638 (with decimals 6 and 7), 639.6, 639.8, 639.9, 671, 673, 674, and 997.2, and ICD-10 codes I26, I80, I81, I82, I63.6, I67.6, K55, H34.8, O08, O22, O87, and O88.[2] VTE events were included if they presented as symptomatic and treatment requiring, and were confirmed by objective diagnostic tests (i.e. ultrasonography, venography, CT scan or perfusion-ventilation scan).

In the DCH Study, VTE events were recorded from December 1, 1993 until April 30, 2008 using the participants' civil registration numbers and linkage to the Danish National Patient Registry and the Danish National Death Registry. The discharge diagnosis codes of relevance were ICD-8 codes 450.99, 451.00, 451.08, 451.09, 451.99 and ICD-10: I26, I80.1–I80.9.[299] Trained personnel reviewed the medical records for each potential VTE case including typical clinical symptoms, laboratory blood tests and further diagnostic procedures. A verified VTE event required clinical symptoms of VTE and a confirmatory diagnostic test (i.e. ultrasonography, echocardiography, venography, CT scan, perfusion-ventilation scan or autopsy).[299] The Danish National Death Registry was used to identify VTE as a cause of death, and only VTE events verified with an autopsy were included.

3.5. Statistical analyses

Statistical analyses for all four papers in the present thesis were performed using STATA version 15.0 and 16.0 (Stata Corporation, College Station, TX, USA). Cancer was entered as a time-varying covariate, and the data was split in relation to the date of cancer diagnosis. For Papers I-III, subjects who developed cancer contributed with person-time as unexposed (i.e. cancer-free) from date of inclusion until six months prior to the cancer diagnosis, and thereafter contributed with person-time as exposed (i.e. active cancer) until two years following the cancer diagnosis. In Paper IV, subjects contributed with person-years as unexposed until one year prior to the cancer diagnosis date, and then as exposed to occult cancer until diagnosed with cancer, and were thereafter censored from the analyses. Cox proportional hazard regression models were used to estimate the hazard ratios (HRs) with 95% confidence intervals (Cis) for incident VTE according to the presence of the different prothrombotic genotypes or categories of genotypes in a genetic risk score and by cancer status. All analyses were adjusted for age, sex and/or BMI.

3.6. Calculation of biological interaction

Interaction refers to the situation where the effect of one exposure on a certain outcome is different across strata of another exposure.[300] Meaning that if interaction between two exposures is present, these exposures are not independent in causing a certain outcome. Biological interaction can be approached in several ways, e.g. by calculating the relative excess risk due to interaction (RERI) or the proportion attributable to interaction (AP) or assessing the synergy index (SI) developed by KJ. Rothman.[301] RERI was calculated in Papers II-IV as HR_{11}-HR_{10}-HR_{01}+1, where HR_{10} was the hazard ratio of one exposure (i.e. cancer) without the presence of the other exposure (i.e. prothrombotic genotypes), H_{01} was the hazard ratio of the presence of prothrombotic genotypes only, and finally, HR_{11} indicated the hazard ratio with both exposures present (cancer and prothrombotic genotypes). RERI values below 0 indicate a negative interaction, whereas values above 0 indicate synergism. AP was calculated as RERI/HR_{11}, and can be interpreted as the proportion of cases in the combined groups that is due to interaction between the two exposures (i.e. the amount of VTE events in subjects with cancer and prothrombotic genotypes that occur due to the biological interaction between cancer and these SNPs). AP values below 0 indicate negative interaction or less than additivity, whereas values above 0 indicate a positive interaction. SI indicates the excess risk from the presence of interaction, relative to the risk from exposure when there is no interaction.[302] Values of RERI and AP equal to 0 and SI equal to 1.0 indicate no interaction.[300]

4. Main results

4.1. Paper I

Genetic variation of platelet glycoprotein VI and the risk of venous thromboembolism

VTE is a frequent complication in patients with cancer. In recent years, several SNPs have been found to influence VTE risk, however, these SNPs have been scarcely studied in cancer. Glycoprotein VI (GP6) rs1613662, is a missense mutation affecting the glycoprotein VI (GPVI) receptor for collagen and consequently affecting platelet adhesion and activation. The impact of GP6 rs1613662 on the risk of VTE has only been investigated in case-control studies, and the combined effect of GP6 rs1613662 and cancer on VTE risk has not been previously investigated. Thus, we aimed to investigate the association of GP6 rs1613662 on the risk of VTE in the general population and stratified by cancer status, using a large case-cohort. Cases with incident VTE (n=1493) and a subcohort (n=13072) were derived from the fourth survey of the Tromsø Study (Tromsø 4) and the second survey of the Nord-Trøndelag Health Study (HUNT 2). Cox regression was used to calculate age-, sex- and body mass index-adjusted hazard ratios for the association between GP6 variation and VTE by the presence of GP6 alleles and cancer status. During the study period, 1536 were diagnosed with cancer, of which 233 (15.2%) experienced a VTE. In cancer-free subjects, the risk of incident VTE decreased with the number of minor alleles, and subjects homozygous for the minor GP6 allele (GG) had 34% decreased risk of incident VTE (HR 0.66, 95% CI 0.43-1.01) compared to subjects homozygous for the major allele (A allele) at GP6. In contrast, cancer patients homozygous for the minor allele (GG) had an increased risk of VTE, particularly PE (HR 1.96, 95% CI 0.78-4.94), compared to cancer patients homozygous for the major allele (AA). The association between homozygosity at the G-allele and VTE risk was moderately attenuated after adjustment for cancer type and stage, as this genetic variant was associated with prothrombotic cancers and advanced stages. In conclusion, the GP6 rs1613662 G-allele displayed a protective effect on VTE risk in cancer-free subjects, while an increased risk of VTE was observed in cancer patients homozygous for the G-allele. Our findings support a role of platelet reactivity in the pathogenesis of VTE, which may differ according to cancer status.

4.2. Paper II

Fibrinogen gamma gene rs2066865 and risk of cancer-related venous thromboembolism

Homozygous carriers of the fibrinogen gamma gene (FGG) rs2066865 have a moderately increased risk of VTE, but the effect of the FGG variant in cancer is unknown. We aimed to investigate the effect of the FGG variant and active cancer on the risk of VTE. Cases with incident VTE (n=684) and a randomly selected age-weighted subcohort (n=3931) were derived from a population-based cohort (the Tromsø 4 Study). Active cancer was defined as six months prior to and two years following the cancer diagnosis date, and thus, VTE events occurring in this timeframe were considered to be cancer-related. Cox regression was used to estimate hazard ratios (HR) with 95% confidence intervals (CI) for VTE according to categories of cancer and FGG. During a mean follow-up of 12.6 years, 854 subjects had active cancer, of whom 167 experienced an incident VTE. In subjects without cancer, homozygosity at the FGG variant was associated with a 70% (HR 1.7, 95% CI 1.2-2.3) increased risk of VTE compared to non-carriers. Cancer patients homozygous for the FGG variant had a two-fold (HR 2.0, 95% CI 1.1-3.6) higher risk of VTE than cancer patients without the FGG variant. Moreover, the six-month cumulative incidence of VTE among cancer patients was 6.4% (95% CI 3.5-11.6) in homozygous carriers of FGG and 3.1% (95% CI 2.3-4.7) in those without risk alleles. A synergistic effect was observed between rs2066865 and active cancer on the risk of VTE (synergy index (SI) 1.81, 95% CI 1.02-3.21), indicating a biological interaction between the FGG genotype and cancer. Estimation of the attributable proportion (AP) due to interaction revealed that 43% (95% CI 0.11-0.74) of the VTE events in study participants with cancer and the FGG variant were due to the interaction between the two exposures. In conclusion, homozygosity at the FGG variant and active cancer yielded a synergistic effect on the risk of VTE.

4.3. Paper III

Combined effects of five prothrombotic genotypes and cancer on the risk of a first venous thromboembolic event

The role of combined prothrombotic genotypes in cancer-related VTE is scarcely studied. We aimed to investigate the impact of a 5-SNP score on the risk of VTE in patients with and without cancer using a population-based case-cohort. Cases with a first VTE (n=1493) and a subcohort (n=13072) were derived from the fourth survey of the Tromsø Study (1994-2012) and the second survey of the Nord-Trøndelag Health Study (1995-2008). All participants were genotyped for the five SNPs in the genetic risk score proposed by de Haan *et al.*, including ABO (rs8176719), F5 (rs6025), F2 (rs1799963), FGG (rs2066865), and F11 (rs2036914). Active cancer was defined as six months prior to and two years following the cancer diagnosis date, and thus, VTE events occurring in this timeframe were considered to be cancer-related. Cox regression were used to calculate hazard ratios (HRs) for VTE according to cancer status, individual prothrombotic genotypes and the number of risk alleles in the 5-SNP score (0-1, 2-3, and ≥4 alleles). During a median follow-up of 12.3 years, 1496 individuals were diagnosed with cancer, of whom 232 experienced VTE. The VTE risk increased with the number of risk alleles in the 5-SNP score among subjects without and with cancer. In cancer-free subjects, the HR was 2.17 (95% confidence interval [CI] 1.79-2.62) for ≥4 versus 0-1 risk alleles. In cancer patients, the corresponding HR was 1.93 (95% CI 1.28-2.91). The combination of cancer and ≥4 risk alleles yielded a 17-fold (HR 17.1, 95% CI 12.523.4) higher risk of VTE compared with cancer-free subjects with 0-1 risk alleles. This combined effect was higher than expected on the basis of the individual effects of cancer and ≥4 risk alleles (RERI 6.72 95% CI 1.17-12.26). The attributable proportion (AP) revealed that 39% of the total VTE events in participants with cancer and ≥4 risk alleles were attributable to the interaction between the two exposures (i.e. cancer and ≥4 risk alleles). In conclusion, the risk of VTE increased with the number of prothrombotic risk alleles in subjects with and without cancer, and the combination of prothrombotic risk alleles and cancer displayed a supra-additive effect on the risk of VTE, indicating a biological interaction between the risk factors. Our findings suggest that the 5-SNP score may be useful for identifying cancer patients at increased risk of VTE.

4.4. Paper IV

Prothrombotic genotypes and risk of venous thromboembolism in occult cancer

Unprovoked VTE can be the first manifestation of an occult cancer, and around 5% of patients with unprovoked VTE are diagnosed with cancer within one year of follow-up. Some SNPs are found to have biological interaction with overt cancer, resulting in a synergistic effect on VTE risk. Whether individual prothrombotic genotypes or number of risk alleles in a genetic risk score (GRS) affect VTE risk in occult cancer have not been addressed. Thus, we aimed to investigate the individual and joint effect of five prothrombotic genotypes and occult cancer on VTE risk in a general population. Occult cancer was defined as one year preceding the cancer diagnosis date, and thus, VTE events occurring in this period were defined as related to occult cancer. Cases with incident VTE (n=2141) and a subcohort (n=14911) were sampled from the Scandinavian Thrombosis and Cancer Cohort (1993-2012). Five SNPs previously reported in a GRS were genotyped: ABO (rs8176719), F5 (rs6025), F2 (rs1799963), FGG (rs2066865) and F11 (rs2036914). Hazard ratios (HRs) for VTE by individual SNPs and GRS were estimated according to non-cancer and occult cancer exposure using Cox regression. During a median follow-up of 12.2 years, 1817 subjects developed occult cancer, and of these, 93 experienced a VTE. The VTE risk was 4-fold higher (HR 4.05, 95% CI 3.28-5.00) in subjects with occult cancer compared to those without cancer. Subjects with an occult cancer and VTE had a higher proportion of prothrombotic and advanced cancers compared to cancer patients without VTE. The VTE risk increased according to individual prothrombotic genotypes and the GRS in cancer-free subjects, while no such effect was observed in subjects with occult cancer (HR for ≥4 versus ≤1 risk alleles in the GRS: 1.14, 95% CI 0.61-2.11). In conclusion, five common prothrombotic genotypes, individual or combined, were not associated with risk of VTE in occult cancer. Our findings suggest that prothrombotic mechanisms related to rapidly advancing cancers at high-risk sites are prominent for VTE risk in occult cancer and supersede the effect of prothrombotic genotypes.

5. General discussion

5.1. Methodological considerations

5.1.1. Study design

The papers (I-IV) in this thesis are based on data from three large, prospective, population-based cohort studies. Further, we used a case-cohort study design for all the four papers. Therefore, both the cohort study design and case-cohort design will be discussed.

Cohort studies and case-control studies are the most commonly used observational study designs in clinical medicine.[303] Observational studies often aim to examine and quantify risk factors for health-related outcomes and to identify preventable causes of a disease or medical condition. Contrary to experimental studies (i.e. trials), the researchers or investigators in observational studies do not intervene but rather observe and assess the strength of the relationship between an exposure and an outcome.[304] In cohort studies, information on exposure and various predefined characteristics are obtained for all study participants at study enrollment, with subsequent follow-up until occurrence of an outcome, or until migration, death or end of study period. Thereafter, the group of exposed subjects and the group of non-exposed subjects are compared with respect to the outcome.

Cohort studies offer several advantages.[305] The prospective study design makes it possible to calculate incidence rates (IR), as a measure of the absolute risk of a disease, and relative risk estimates of disease in exposed and unexposed individuals. Compared to other observational studies, the nature of the cohort study design also allows several exposures and outcomes to be investigated simultaneously. Furthermore, if the cohort study is based on a large sample size and a defined and well-characterized population (representative sample), with a low degree of bias and confounding (high internal validity), the results can be extrapolated beyond the source population to similar populations (i.e. external validity). Additionally, as the information on exposure is collected prior to the outcome in cohort studies, the criteria of temporality is met. The criteria regarding the temporal sequence between an exposure and an outcome is one of the criteria needed to provide epidemiologic evidence for causality.[306] Other criteria for determination of causality are for instance strength of the association, a plausible mechanism between exposure and outcome, a biological gradient (dose-response relationship), reversibility and consistency with other studies.[306]

Results from one cohort study are not sufficient to establish causality. For this matter, a randomized controlled trial (RCT) would be preferable. In RCTs, comparison groups are similar in all characteristics except from the exposure or intervention, and thus considerably reducing confounding and bias. However, even though RCTs are the most suitable study design to conclude on causality, RCTs

require a lot of resources and are both time-consuming and expensive. Further, RCTs may rise ethical concerns and may be impossible to perform. For example, it would be unethical and impossible to inflict cancer on study participants for the purpose of investigating the association between cancer and VTE. Therefore, a cohort study design would be a suitable approach to investigate cancer-related VTE. Some limitations of the cohort study design require attention. Cohort studies and other observational study designs are prone to bias and confounding, which can cause over- and/or underestimates or even false assumptions if not taken into consideration. Moreover, the cohort design is poorly suited to investigate rare outcomes with low incidence rates, since the design requires large populations and a long-term follow-up for enough outcomes to occur and yield an adequate statistical power. However, the outcome of interest in this thesis (VTE) occurs frequently in the general population.

A ***case-cohort design***, a variation of the cohort study design, was used for all the papers in this thesis. A case-cohort design includes only the selected cases of an outcome (e.g. VTE) and a random age-weighted sample of subjects, a sub-cohort, derived from the entire cohort study (Tromsø 4, HUNT 2 or the STAC cohort) independent of outcome status (Figure 8). The sub-cohort is meant to reflect the occurrence of the exposure in the original cohort population.[307,308] The case-cohort design is often used when large cohorts are needed to observe a sufficient amount of cases, but it is not feasible to collect data on covariates for the whole cohort. Prothrombotic genotypes were exposure variables in all the papers (I-IV), and genotyping had to be done for all subjects included in the different studies. Genotyping is both time-consuming and costly. Therefore, we chose the case-cohort design to limit the costs and time required for genotyping, as the full covariate data (e.g. genotyping) in case-cohort designs is only required for cases and sub-cohort members.

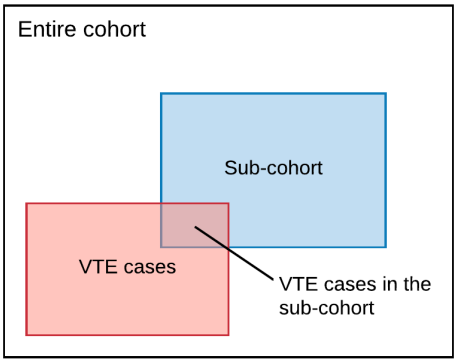

Figure 8. Case-cohort study design.

Similar to the cohort study design, the case-cohort study has the temporal sequence of exposure and outcome, reducing the risk of recall bias or reverse causation. Further, the temporality also limits reverse causation, a type of temporal bias where the outcome influences exposure status. Like cohort studies, the incidence rates, absolute risks and relative risks can be calculated in case-cohort studies. Every person in the original cohort, including the cases, has the same probability of being selected to the sub-cohort regardless of how much person-time this participant has contributed with or whether the person has experienced an outcome or not.[309] With appropriate sampling and analyses, the risk estimates in the case-cohort are similar to those in the original cohort.[307] The case-cohort study design also carries some limitations. The cases are often overrepresented in the case-

cohort sample, which should be taken into consideration and accounted for. [307] In our studies, the sub-cohort samples are large, thus limiting overrepresentation of cases.

5.1.2. Validity and generalizability

Validity refers to how accurately the results of a study represent the true findings and are free from bias. The validity of a study can be separated into two domains, internal and external validity, both essential in epidemiological research.[309] ***Internal validity*** is the extent to which an observed association is true for the population studied, where bias and confounding are minimized and any differences between the studied groups can be truly attributable to the exposure.[309,310] ***External validity***, often referred to as ***generalizability***, is the extent to which the study results can be generalized to populations outside the study population.[309,310] Further, external validity is reliant on internal validity, however, the presence of external validity does not guarantee internal validity (i.e. the study participants are representative for the study population, but there is a presence of confounding or bias affecting the association between the exposure and the outcome).[311] Most violations of internal validity are due to confounding, selection bias and/or information bias.[309] Though RCTs are designed for minimizing bias and confounding, ensuring high internal validity, the strict inclusion criteria for participants in most RCTs limits the external validity of the trials.[312] In cohort studies derived from general populations with well-defined inclusion and exclusion criteria, high attendance rates and minimal loss to follow-up enhances the chance of high external validity.[313]

5.1.3. Bias and misclassification

In epidemiological research, errors in estimation can occur either *random* or *systematic*. The random errors can be minimized by the increase in the sample size, but the systematic errors will remain despite a large sample size. Bias is the term for *systematic errors* in the study design, analysis or conduct of the study that results in incorrect estimates of the true association between an exposure and an outcome.[309] Bias can lead to either over- or underestimation of the risk estimates, depending on the type of systematic error. Further, bias may influence both internal and external validity. There are several types of biases and several classifications of bias with some overlap.[311] However, most biases in epidemiology occur under the definition as either selection bias or information bias.[311,314]

Selection bias refers to any systematic error in the recruitment and/or retention of study participants, which occur when the exposure or outcome status of an individual influence the probability of participating in the study.[173] Selection bias is mainly a problem in case-control studies,

however, rarely lead to erroneous associations in prospective cohort studies as participants in cohort studies are recruited prior to the outcome event.[313] Cohort studies are prone to non-response bias (or self-selection/participation bias), a type of selection bias which is introduced when subjects who volunteer to participate differ from those who do not. In cohort studies, the non-responders are often less concerned about health, more often institutionalized elderly or patients suffering from different medical conditions than the responders. Overall, participation in epidemiological studies have declined over the past years, and attenders are more likely to have higher socioeconomic status and education, be female and married.[315] This is also seen in the latest surveys of the Tromsø Study, where the attendance rate has decreased from approximately 83% in Tromsø 1-3 to 66% in Tromsø 6, and the non-responders being younger, more often unmarried and men.[293]

In the largest survey of the Tromsø Study, Tromsø 4 (Papers I-IV), all inhabitants aged 25 or older were invited, and the attendance was 77%.[293] The HUNT 2 survey (Papers I, III and IV) had a similarly high attendance rate of 71%, with participants aged above 19 years and living in Nord-Trøndelag.[294] As the participation rates were high, selection bias was likely minimized. However, the non-responders will always be an issue. In both Tromsø 4 and HUNT 2, some groups were less represented than others. Participation rates were lower among those under the age of 40 years and above 80 years, threatening the generalizability in the youngest and oldest populations.[293,294] Further, men were less represented than women in both studies.[293] However, the age-specific IRs of cancer in both men and women in Tromsø 4 and HUNT 2, were similar to those reported nationally in Norway,[316] indicating that our study populations are representative for the inhabitants of Norway. In our studies, the generalizability of the youngest would not be a large issue, as the incidence of VTE and cancer in this age group is low. However, it could be a concern for the oldest populations as VTE and cancer occur frequently in elderly, and an underrepresentation of this population would diminish the generalizability of the results for these subjects. A study on non-responders of HUNT 2 revealed that the main reason for not attending among the youngest was lack of time or having moved to another country, and for the oldest the main reason were already having many health-related visits or medical consultations and no need for further examinations.[294] Around 10% did not attend the health survey due to medical immobilization.[294].

For Paper IV, we also used data from the DCH Study. In the DCH Study, people from urban areas of Copenhagen and Aarhus aged 50 to 64 years were invited to partake, and the attendance rate was 35%.[295] It is to be noted that age standardized IRs for cancer in Denmark and in the DCH Study were comparable.[296] The DCH Study had high proportions of participants with high education level and high socioeconomic status.[296] Cancer is thought to occur more frequent in individuals with lower socioeconomic status, and an underrepresentation of participants with low socioeconomic status

could result in a lower incidence of cancer. This could indicate that participants who develop cancer in our study are more health-aware than cancer patients in general, being more aware of symptoms, resulting in earlier cancer detection, earlier treatment and thereby lowering the risk of VTE. However, some cancers develop with vague symptoms or with late developing symptoms, and so, symptoms are presented first in advanced cancer stages and late detection despite increased health-awareness. Nevertheless, as the age standardized incidence rates (SIR) of cancer in the DCH Study did not differ from the general Danish population,[296] the low participation rate in subjects with lower socioeconomic status should not have considerable impact on the validity in this cohort study. The equal SIRs of cancer seen in study participants of the DCH Study and the general population of Denmark might be due to the small differences between social classes in Scandinavian countries, compared to the rest of the world.

The incidence of VTE ranges from 1 to 2 per 1000 person-years in other studies.[1,2,122] The IRs of VTE per 1000 person-years in Tromsø 4 (IR 1.7, 95% CI 1.6-1.9), HUNT 2 (IR 1.5, 95% CI 1.4-1.6) and the DCH Study (IR 1.2, 95% CI 1.1-1.3)[295] are approximately of the same size, and thus, our cohorts are comparable to other Western populations. Additionally, the incidence of cancer in all the cohorts used in this thesis seems to be similar to reports from the Norwegian Cancer Registry and Danish Cancer Registry.[295,316] All of these findings indicate that the study populations in our studies are representative for the general Norwegian and Danish population. However, the majority of the study participants in our cohorts are of Caucasian ethnicity, and consequently, generalizing our results to populations with other ethnicities must be done with caution.

In this thesis, we used genotyping information for the following prothrombotic SNPs: rs1613662 in GP6, rs2066865 in FGG, rs8176719 (non-O blood type) in ABO, rs6025 (FVL) in F5, rs1799963 (prothrombin G20210A) in F2, and rs2036914 in F11. As there are large global variations in the human genome, the distribution of prothrombotic genotypes may vary greatly.[317] Yet, the allele frequency of the different SNPs investigated in our studies (Papers I-IV), is essentially similar to other Western reference populations, indicating that our results on genetics and VTE risk could be generalizable to both Scandinavian and other Western populations.[163]

Differential losses to follow-up is another type of selection bias, that occurs when the participants lost to follow-up (i.e. censored or dropped out) differ from those who remain in the study.[173] This type of selection bias is always a concern in cohort studies,[311] especially for cancer and VTE, where ***competing risk of death*** has been found to overestimate the associated risk for some cancers.[318] In survival analysis, death is handled as a censoring event the same way as for migration, meaning that people who move or die do not contribute with person-time after this date because it is unknown whether the outcome occurs in these subjects or not. An assumption of survival analysis is

that participants who are not censored, have the same probability or risk of the outcome as those who are censored (also called non-informative or independent censoring). In all of the four papers in this thesis, subjects were censored for migration or death. Censoring for migration rise no concerns, as there is no reason to believe that those who moved from either Tromsø, Nord-Trøndelag, Copenhagen or Aarhus had a different risk of cancer or VTE than those who remained in the areas. However, death naturally prevents future outcomes to occur, and when mortality differs between groups, death is consequently considered a competing event. As cancer patients have higher mortality than people who do not develop cancer, they also have a higher probability of being censored by death. Thus, the censoring affects exposed (cancer patients) and non-exposed (cancer-free subjects) differently, and further, could result in less person-time of cancer patients at risk and an overestimation of the risk of cancer-related VTE. One way of dealing with competing risk of death is the statistical method introduced by Fine and Gray where death is considered a competing event rather than a censoring event.[319] This method presents the 'true' probability of an outcome to occur, regardless of competing mortality, theoretically resulting in unbiased and meaningfully interpretable results.[320] Ay and colleagues performed a study using the CATS cohort, comparing the performance of traditional analysis (e.g. Kaplan-Meier and Cox regression) to competing risk of death analysis on the risk of VTE in cancer patients.[318] They found that VTE risk was overestimated when using the traditional analysis compared to competing risk of death regression in cancers with high early mortality (e.g. pancreatic, gastric and lung cancer). However, minimal differences between the analyses were found for cancers with lower mortality rates (e.g. lymphoma and breast cancer). This study conclude that competing risk of death should be taken into account for biomarker studies with high mortality, RCTs with interventions in groups with different mortality rates, non-randomized trials with differences in risk factors for death between groups, and in prognostic studies (e.g. studies on risk prediction models) that can have an impact on clinical decision making.[318] However, when investigating causality between an exposure and an outcome (etiological research), the exposed and unexposed individuals alive and actually at risk of an outcome to occur are compared. In these situations, the censored participants contribute with exposed or unexposed person-time before the censoring event, and do not affect the hazard ratio after being censored.[321] In other words, in studies investigating etiological questions, traditional analysis methods can be used. This is what we have done for all of the four papers included in the present thesis, with subjects contributing with non-exposed person-time and exposed person-time (when diagnosed with cancer). Further, we did Cox regression analysis stratified by cancer status, with cancer patients carrying genetic risk factors being compared to cancer patients without risk alleles, and cancer-free carriers compared to cancer-free non-carriers. The presence of the prothrombotic SNPs is not known to be associated with mortality. Consequently, we would not expect the risk of death to differ in cancer patients with and without the presence of the SNPs. In Paper I, we

implemented the competing risk of death analysis. Not surprisingly, the hazard ratios (Cox regression) and sub-distribution hazard ratios (competing risk regression) were nearly identical for cancer patients with and without the presence of GP6 rs1613662.

In Paper VI, we investigated the association between prothrombotic genotypes and VTE in occult cancer. In order to be registered with occult cancer, patients had to survive until their date of a cancer diagnosis. Thus, subjects with occult cancer who died from either a VTE, a underlying cancer or other causes before a cancer was diagnosed would be misclassified as cancer-free (immortal time bias). Such misclassification could cause an underestimation of the VTE risk in occult cancer. However, the five prothrombotic SNPs are not expected to increase the death-rate in the general population [322,323], and thus, we believe that such misclassification, if present, would have negligible impact on our results.

Information bias refers to an error in the methods used for data collection on the study participants that leads to in inaccurate or erroneous information about the exposures or outcome. Information bias may result in ***misclassification***, which occurs when the study participants are placed in the wrong exposure or outcome category. There are two types of misclassification: differential/non-random and non-differential/random misclassification.[309] Differential misclassification occurs when the misclassification is dependent on the exposure or outcome status, resulting in one of the groups more often being misclassified than the comparison group, altering the degree of association between an exposure and an outcome.[173] Differential misclassification can result in either overestimation or underestimation of the true association. In the three cohorts used in this thesis, the study participants were included prior to the exposure and outcome assessment, and thus, differential misclassification is unlikely. Non-differential misclassification is a misclassification of the exposure that is independent of the outcome, which results in equal probability of misclassification in cases and non-cases. Non-differential misclassification tends to bias the association towards the null hypothesis, underestimating the true association. Several variables used in our studies are self-reported through questionnaires (e.g. smoking habits, physical activity, diabetes), which may introduce misclassification. However, the main exposures (prothrombotic genotypes and cancer) and outcome variables (VTE) in the present thesis are extracted from the patients' medical records by trained personnel (VTE), derived from well-validated national registries (cancer) or laboratory testing (genetics). Therefore, the degree of misclassification should be limited. Even though information in medical records rely on reporting from doctors, nurses and health care professionals, the main exposure and outcome variables in this thesis are major clinical events less likely to be unreported and thereby misclassified. Further, several Cox regression models in this thesis were adjusted for age, sex and BMI, which are variables not likely to be wrongly reported. In addition, BMI was calculated from trained professionals measuring height and body weight in participants wearing light clothing and no shoes.

In Papers I-IV, we performed genotyping of different exposure variables of prothrombotic SNPs. As for all laboratory testing, there is always some risk of technical errors during the process of measuring and testing. To minimize misclassification, DNA testing were repeated if the cell rates were low. Further, we excluded SNPs that were out of the Hardy-Weinberg Equilibrium for allele frequencies or with allele frequencies inconsistent with those previously reported. If measurement errors did occur in this process, it would be by chance and thus, defined as random errors and not systematic. The other main exposure in our studies was cancer. Incident cancer diagnoses were identified by linkage to the Cancer Registry of Norway (CRN) (Papers I to IV) and the Danish Cancer Registry (Paper IV). Cancer registration and reporting cancer cases is mandatory by law in Norway and Denmark,[297,298] and reports have found both cancer registries complete and valid, with a completeness of 98.8% and 95-98% in Norway and Denmark, respectively.[297,298] Further, a cancer diagnosis is regarded as more accurate if it is based on morphological verification, and the percentage of microscopically confirmed diagnoses in the registries has been found to be 94% in Norway and 93% in Denmark.[297,298]

In all the four papers in the present thesis, VTE was the main outcome variable of interest. Misclassification of VTE cases as false-positives in our studies were largely avoided by the use of strict validation criteria. The four criteria included radiological evidence, signs and symptoms of VTE that resulted in a clinical diagnosis and treatment of DVT or PE. We therefore assume that there is a limited amount of misclassification concerning the most important variables in our studies. Thus, information bias would only have a minor influence on the risk estimates.

Medical surveillance bias refers to when a exposure variable leads to closer surveillance and increased probability of detection of the outcome of interest.[324] This is especially a potential problem when the outcome is subclinical (i.e. symptoms develop over time or there are no symptoms at all) and exposed people are more frequently examined. This type of bias often result in an overestimation of the risk. As the risk of cancer increase the risk of VTE and vice versa, one of them occurring increases the probability of the other to be detected. Cancer patients are frequently examined by health personnel, and undergo frequent imaging, and thus, the close medical surveillance increases the probability of VTE detection. For instance, patients with suspected PE are examined with CT, which may also detect lung cancer. Further, guidelines recommend cancer screening in patients with unprovoked VTE that could result in cancer detection, which may have overestimated the IR and HR according to the exposure in our studies. Another consideration is the detection of asymptomatic VTE cases when subjects for instance undergo imagining (e.g. CT of the thorax) due to an underlying cancer. However, to avoid differential bias, the asymptomatic VTEs were not included as VTE cases in our cohorts because this could have overestimated the VTE events in cancer patients compared to cancer-free subjects, as cancer-free subjects would not have had the same medical surveillance and imaging.

Thus, the thorough validation process and strict criteria of VTE events were important to avoid wrongful classification of VTE events. There are however, some cases where it might be difficult to differentiate symptoms of an underlying cancer and symptoms of VTE, for instance when they occur from the same organ. An example of such a situation is when a person with lung cancer undergoes imaging due to dyspnea. Dyspnea could be a symptom of both the lung cancer itself and a newly developed PE, and thus, a PE may be detected as the patient are being examined and undergo imaging. In these situations it might be difficult to define the causes of dyspnea (i.e. lung cancer or the PE event), and the classification of the PE events as either asymptomatic or symptomatic.

5.1.4. Biological interaction

Biological interaction refers to when the combined effect of two or more variables on a given outcome results in deviance from an additive effect.[325] Interaction on an additive scale means that the combined effect of two exposures is larger (or smaller) than the sum of the individual effects of the two exposures, whereas interaction on a multiplicative scale means that the combined effect is larger (or smaller) than the product of the individual effects.[300] Several epidemiologists have argued that biological interaction should be assessed on an additive scale rather than a multiplicative scale.[300] A departure or deviance from additivity implies that the number of cases attributable to the joint effect of exposure variables is more or less than the sum of the number of cases that would be caused by each exposure variable separately.[326] In other words, the observed combined effect of the exposure variables on an outcome differs from what is expected based on their independent effects.[173] Most importantly, this would imply that in absence of bias, some subgroups would obtain a greater absolute risk reduction from the intervention than others would.[326]

We assessed the presence of synergism, a positive biological interaction, in subjects with different prothrombotic genotypes and cancer on VTE risk in Papers II, III and IV. Synergism was assessed by calculating the relative excess risk due to interaction (RERI), the proportion attributable to interaction (AP), and assessing the synergy index (SI). These measures of interaction (i.e. RERI, AP and SI), were developed by K.J. Rothman to assess biological interaction for risk factors rather than preventive factors, and the measures are designed for estimates pointing in the same direction.[300] Risk factors meaning that the relative risk of the factor with the outcome is larger than one (i.e. from one to infinite), and preventive factors meaning that the relative risk of the factor with the outcome is smaller than one (i.e. ranging from zero to one).[300] These scales, which are 0-1 and 1-infinite, are not suitable to be combined. In a study by Knol *et al.*, they showed that calculating measures of interaction

on an additive scale using preventive factors can give inconsistent results, and they suggested that preventive factors should be recoded to risk factors before calculating RERI, AP and SI.[300]

In Paper II, we investigated a genetic variant of FGG and active cancer on the risk of VTE. In this paper, both hetero- and homozygosity of the FGG SNP, as well as cancer, displayed an increased risk of VTE and thus, we were able to calculate biological interaction. The risk of VTE due to the combined effect of active cancer and the FGG SNP were 22.2-fold (HR 22.2, 95% CI 12.9-38.1) increased when compared to cancer-free subjects without the presence of the FGG SNP. The 22.2-fold increased risk was much more than what would be expected from summarizing the individual effects of active cancer (HR 11.9, 95% CI 9.3-15.2) and presence of the FGG SNP (HR 1.7, 95% CI 1.2-2.3). In Paper I, we did not aim to assess biological interaction. However, it would not be possible to do measures of biological interaction for the GP6 SNP and cancer, as the risk estimates of the GP6 SNP rs1613662 pointed in different directions for subjects with and without cancer. Further, the risk estimates also differed in heterozygous (AG) and homozygous (GG) subjects of the GP6 SNP in cancer patients, being protective of VTE by the presence of one G-allele, and increasing the VTE risk by the presence of two.

It is also to be noted that, RERI, AP and SI are all measurements based on the risk estimates (i.e. hazard ratios) of the exposures. This indicates that if the hazard ratios calculated for the different exposures have wide confidence intervals and lack of statistical power, it results in wide confidence intervals also for the measurements of interaction. In Paper II, the hazard ratios for VTE by the presence of cancer and the presence of two risk alleles of the FGG SNP, were 11.9 (95%CI 9.3-15.2) and 1.7 (95% CI 1.2-2.3), respectively. In the same paper, calculations of RERI had low statistical power as the confidence intervals ranged from -2.4 to 21.6 (RERI 9.6). Even though there are some uncertainties, the effect size and not the statistical significance level, should be the main focus. However, it is to be noted the results must be interpreted with caution.

5.1.5. Confounding

Confounding represents a threat to the evaluation of causal relationships in cohort studies.[173] In epidemiology, confounding refers to a situation where a non-causal association between an exposure and an outcome is observed as a result of the influence of a third, known or unknown, variable (Figure 9).[173] The confounding variable (i.e. a confounder) has to be associated

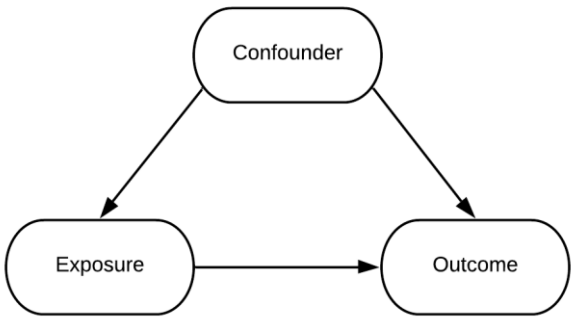

Figure 9. The concept of confounding.

with both the exposure and the outcome, to be unevenly distributed between the groups of interest and cannot be an intermediate variable in the causal pathway from the exposure to the outcome.[173,309,327] A variable that represents an intermediate step in the causal chain between the exposure and the outcome should not be handled as a confounder but treated as an intermediate factor (i.e. a mediator).[309,327] Confounding may strengthen, weaken or even change the direction of the true association. In RCTs, the participants are randomly assigned to intervention groups, and all potential confounders are thought to be evenly distributed among the comparison groups, not affecting the risk estimates.[327] Unlike RCTs, cohort studies and other observational studies are not able to randomize the exposure variables and are particularly vulnerable to residual confounding. Accordingly, all associations observed in cohort studies must be assessed for possible confounders in the statistical analysis. There are several strategies to deal with confounding in cohort studies.

Common strategies to minimize confounding in epidemiological studies include regression techniques and stratification.[328,329] ***Regression techniques***, adjustments for confounders, is the most frequently used method to reduce confounding in cohort studies.[328] In such regression models, confounding variables are included as covariates in multivariable analysis.[173] Further, regression models use data to estimate how confounders are related to the outcome and are used to minimalize the effect of confounders on the risk estimates.[173] Several types of regression exist, such as linear, logistic-, and Cox regression. The main advantage of this approach compared to stratification, is that data from all subjects are taken into account, and thus, the statistical power of the study is not reduced.[328] In Papers I-IV in the present thesis, the hazard ratios of the outcome (VTE) were adjusted for age and sex in Cox regression models, and additionally, more complex multivariable models including age, sex, BMI and other potential confounders were also used. In Paper I, we investigated the effect of the genetic variation in glycoprotein VI (GP6 rs1613662) on the risk of VTE in subjects with and without cancer. In cancer patients, the presence of advanced stages could potentially be a confounder, as higher cancer stages are associated with increased VTE risk.[15] In this study, the percentage of distant metastasis were higher in subjects heterozygous for the G-allele at GP6 compared to those without G-alleles. Furthermore, carriers of the G-allele were found to be associated with more prothrombotic cancers (i.e. lung, colorectal, hematological cancer and lymphomas). However, including the variables 'cancer type' and 'cancer stage' (i.e. localized disease, regional spread and distant metastasis) in a multivariable regression model the risk estimates were only moderately attenuated, and accordingly, cancer type and cancer stage were not considered as confounders. Some variables in cancer patients, such as chemotherapy and hospitalization, may also be intermediates in the causal pathway. Adjusting for such mediators could result in an overestimation and also potentially obscure the results.

As mentioned, ***stratification*** is a second method to reduce confounding in cohort studies.[173] In stratified analyses, the study population is divided into strata, or sub-groups, of the confounder, and the effect of the exposure is estimated in each separate sub-group. However, stratification is not always practical if there are small numbers of outcome cases in the sub-groups, which could reduce the statistical power. Another disadvantage of stratification is the possible additional confounding of other variables that suddenly differ between the sub-groups by stratification. It is of importance to specify that even though we used both regression techniques and stratification to minimize confounding, unknown confounding factors may still be present and result in residual confounding.

Matching could be viewed as a type of stratification, where study participants are as equal as possible between groups. A case-cohort design was used in the present thesis for all the four papers. When creating the case-cohorts, the sub-cohorts were randomly sampled from the original cohorts (i.e. Tromsø 4, HUNT 2 and the DCH Study). The sub-cohort members were matched to the VTE cases by five-year age categories. This matching was done as the incidence of VTE differ in age groups, and the average age of people with a VTE event is higher than the mean age of the populations in the original cohorts.

Even though various methods for dealing with confounding factors were applied in our analysis, residual confounding is always a concern. This type of confounding refers to a situation where the effect of a confounder is not fully resolved due to incomplete handling of confounding.[173] Residual confounding in cohort studies can occur when confounders are unknown, misclassified or unmeasured, or when the stratification leads to imbalance of characteristics between sub-groups. Due to the randomization of exposure/intervention in RCTs, they are the golden standard for minimizing confounding and establishing a causal relationship. Unfortunately, residual confounding will always be a challenge in observational studies.

5.1.6. Statistical power and precision

Statistical power can be defined as the probability of rejecting a false null hypothesis (H_0). A null hypothesis implies that no difference or association exists on the outcome variable of interest between the groups being compared.[330] An alternative hypothesis (H_a), the opposite of the null hypothesis, typically implies that a difference in the population studied does exist between groups that are compared on the outcome of interest.[330] Epidemiological studies often seek to either reject or confirm the null hypothesis (i.e. statistical hypothesis testing), based on the belief that the alternative hypothesis might be true. A type I error (also known as false positive) is the incorrect rejection of a

true null hypothesis, whereas type II error (also known as false negative) is the non-rejection of a false null hypothesis.

Statistical tests were developed in the early 1900s and became popular, especially in the field of medicine, because they provided clear-cut conclusions about whether an association existed or not.[331] Since the 1980s, an opposition against the use of statistical tests and clear cutoff p-values has been growing.[331] Several papers have debated on the division of results as "significant" or "non-significant" according to the commonly used threshold of P-values of 0.05.[331] The P-value is a function of the strength of the true association and the sample size.[331] This leads to a potential problem both in studies based on large sample sizes and studies based on small sample sizes. In an extremely large sample size, even the smallest differences becomes significant. Further, in a small sample size, large effects might not reach significant levels and thus, misleadingly, not be considered to be a true association. For instance, an intervention which reduces VTE risk by 1% could provide statistically significant results when tested in a large sample size. Contrary, an intervention that reduces the risk of VTE by a total of 50% tested in a small sample size could display a non-significant p-value, and thus, not be considered for clinical use. Thus, interpretation of the results in light of effect size and sample size is essential.

In the four papers in the present thesis, we analyzed our data using Cox regression models. The statistical power provided by Cox regression models depends on the number of events (i.e. VTE events). In all papers, we investigated different prothrombotic genotypes and the risk of VTE, also in relation to cancer status. For subgroups analysis, especially for rare genetic variations further categorized by cancer status, the number of VTE cases were low. For instance, there were only 10 VTE events in cancer patients homozygous for the GP6 SNP in Paper I. Thus, the analyses did not reach statistical significance, and the confidence intervals ranged from 0.85 to 3.07. The low statistical power is unfortunately seen in all papers, resulting in broad and overlapping confidence intervals. Our limited statistical power increases the risk of type II errors, a wrongfully rejection of the alternative hypothesis. Let's say that the finding of a 22.2-fold increased VTE risk by homozygosity of the FGG SNP and cancer investigated in Paper II, is in fact true. A low statistical power and a rejection of this finding could result in the prothrombotic genotype not to be further investigated or included in future prediction models. For small studies, confidence intervals can remind us that results are consistent with both the null hypothesis and the alternative hypothesis, indicating that both hypotheses can reflect the actual truth.

In a paper by J. Sterne and G.D. Smith, the authors describe how results of medical research should not be reported as "significant" or "non-significant" but should rather be interpreted in the context of the type of study and other available evidence.[332] In the paper "No P Please" by F.R. Rosendaal and P.H. Reitsma, the authors encourage to show effect sizes with confidence intervals and

limit the use of statistical hypothesis tests.[331] Several publications support their point of view, that presentation of statistical analysis should be done by presenting confidence intervals in addition to, or in place of, P-values, moving away from a mechanistic accept-reject dichotomy.[332] As stated in all the articles in the present thesis, the results should be interpreted with caution due to the wide confidence intervals. However, the low statistical power is a result of a low number of cases in subgroup analysis, and we consider the risk estimates to be representative. We therefore chose to focus on the risk estimates and the effect size and not only the significance levels as one should be seen and interpreted in the light of the other.

5.2. Discussion of main results

5.2.1. Effect of prothrombotic genotypes on venous thromboembolism in overt cancer

Prior to the present thesis, family and twin studies indicated that heritability explains up to 60% of VTE events,[155] and in recent years, several genetic variations have been found to influence the risk of VTE.[36] The majority of the genetic studies have, however, excluded individuals with cancer and thus, several of the known prothrombotic SNPs have never been investigated in cancer patients. Further, studies investigating different SNPs on VTE risk in cancer, are often small, with case-control designs and of selected populations, limiting the validity and generalizability of the results.

In Paper I, we reported that the risk of VTE decreased with the number of minor alleles at GP6 rs1613662 in the total case-cohort (i.e. including both subjects with and without cancer), with a 21% decreased risk of incident of VTE in carriers of the minor allele (i.e. AG/GG) at GP6. In cancer-free subjects, we found that homozygous carriers (GG) had a 34% decreased risk of incident VTE compared to subjects homozygous for the major allele (AA) at GP6. These findings are similar to previous observational case-control studies which have consistently demonstrated that carriers of the G-allele at GP6 have a 20% decreased VTE risk and inversely, that A-allele carriers are at a 15% higher risk of VTE than non-carriers. [177,178] In 2008, Bezemer and colleagues reported an OR of 1.15 (95% CI 1.01-1.30) for DVT risk for A-allele carriers of the GP6 SNP in the MEGA Study, including 1314 cases and 2877 controls.[172] In a report published in 2009, Tregouet *et al.* evaluated the effect of the GP6 SNP (rs1613662) on VTE risk in the three following case-control studies: The Marseille Thrombosis Association Study (MARTHA), the FARIVE Study, and a GWAS screening of 453 cases and 1327 controls recruited from four different French centers.[177] The OR of VTE according to the presence of the A-allele at the GP6 SNP in the three case-control studies ranged from 1.08 to 1.27.[177] Further, in a case-control study using data from the Genetic Attributes and Thrombosis Epidemiology (GATE) Study, Austin *et.al*

reported an OR of 1.04 for VTE by the presence of the A-allele at GP6 in 546 VTE cases and 663 controls of white Americans.[178]

GP6 rs1613662 distinguishes isoforms of the GP6 gene, which encodes the platelet receptor GPVI. GPVI is considered to play a crucial role in platelet adhesion and activation.[333] Platelets carrying the minor allele (G-allele/Pro219) at GP6 rs1613662 express fewer GPVI receptors,[175] which leads to attenuated platelet adhesion and activation.[176] Experimental animal studies have shown that platelets play a fundamental role in the formation of venous thrombi.[334-336] Using the Tromsø Study, Brækkan *et al.* have demonstrated that subjects with high MPV, a phenotype associated with increased platelet reactivity,[337-340] had 30% and 50% increased risk for total- and unprovoked VTE, respectively.[224] Further, Tsai *et al.* have reported that subjects with high levels (the highest quartile) of von Willebrand factor in plasma, a factor instrumental for adhesion of platelets to the vascular wall, have an almost 5-fold higher risk of future VTE compared to subjects in the lowest quartile.[222] In addition, randomized clinical trials have shown that treatment with the platelet inhibitor aspirin is associated with 20-30% reduced risk of recurrent VTE. [341,342] Our findings that carriers of the G allele(s) at GP6 have lowered risk of incident VTE provide further evidence that platelet function is involved in thrombus formation in VTE.

To the best of our knowledge, we were the first to investigate the effect of GP6 rs1613662 on VTE risk in cancer. We found that among cancer patients, subjects heterozygous for the minor GP6 allele (AG) exhibited decreased VTE risk of similar magnitude to that found in subjects without cancer. In contrast, cancer patients homozygous for the G-allele displayed an increased risk of VTE, when compared to cancer patients homozygous for the A-allele (AA). We also reported that the G-allele was associated with more prothrombotic cancers (i.e. lung, colorectal, hematological cancer and lymphomas) and presented with more severe stages of cancer (i.e. distant metastasis), when compared to subjects heterozygous or homozygous for the A-allele. The inverse effect may be explained by a differential impact of the G-allele at GP6 rs1613662 on the cancer type, cancer stages and mortality rates. However, variants at the GP6 rs1613662 may also have differential impact on platelet reactivity under various conditions. When we adjusted for cancer types and stages, the risk estimates were only moderately attenuated, indicating that the prothrombotic and metastatic cancers cannot be the whole explanation for the opposite effects observed in hetero- and homozygous carriers of the GP6 SNP.

Our findings support a role of platelet reactivity in the pathogenesis of VTE, which may differ according to cancer status. Elevated platelet counts are frequently observed in cancer patients, have been found to be associated with decreased survival,[113,115] and have been shown to predict future risk of cancer-related VTE events. [226,343] In contrast, no association between platelet count and the VTE risk has been observed in general populations. [139,223,224,226] In addition, mean platelet volume (MPV) has

shown differential association with VTE risk in subjects without and with cancer. Whereas high MPV is associated with an increased risk of VTE in the general population,[224] it is associated with a lower VTE risk and improved survival in cancer patients.[344] The mechanism(s) underlying the combined, but apparent opposite effect of homozygosity at the G-allele of GP6 rs1613662 and cancer on VTE risk remains elusive. Of note, we cannot rule out the possibility of a chance finding, as there are only 10 VTE cases in cancer patients homozygous for the G-allele at GP6.

Even though the role of prothrombotic genotypes in the pathogenesis of VTE in malignant disease have been scarcely studied, previous studies have found that some prothrombotic genotypes (e.g. factor V Leiden and prothrombin G20210A) are associated with increased risk of VTE in cancer patients.[264,266,271] Further, the combined effect of cancer and factor V variants (e.g. factor V Leiden rs6025 and rs4524) has been found to exceed the sum of the individual effects of the genetic variants and cancer, implicating a biological interaction on the risk of VTE.[43,267] Due to the findings of a synergistic and supra-additive effect of prothrombotic genotypes and cancer, we were interested in investigating the joint effect of other prothrombotic SNPs and cancer of the risk of VTE.

In paper II, we reported that homozygosity of the risk allele (C-allele) at FGG rs2066865 was associated with an increased risk of VTE. In 2005, Uitte de Willige *et al.* were the first to propose FGG rs2066865 as a novel risk factor for VTE in the Leiden Thrombophilia Study.[168] In this study, they demonstrated that the FGG polymorphism influences VTE risk merely in Caucasian populations, and not in African-Americans.[168] Further, the FGG SNP has not been found to influence VTE risk in Chinese populations.[345] Since 2005, several observational studies have reported an association between homozygosity of the FGG SNP risk alleles and an increased risk of VTE in Caucasians.[168,169,346] In a meta-analysis of seven observational studies, the OR was 1.61 for VTE in subjects with two risk alleles at the FGG SNP.[169] In correspondence with these results, we reported that subjects without cancer had a HR for VTE of 1.7, when compared to cancer-free subjects with zero risk alleles.

We also reported the combined effect of the FGG SNP and cancer on VTE. The combination of homozygosity (CC) at rs2066865 and active cancer displayed a synergistic effect on VTE risk on an additive scale, and the effect was particularly increased for the risk of PE. The finding might indicate that the FGG variant plays a more important role in the pathogenesis of PE than DVT in the presence of cancer. The underlying mechanism(s) for the latter observation is unknown, but may imply that rs2066865 is associated with fragile thrombi, which are more prone to embolization and manifest clinically as PE rather than DVT in cancer patients.

The mechanism by which the rs2066865 affects susceptibility to VTE is not fully elucidated. However, the current hypothesis is that it acts through a phenotype with altered fibrinogen

composition and formation. The FGG rs2066865 is thought to tag the FGG-H2 haplotype, meaning that the prothrombotic SNP is inherited along with other specific genotypes. Carriers of the FGG-H2 haplotype are found to have lower levels of γ' fibrinogen and γ' fibrinogen/total fibrinogen,[168] without altering the total fibrinogen level.[347] Fibrinogen γ' inhibits thrombin activity and thus, lower levels of γ' fibrinogen results in less inhibition of thrombin-mediated activation of FVIII, FV and platelets.[348-351] In addition, fibrinogen γ' has been found to increase the sensitivity for activated protein C (APC),[352] and thus, presence of FGG SNP resulting in lower levels of γ' fibrinogen, results in a reduced APC sensitivity.

Similarly to the findings of Gran *et al.* in a case-cohort of the Tromsø Study, where the two SNPs of F5 (FVL and rs4524) had a strong impact on cancer-related VTE directly surrounding the cancer diagnosis date,[267] we found that the cumulative incidence of VTE was substantially increased the first six months following the cancer diagnosis in subjects homozygous for the FGG SNP. These findings are in accordance with the thrombosis potential model (Figure 5), where several risk factors need to be present for a VTE event to occur. Alone, inherited risk factors may only mildly increase VTE risk, however, in the presence of cancer, the thrombosis potential is further increased and may results in a VTE event. In the time period following a cancer diagnosis, the patients often undergo treatment, such as surgery, radiation and/or chemotherapy, and treatment-related complications (e.g. acute infections and immobilization) occur frequently. The occurrence of these additional risk factors might explain some of the substantial increase in VTE incidence the first months following the cancer diagnosis date. However, studies have shown a substantially increased risk of VTE already six months prior to the cancer diagnosis, which cannot be explained by treatment-related risk factors.[185] The increased VTE risk prior to cancer diagnosis might be a result of an interplay between prothrombotic genotypes and the prothrombotic state of the cancer itself.

In Paper III, we investigated the risk of VTE in patients with overt cancer by the presence of prothrombotic genotypes, both as individual SNPs and as categories of the 5-SNP score (0-1, 2-3, and ≥4 risk alleles).[181] Further, we investigated whether the combination of cancer and prothrombotic genotypes displayed a biological interaction (i.e. synergistic effect) on VTE risk. For each prothrombotic genotype, ABO (rs8176719), F5 (rs6025), F2 (rs1799963), FGG (rs2066865), and F11 (rs2036914), the VTE risk increased in subjects with and without cancer. The risk of VTE was particularly increased for the genotypes in F5, FGG and ABO, where we reported a more than additive effect in combination with cancer. These findings were in accordance with previously published studies on prothrombotic genotypes and risk of cancer-related VTE. Factor V Leiden,[43,267] ABO rs505922 and rs8176746,[280] ABO rs8176719,[283] prothrombin G20210A,[263,274-276] and FGG rs2066865[353] have all been found to increase the risk of VTE in cancer patients. Genotypes of F5 (FVL and rs4525) and FGG have previously been found to exert a more than additive effect on VTE risk when combined with cancer.[267,353] Accordingly,

we found a positive RERI for the genotypes of ABO, FVL and FGG, indicating a biological interaction between these SNPs and cancer on VTE risk.

The 5-SNP score by de Haan and colleagues has been found to be associated with VTE risk in the general population,[181] and in subjects with ischemic stroke.[290] When we applied the 5-SNP score in Paper III, we found a dose-response relationship between the number of risk alleles and VTE risk in subjects with and without cancer. Further, the combined effect of cancer and the high-risk category of the GRS (≥4 risk alleles) yielded a more than additive effect on VTE risk, with a HR of 17 compared to those without cancer in the low-risk category (0-1 risk alleles). Measures of biological interaction revealed that 39% of the VTE events occurring among cancer patients with ≥4 risk alleles could be attributed to the interaction between the risk factors (i.e. cancer and SNPs). Our findings suggest that the GRS of the five prothrombotic SNPs could be a useful tool for identifying cancer patients at high risk of VTE. However, the predictive performance of the GRS in cancer patients remains to be determined.

Further, the finding of a more than additive effect on VTE risk when combining cancer and the SNPs, could indicate that the prothrombotic genotypes act through pathophysiological pathways that further increases the procoagulant state of malignancy. The five prothrombotic SNPs are all related to functions of the coagulation system and enhance its performance. Malignant tumors release cell-free DNA, tissue factor and growth factors that promote the release of neutrophil extracellular traps (NETs) from neutrophils.[112] These are main triggers of the intrinsic and extrinsic pathways of the coagulation system that in combination with prothrombotic genotypes will facilitate downstream coagulation activation with subsequent increased risk of thrombus formation. Moreover, acquired resistance to activated protein C is common in cancer patients, and may contribute to further increase the risk in patients with FVL.[265]

5.2.2. Effect of prothrombotic genotypes on venous thromboembolism in occult cancer

Studies have shown that the risk of VTE is increased already one year prior to a cancer diagnosis. [24,185] This finding might be explained by the prothrombotic state of the occult cancer alone, or the combination of occult cancer and other patient-related predisposing factors for VTE. It is not known to what extent individual prothrombotic genotypes and/or a GRS of five prothrombotic genotypes affect the risk of VTE in occult cancer as no study prior to ours have investigated this topic.

In Paper IV, we reported the risk of VTE by the presence of ABO rs8176719 (non-O blood type), F5 rs6025 (Factor V Leiden), F2 rs1799963 (prothrombin G20210A), FGG rs2066865 and F11 rs2036914, both as individual SNPs and number of risk alleles in a GRS in subjects with and without

cancer using the STAC cohort. Similar to Paper III and previous studies,[181-183] we confirmed that the risk of VTE increased by the individual SNPs and the number of risk alleles in the GRS in subjects without cancer.[181] In contrast, the prothrombotic SNPs were not associated with the risk of VTE in subjects with occult cancer, and accordingly, the combination of occult cancer and individual risk alleles or number of risk alleles in the GRS, did not display any biological interaction or synergism on the risk of VTE. The risk estimates were similar in sensitivity analyses where we altered the occult cancer period to six months and two years before the cancer diagnosis.

Previous studies have shown that VTE patients with occult cancer are more often diagnosed with prothrombotic cancers such as pancreatic, lung, gastrointestinal and hematological cancers,[24,29,35,79,91,354] and more advanced stages (higher degree of regional and distant metastasis) at the time of cancer diagnosis.[24,91,355] Accordingly, we reported that subjects who developed VTE during the occult cancer period had a higher proportion of regional and distant metastasis, as well as a higher proportion of cancers at high-risk sites, when compared with those who did not develop VTE during the occult cancer period. The mechanisms for VTE in cancer are multifactorial and involve overlapping pathways related to the cancer itself and patient-related factors.[249]

Our findings might indicate that the prothrombotic mechanisms related to VTE in occult cancer (i.e. aggressive and prothrombotic cancers) supersede the effect of the prothrombotic genotypes. Upregulation of tissue factor, which is found to be involved in cancer-progression, has been reported in advanced cancer stages,[105] as well as in high-risk sites for VTE.[356,357] In addition to TF expression, inflammatory responses with increased levels of circulating proinflammatory cytokines,[106-108] inhibition of fibrinolytic activity through expression of plasminogen activator inhibitor-1 (PAI-1),[109-111] and formation of NETs from neutrophils,[112] may substantially contribute to a prothrombotic state in rapidly developing, aggressive, occult cancers. Thus, the combination of several prothrombotic pathways induced by an advancing occult cancer, is likely sufficient to push an individual's thrombotic potential above the threshold for thrombus development, regardless of the presence of inherent prothrombotic risk factors.

6. Conclusions

- The G-allele at GP6 rs1613662 displayed a protective effect on VTE risk in cancer-free subjects, while an increased risk of VTE was observed in cancer patients homozygous for the G-allele. Our findings support a role of platelet reactivity in the pathogenesis of VTE, which may differ according to cancer status.

- Homozygosity at FGG rs2066865 was associated with an increased risk of VTE in both subjects with and without cancer. The combination of homozygosity for the rs2066865 genotype and active cancer displayed a synergistic effect on VTE risk on an additive scale, particularly on the risk of PE. Our findings may suggest that FGG is an attractive gene biomarker to pursue in future research on prediction models for VTE risk in cancer patients.

- For each of the five prothrombotic genotypes, ABO (rs8176719), F5 (rs6025), F2 (rs1799963), FGG (rs2066865), and F11 (rs2036914), the VTE risk increased in both cancer-free subjects and cancer patients. When the 5-SNP score was applied, we found a dose-response relationship between number of risk alleles and VTE risk in subjects with and without cancer. Further, the combined effect of cancer and the high-risk category of the GRS (≥4 risk alleles) yielded a supra-additive effect of VTE risk, indicating a biological interaction between the risk factors. These findings may suggest that the 5-SNP score may be useful for identifying cancer patients at increased risk of VTE.

- Five well-established prothrombotic genotypes (ABO (rs8176719), F5 (rs6025), F2 (rs1799963), FGG (rs2066865), and F11 (rs2036914)), alone or in combination, were not associated with the risk of VTE in subjects with occult cancer. Subjects with VTE in the occult cancer period had a higher frequency of prothrombotic cancer types and more advanced cancers at the time of cancer diagnosis. These findings might indicate that the potency of prothrombotic mechanisms related to rapidly advancing cancers at high-risk sites in occult cancer supersede the effect of prothrombotic genotypes on VTE risk.

7. Future perspectives

Malignancy is one of the most important risk factors for VTE.[15] Cancer patients who develop VTE have shortened life expectancy compared to cancer patients without VTE.[20,21] Further, the clinical consequences such as recurrent VTE, post-thrombotic syndrome and bleeding complications are typically more common and more severe in cancer patients suffering a VTE event compared to cancer-free VTE patients.[64-66] VTE is a potentially preventable disease by the use of anticoagulant treatment, however, current guidelines do not recommend routine thromboprophylaxis to all cancer patients due to the high risk of bleeding and uncertain benefit-to-harm ratio in these patients.[37-39] The severe complications and potentially fatal outcome of VTE in cancer stresses the need and importance of identifying high-risk subjects, to determine who would benefit from targeted prevention. In our studies, we found that the combination of established genetic risk factors, individual or combined in a GRS, and cancer displayed a supra-additive effect on the risk of VTE. This implies that genetic variations might be genetic biomarkers to pursue in future research on prediction models for VTE in cancer patients. As some biomarkers, such as platelet count[139,207,223,224,226] and MPV[224,344] behave differently as risk markers of VTE in cancer and non-cancer, future studies should search for novel and specific genetic variants associated with cancer-related VTE.

Several risk prediction models for VTE risk in cancer have been proposed, such as the Khorana score,[207] the Vienna CATS score,[252] the PROTECHT score,[250] and the CONKO score.[251] However, these risk scores focus mainly on clinical risk factors and modifiable biomarkers, and currently, they are not recommended in international guidelines due to their inadequate ability to discriminate between subjects of high and low VTE-risk and their unsatisfying performances in validation studies. As prothrombotic genotypes are fixed, not influenced by disease, interventions or complications, they are attractive candidates as biomarkers for VTE in cancer. New prediction models combining acquired and genetic risk factors for VTE may improve risk stratification.

In 2018, the TiC-Onco risk score was proposed for cancer patients of VTE risk, being the only score including prothrombotic genotypes combined with clinical factors.[358] The GRS of the TiC-Onco score is based on rs2232698, rs6025, rs5985 and rs4524, and the clinical factors included were high BMI (>25kg/m^2), family history of VTE and primary tumor site.[358] A recent study including 71 VTE events, showed that the TiC-Onco risk score performed significantly better at identifying high-risk patients of VTE than the Khorana risk score (AUC 0.73 vs 0.58, sensitivity 49 vs 22%, specificity 81 vs 82%, PPV 37 vs 22% and NPV 88 vs 82%).[358] Future studies should investigate if the genetic risk factors found to display a synergistic effect on VTE risk in combination with cancer, could improve the discriminatory ability of already established prediction models of VTE in cancer patients.

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Paper I

Genetic variation of platelet glycoprotein VI and the risk of venous thromboembolism

Family studies have indicated that heritability explains 50-60% of the venous thromboembolism (VTE) events,[1] and in recent years, several single nucleotide polymorphisms (SNP) have been found to influence the VTE risk.[2] Glycoprotein VI (*GP6*) *rs1613662*, also known as T13254C, is an A/G single nucleotide variation in amino acid 219, which results in a serine to proline substitution affecting the glycoprotein VI (GPVI) receptor for collagen.[3] Platelets carrying the minor allele (G-allele/Pro219) at *GP6 rs1613662* express fewer GPVI receptors,[4] which leads to attenuated platelet adhesion and activation.[5] Previous observational studies in selected populations have consistently demonstrated that carriers of the A-allele at *GP6* have a 15% higher risk of VTE than non-carriers, and inversely, that G-allele carriers have a 20% lower VTE risk.[6,7]

Cancer is associated with a highly increased risk of VTE,[8] and the complex interactions between inherited and acquired risk factors on VTE risk (*e.g.* genetic alterations and platelet count) are more compound and often different in malignancy.[9,10] The impact of *GP6 rs1613662* on the risk of VTE has only been investigated in case-control studies, and the combined effect of *GP6 rs1613662* and cancer on VTE risk has not been previously investigated. We therefore aimed (i) to investigate the association between *GP6 rs1613662* and VTE risk in the general population and stratified by cancer status, and (ii) to explore the combined effects of *GP6 rs1613662* and active cancer on the risk of VTE, using a large case-cohort recruited from the general population.

Cases with symptomatic, incident VTE (n=1,493) and a subcohort (n=13,072) were recruited from the fourth survey of the Tromsø Study and the second survey of the Nord-Trøndelag Health Study (HUNT), conducted in 1994-1995 and 1995-1997, respectively. Detailed descriptions of both studies have been published previously.[11,12] The Regional Committee of Medical Health Research Ethics approved the studies, and all participants gave their informed written consent to participate. VTE events were identified by broad searches at the hospitals providing health care for the two regions and thoroughly validated by review of medical records, as previously described in detail.[13,14] We excluded participants not registered as inhabitants of Tromsø or Nord-Trøndelag at study inclusion (n=3), subjects with a cancer diagnosis prior to inclusion (n=573) or with missing values for *GP6 rs1613662* (n=7). Eventually, the case-cohort consisted of 13,982 participants: 1,395 VTE cases and 12,587 subjects in the subcohort. *GP6 rs1613662* was genotyped with the Sequenom platform as previously described elsewhere.[10] The HUNT study performed genotyping using the Illumina HumanCore Exome array. Information regarding date of cancer diagnosis, primary site of malignancy (ICD-7-codes 140-205) and cancer stage during the entire follow-up, was obtained by linkage to the Cancer Registry of Norway.

Statistical analyses were performed using STATA version 15.0 (Stata Corporation LP, College Station, TX, USA). Cancer was entered as a time-varying covariate, and the data was split in relation to the date of cancer diagnosis. A VTE were considered cancer-related if it occurred within six months prior to and up to 2 years after the date of cancer diagnosis. Subjects with cancer who were alive and VTE-free at the end of the active cancer period were censored from this date onwards. Age-, sex- and body mass index (BMI)-adjusted hazard ratios (HR) with 95% confidence intervals (CI) for VTE were calculated according to variants at *GP6 rs1613662* subjects with and without cancer. Subjects homozygous for the major allele at *GP6* (*i.e.* AA) were used as the reference group in the categorized analyses. The proportional hazard assumption was confirmed by the use of Schoenfeld's global test. The Fine–Gray model[15] was applied in a sensitivity analysis to account for mortality as a competing event, and subdistribution hazard ratios (SHR) were estimated. Mortality rates and HR for death by categories of *GP6* alleles were estimated for disease free subjects, subjects with cancer only, subjects with VTE only, and subjects with cancer-related VTE. The distribution of cancer sites and cancer stages were displayed across *GP6* variants (*i.e.* AA, AG and GG).

Table 1. Baseline characteristics of study participants.

	Entire Case-Cohort	Active Cancer
Subjects	13,982	1,536
Age (years)	51.2±16.5	61.9±12.4
Sex (males)	47.4 (6,625)	52.0 (800)
BMI (kg/m^2)	26.3±4.2	26.8±4.3
Daily smoking	29.4 (4,113)	32.9 (505)
Self-reported CVD	8.3 (1,161)	12.6 (193)
*GP6 rs1613662**	0.17	0.17
Heterozygous (AG)	3,936	425
Homozygous (GG)	419	45

Values are numbers or percentages with numbers or means ± standard deviation (SD) in parenthesis. Active cancer: period from six months before a cancer diagnosis until 2 years after; BMI: body mass index; CVD: cardiovascular disease (stroke, angina, myocardial infarction); *: allele frequency (G allele).

Baseline characteristics in the entire cohort and in subjects with active cancer are presented in Table 1. The allele frequency of *GP6* was 0.17 in both the entire case-cohort and in the active cancer group, which is similar to the frequency reported in reference populations.[2,3]

Of the 1395 VTE events, 819 (58.7%) were deep vein thrombosis (DVT) and 576 (41.3 %) were pulmonary embolism (PE). The HR for VTE, DVT and PE adjusted for age, sex and BMI by categories of *GP6* alleles and cancer-status are presented in Table 2. The HR for incident VTE was 21% (HR 0.79, 95% CI: 0.70-0.89) lower in subjects with ≥1 G-alleles at *GP6*, when compared to subjects homozygous for the A-allele. The VTE risk was essentially similar for heterozygous (AG) and homozygous (GG) subjects compared to those homozygous for the major allele (AA). The number of G-alleles appeared to have a differential effect on the risk of DVT and PE. However, these findings should be interpreted with caution, as the number homozygous carriers in each category was low. The mortality rates for the entire case-cohort did not differ across the *GP6* variants, and coherently, the risk estimates from the competing risk by death model were similar to those obtained with the traditional Cox regression model (*data not shown*).

In cancer-free subjects, the risk of incident VTE decreased with the number of minor alleles, and subjects homozygous for the *GP6* allele (GG) had 34% decreased risk of incident VTE (HR 0.66, 95% CI: 0.43-1.01) compared to subjects homozygous for the major allele (A allele) at *GP6*.

There were 1,536 patients with active cancer of which 233 (15.2%) experienced a VTE. Among cancer patients, subjects heterozygous for the minor *GP6* allele (AG)

exhibited decreased VTE risk (HR 0.82, 95% CI: 0.60-1.11), which was of similar magnitude to that found in subjects without cancer. In contrast, cancer patients homozygous for the G-allele had a higher risk of VTE, DVT and PE, when compared to cancer patients homozygous for the A-allele (AA). In cancer patients, the risk of PE was particularly high in homozygous carriers of the G-allele (HR 1.96, 95% CI: 0.78-4.94).

The G-alleles were found to be associated with more prothrombotic cancers (*i.e.* lung, colorectal, hematological cancer and lymphomas) and presented with more severe stages of cancer (*i.e.*distant metastasis), when compared to subjects heterozygous or homozygous for the A-allele (Table 3). To test whether the association between homozygosity for the G-allele and increased risk of cancer-related VTE could be explained by variant-dependent differences in cancer types, cancer stages, and mortality rates, additional adjustments of age, sex, BMI, cancer types and stages as well as the Fine-Gray model were applied (Table 2). When cancer types and stages were included in the adjusted model, the risk estimates were moderately attenuated. Moreover, the risk estimates for cancer-related VTE in homozygous carriers of the G-allele remained essentially unchanged when competing risk by death was taken into account (data not shown).

Table 2. Hazard ratios with 95% confidence intervals for venous thromboembolism, deep vein thrombosis and pulmonary embolism by categories of *GP6 rs1613662* alleles and cancer.

GP6 alleles	All subjects Events	All subjects HR (95% CI)*	Cancer-free Events	Cancer-free HR (95% CI)*	Active cancer Events	Active cancer HR (95% CI)*	Active cancer HR (95% CI)†
VTE							
AA	1,039	Ref.	807	Ref.	168	Ref.	Ref.
AG	321	0.80 (0.70-0.89)	242	0.77 (0.66-0.88)	55	0.82 (0.60-1.11)	0.84 (0.62-1.15)
GG	35	0.83 (0.59-1.17)	22	0.66 (0.43-1.01)	10	1.61 (0.85-3.07)	1.39 (0.73-2.65)
AG/GG	356	0.79 (0.70-0.89)	264	0.76 (0.66-0.87)	65	0.88 (0.66-1.18)	0.90 (0.67-1.20)
DVT							
AA	593	Ref.	457	Ref.	102	Ref.	Ref.
AG	209	0.89 (0.76-1.05)	158	0.88 (0.73-1.05)	37	0.91 (0.62-1.33)	0.95 (0.65-1.39)
GG	17	0.70 (0.43-1.13)	10	0.52 (0.28-0.98)	5	1.33 (0.54-3.28)	1.13 (0.46-2.79)
AG/GG	226	0.87 (0.75-1.02)	168	0.84 (0.71-1.01)	42	0.94 (0.66-1.36)	0.97 (0.67-1.39)
PE							
AA	446	Ref.	350	Ref.	66	Ref.	Ref.
AG	112	0.65 (0.53-0.80)	84	0.62 (0.49-0.79)	18	0.68 (0.41-1.16)	0.70 (0.41-1.18)
GG	18	1.01 (0.63-1.62)	12	0.85 (0.48-1.50)	5	1.96 (0.78-4.94)	1.69 (0.67-4.28
AG/GG	130	0.68 (0.56-0.83)	96	0.64 (0.51-0.80)	23	0.80 (0.50-1.29)	0.80 (0.50-1.30)

HR: hazard ratio; CI: confidence interval; *: adjusted for age, sex and body mass index (BMI); †: adjusted for age, sex, BMI, cancer site and cancer stage; Active cancer: period from six months before a cancer diagnosis until two years after; Events: the number of venous thromboembolism (VTE), deep vein thrombosis (DVT), or pulmonary embolism (PE) in each category.

Table 3. The distribution of cancer types and stages across genotypes at *GP6 rs1613662* alleles (AA, AG, GG).

Cancer site	AA (n=1,723)	AG (n=702)	GG (n=75)	Total (n=2,500)
Colorectal	283 (16.4)	105 (15.0)	14 (18.7)	402 (16.1)
Upper GI tract	92 (5.3)	34 (4.8)	4 (5.3)	130 (5.2)
Pancreatic	53 (3.1)	15 (2.1)	2 (2.7)	70 (2.8)
Lung	178 (10.3)	85 (12.1)	15 (20.0)	278 (11.1)
Breast	176 (10.2)	86 (12.3)	2 (2.7)	264 (10.6)
Gynecologic	102 (5.9)	45 (6.4)	1 (1.3)	148 (5.9)
Prostate	279 (16.2)	119 (17.0)	14 (18.7)	412 (16.5)
Urologic	155 (9.0)	57 (8.1)	6 (8.0)	218 (8.7)
CNS	67 (3.9)	33 (4.7)	1 (1.3)	101 (4.0)
Hematologic and lymph	154 (8.9)	45 (6.4)	9 (12.0)	208 (8.3)
Remaining cancers*	184 (19.7)	78 (11.1)	7 (9.3)	269 (10.8)
Cancer stage				
Localized disease	586 (34.0)	277 (39.5)	18 (24.0)	881 (35.2)
Regional spread	399 (23.2)	158 (22.5)	17 (22.7)	574 (23.0)
Distant metastasis	318 (15.5)	126 (17.9)	19 (25.3)	463 (18.5)

CNS: central nervous system; GI: gastrointestinal; *: ear, nose throat, melanomas, endocrine, sarcomas and unknown sites; N: indicates the number of cancers in each category and the percentages are presented in parentheses.

Our findings support the notion that platelet function is involved in the pathogenesis of VTE. The mechanism(s) underlying the combined, but apparent opposite effect of homozygosity at the G-allele of *GP6 rs1613662* and cancer on VTE risk remains elusive. The inverse effect may be explained by a differential impact of the G-allele at *GP6 rs1613662* on the cancer type, cancer stages and mortality rates. However, variants at the *GP6 rs1613662* may also have differential impact on platelet reactivity under various conditions.

In contrast to cancer-free subjects where the VTE risk decreased with the number of G-alleles, cancer patients homozygous for the G-allele at *GP6 rs1613662* displayed an increased VTE risk. Our findings imply that measures of platelet function may have differential impact on the VTE risk in cancer-free subjects and cancer patients. Elevated platelet counts are frequently observed in cancer patients, are associated with decreased survival,[16] and have been shown to predict future cancer-related VTE events.[9] In contrast, no association between platelet count and the VTE risk has been observed in general populations.[9] In addition, mean platelet volume (MPV), a marker of platelet reactivity, has shown differential association with VTE risk in subjects without and with cancer. Whereas high MPV is associated with an increased risk of VTE in the general population,[17] it is associated with a lower VTE risk and improved survival in cancer patients.[18]

The main strengths of our study are the prospective design and the validation of VTE events and cancer diagnoses. The high attendance rate and wide age distribution in the parent cohorts reduces the chance of selection bias in the subcohort. Some limitations merit consideration. Unfortunately, cancer treatment modality was not available and restricted the possibility to evaluate the relationship between treatment related factors and genetics. Moreover, our study had limited statistical power, particularly in some subgroups. This resulted in wide CI, and our risk estimates should therefore be interpreted with caution.

In conclusion, the *GP6 rs1613662* G-allele displayed a protective effect on VTE risk in cancer-free subjects, while an increased risk of VTE was observed in cancer patients homozygous for the G-allele. Our findings support a role of platelet reactivity in the pathogenesis of VTE, which may differ according to cancer status.

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Paper II

ARTICLE

Fibrinogen gamma gene *rs2066865* and risk of cancer-related venous thromboembolism

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ABSTRACT

Venous thromboembolism (VTE) is a frequent complication in patients with cancer. Homozygous carriers of the fibrinogen gamma gene *(FGG) rs2066865* have a moderately increased risk of VTE, but the effect of the *FGG* variant in cancer is unknown. We aimed to investigate the effect of the *FGG* variant and active cancer on the risk of VTE. Cases with incident VTE (n=640) and a randomly selected age-weighted sub-cohort (n=3,734) were derived from a population-based cohort (the Tromsø study). Cox-regression was used to estimate hazard ratios (HR) with 95% confidence intervals (CI) for VTE according to categories of cancer and *FGG*. In those without cancer, homozygosity at the *FGG* variant was associated with a 70% (HR 1.7, 95% CI: 1.2-2.3) increased risk of VTE compared to non-carriers. Cancer patients homozygous for the *FGG* variant had a two-fold (HR 2.0, 95% CI: 1.1-3.6) higher risk of VTE than cancer patients without the variant. Moreover, the six-months cumulative incidence of VTE among cancer patients was 6.4% (95% CI: 3.5-11.6) in homozygous carriers of *FGG* and 3.1% (95% CI: 2.3-4.7) in those without risk alleles. A synergistic effect was observed between *rs2066865* and active cancer on the risk of VTE (synergy index: 1.81, 95% CI: 1.02-3.21, attributable proportion: 0.43, 95% CI: 0.11-0.74). In conclusion, homozygosity at the *FGG* variant and active cancer yielded a synergistic effect on the risk of VTE.

Introduction

Venous thromboembolism (VTE), a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease associated with substantial short- and long-term morbidity and mortality.[1,2] The incidence of VTE is 1-2 in 1,000 people/ year, and it increases steeply with age.[3] Malignant disease is associated with a four- to seven-fold increased risk of VTE, and 20-25% of all first lifetime VTE-events are cancer-related.[4,5] VTE, particularly in cancer, leads to prolonged and more frequent hospitalizations, and has a substantial impact on quality of life.[6,7] Complications of VTE, such as recurrence, post-thrombotic syndrome and treatment-related bleeding, occur more frequently in cancer patients,[6,8,9] and the risk of death is higher in cancer patients with than without VTE.[10,11]

Family and twin studies suggest that VTE is highly heritable, and likely results from an interplay between inherited and environmental factors.[12,13] Fibrinogen, the precursor of fibrin, is an essential component in the final stage of the coagulation cascade. The fibrinogen molecule has three subunits called Aα, Bβ and γ, which occur in pairs for a total number of six subunits. The γ chain, transcribed from the

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fibrinogen gamma gene (*FGG*) located on chromosome 4, has two isoforms, γA and γ'. In the Leiden Thrombophilia Study, the *FGG rs2066865* single nucleotide polymorphism (SNP) was first proposed as a risk factor for VTE by reducing fibrinogen γ' levels.[14] Several later genotyping[15,16] and genome-wide association studies (GWAS)[17,18] confirmed an association between *rs2066865* and VTE risk, whereas two cohort studies found no significant association.[19,20] In a recent meta-analysis including seven studies, *rs2066865* was associated with an increased risk of VTE (OR 1.61, 95% CI: 1.34-1.93).[21]

The majority of the genetic studies have excluded individuals with cancer-related thrombosis. However, as prothrombotic genotypes are fixed, and not influenced by disease, interventions and complications, they may be attractive candidates as biomarkers of VTE risk in cancer patients. Recent studies have suggested that interactions between cancer and other prothrombotic genotypes (factor V variants *rs6025* and *rs4524* and prothrombin *G20210A*) have synergistic effects on the risk of VTE.[22-25] To the best of our knowledge, no study has investigated the impact of *rs2066865* on the risk of VTE in cancer patients. Therefore, we aimed to investigate the joint effect of *rs2066865* and active cancer on the absolute and relative risks of VTE in a population-based case-cohort.

Methods

Study population

The Tromsø Study is a single-center population-based cohort, following residents of the municipality of Tromsø, Norway, with repeated health surveys. The case-cohort was derived from the fourth survey (Tromsø 4), which included 27,158 participants aged 25-97 years. A detailed cohort profile of the Tromsø study has been published previously.[26] The study was approved by the Regional Committee for Medical and Health Research Ethics in Northern Norway, and all participants provided informed written consent to participation. From enrolment in Tromsø 4 (1994/95), subjects were followed until December 31, 2012. Detailed information regarding identification and validation of VTE-events are described in the *Online Supplementary Material and Methods.*

In total, 710 participants developed VTE during follow-up. Of these, 26 did not have blood samples available or of sufficient quality for DNA analyses. The remaining 684 subjects were included as the cases in our study. A subcohort (n=3,931) was composed by randomly sampling individuals from Tromsø 4 weighted for the age distribution of the cases in 5-year age-groups. Due to the nature of the case-cohort design, where each participant has the same probability of sampling, 72 of the cases were also in the subcohort. Subjects with a history of cancer prior to inclusion (n=232) and subjects with missing information on *rs2066865* (n=9) were excluded from the analysis. The final case-cohort consisted of 4,374 subjects, with 640 cases and 3,734 in the subcohort. A flow chart of the case-cohort is displayed in Figure 1.

Baseline measurements and genotyping

Baseline measurements and genotyping methods are described in the *Online Supplementary Materials and Methods.*

Cancer exposure

Cancer assessment is described in the *Online Supplementary Materials and Methods*. Previous studies have shown a strong temporal relation between cancer diagnosis and incident VTE, and up to 50 % of cancer-related VTE events presents within a 2.5-year interval (from six months preceding the cancer diagnosis until 2 years following the cancer diagnosis).[27,28] Therefore, a VTE was defined as related to active cancer if it occurred within this time period.

Subjects who survived the active cancer period without a VTE were censored at the end of the active cancer period (*i.e.* 2 years after cancer was diagnosed). The censoring was performed because information regarding remission and relapse of cancer was unavailable, and extension of the observation period of cancer could result in the dilution of the estimates due to inclusion of VTE cases not necessarily caused by cancer. This approach resulted in censoring of 14 VTE cases that occurred after the active cancer period. Thus, 626 VTE cases were included in the final analyses.

Statistical analysis

Statistical analyses were performed using STATA version 15.0 (Stata Corporation LP, College Station, TX, USA). Cox proportional hazards regression models were used to obtain age- and sex-adjusted HR with 95% CI for VTE across categories of cancer status (no cancer/active cancer) and FGG risk alleles. Cancer was assessed as a time-dependent covariate in the model. Subjects who developed cancer contributed person-time as unexposed from the inclusion date until six months prior a cancer diagnosis, and thereafter contributed person-time in the active cancer group as exposed. Absolute incidence rates (IR) were calculated based on person-time from the original cohort (n=27,128). To calculate joint effects conferred by active cancer and FGG risk alleles, subjects with no cancer and no risk alleles were used as the reference group in the Cox model. Based on the total active cancer person-time at risk derived from the source cohort, 1-Kaplan-Meier curves were used to estimate the cumulative incidence of VTE in subjects with active cancer according to the presence of *FGG* risk alleles. Methods for assessing synergism between *FGG* and active cancer on the risk of VTE are described in detail in the *Online Supplementary Materials and Methods.*

Results

The mean follow-up of the case-cohort was 12.6 years. In total, 854 subjects had active cancer, of which 167 experienced an incident VTE. The baseline characteristics of

Table 1. Baseline characteristics in the entire case-cohort and in the active cancer group.

	Entire case-cohort	Active cancer
Subjects (n)	4374	854
Age (years)	58 ± 13	62 ± 10
Sex (males)	47.0 (2,048)	53.0 (456)
BMI (kg/m^2)	26.0 ± 4	26.0 ± 4
Daily smoking	34.5 (1,464)	43.5 (364)
WBC count (10^9/L)	7.1 ± 1.8	7.2 ± 1.8
Platelet count (10^9/L)	251 ± 60	250 ± 58
*rs2066865**	0.26	0.26
1 risk allele	1,723	334
2 risk alleles	289	51

Values are numbers or percentages with numbers in parenthesis or means ± standard deviation (SD). Active cancer: period from six months before a cancer diagnosis until two years after; BMI: body mass index; Daily smoking indicates smoking at the time of enrollment; WBC: white blood cell; *: allele frequency.

the entire case-cohort and in those with active cancer during follow-up are presented in Table 1. Subjects who developed active cancer were slightly older (61±10 years *vs.* 58±13 years) and reported a higher frequency of daily smoking (46% *vs.* 35%) compared to the entire case-cohort. The minor allele frequency of *rs2066865* was 0.26, which is comparable to reference populations.[14,29] The homozygous variant of the *FGG* was present in 289 (6.6%) subjects, the heterozygous variant in 1,723 (39.4%) subjects, while 2,362 (54.0%) subjects were non-carriers of the *FGG* variant. The allele frequency was essentially similar in subjects who developed cancer. Expected *versus* observed proportions of hetero- and homozygous individuals in the subcohort according to the Hardy-Weinberg equilibrium are presented in *Online Supplementary Table S1*.

The clinical characteristics of the VTE events stratified by the presence of active cancer are shown in Table 2. Compared to the non-cancer-related VTE, cancer-related VTE were more often a DVT (59.2% *vs.* 55.5%) than a PE (40.7% *vs.* 44.4%). The prevalence of provoking factors such as acute medical conditions, immobilization and surgery were essentially similar between the two groups, as were the total proportion of VTE with one or more concurrent provoking factors (44.3% *vs.* 44.7%). Non-cancer related VTE were more likely to be associated with traumas (9.6% *vs.* 2.4%) while other provoking factors *(i.e.* venous catheters) were more frequent in cancer-related VTE (8.4% *vs.* 3.7%).

In participants without cancer, the IR of VTE increased from 1.2 (95% CI: 1.1-1.4) per 1,000 people/year among non-carriers of *FGG rs2066865* to 2.0 (95% CI: 1.5-2.7) per 1,000 people/year among those with two risk alleles. Accordingly, the risk of VTE was 70% (HR 1.7, 95% CI: 1.2-2.3) higher in those with two risk alleles at FGG compared to non-carriers (Table 3). In subjects with active cancer, the risk was 12-fold higher (HR 11.9, 95% CI: 9.3-15.2) in those with no *FGG* risk alleles, and 22-fold higher (HR 22.2, 95% CI: 12.9-38.1) in those with two *FGG* risk alleles, compared to cancer-free subject without risk alleles. Cancer patients with two risk alleles at *FGG* had a two-fold higher (HR 2.0, 95% CI 1.1-3.6) risk of VTE compared to cancer patients without risk alleles. In sub-analyses, the effect of active cancer and homozygosity at FGG yielded higher risk estimates for PE (HR 2.9, 95% CI: 1.3-6.6) than for DVT (HR 1.6, 95% CI: 0.7-3.5).

The cumulative incidence of VTE during the active cancer period is shown in Figure 2. The cumulative incidence of VTE increased particularly during the first six months following a cancer diagnosis, where we found a substantially steeper incline in the incidence curve for subjects with two risk alleles at *FGG rs2066865*. The cumulative incidence of VTE among homozygous carriers was 5.0% (95% CI: 2.4-9.6), 6.4% (95% CI: 3.5-11.6), and 8.0% (95% CI: 4.6-13.9) at three months, six months and 24 months after cancer diagnosis, respectively. The corresponding figures for cancer patients who were non-carriers were 2.1% (95% CI: 1.5-3.0), 3.1% (95% CI: 2.3-4.7), and 4.8% (95% CI: 3.8-6.2), respectively.

A supra-additive effect on the risk of VTE was observed for the combination of homozygosity at the *FGG* variant and active cancer (Table 4). The Relative excess risk by

Table 2. Characteristics of subjects with cancer-related and non-cancer-related first venous thromboembolism.

	Cancer-related VTE	
	Yes (167)	No (459)
Age at VTE diagnosis (years)	69 ±11	68±14
Sex (Males)	44.9 (75)	47.3(217)
VTE type		
Deep vein thrombosis	59.2 (99)	55.5 (255)
Proximal upper limb	5.1 (5)	2.0 (5)
Distal upper limb	1.0 (1)	0 (0)
Proximal lower limb	62.6 (62)	65.9 (168)
Distal lower limb	12.1 (12)	28.2 (72)
Other localizations	19.1 (19)	3.9 (10)
Pulmonary embolism	40.7 (68)	44.4 (204)
Unprovoked event	NA	54.9 (252)
Provoking factors		
Surgery[a]	12.6 (21)	15.3 (70)
Trauma[a]	2.4 (4)	9.6 (44)
Acute medical condition[b]	15.0 (25)	14.2 (65)
Immobilization[c]	20.4 (34)	20.0 (92)
Other provoking factor[d]	8.4 (14)	3.7 (17)
Total provoked[e]	44.3 (74)	44.7 (205)

Values are numbers or percentages with numbers in parenthesis or means ± standard deviation (SD); VTE: venous thromboembolism; NA: not applicable; [a]within eight weeks before the VTE-event; [b]myocardial infarction, ischemic stroke of major infectious disease; [c]bedrest >3 days, wheelchair, long haul travel >4 hours in the past 14 days; [d]presence of other provoking factors noted by the physician (*e.g.* intravenous catheters); [e]one or more provoking factor above

Table 3. Age and sex adjusted hazard ratios for venous thromboembolism according to categories of fibrinogen gamma (FGG) risk alleles and cancer status.

		VTE			PE			DVT		
	Risk Alleles	Events	HR (95% CI)	HR (95% CI)	Events	HR (95% CI)	HR (95% CI)	Events	HR (95% CI)	HR (95% CI)
No cancer	0	242	Ref.	–	112	Ref.	–	130	Ref.	–
	1	170	1.0 (0.8-1.2)	–	70	0.9 (0.6-1.2)	–	100	1.1 (0.8-1.4)	–
	2	47	1.7 (1.2-2.3)	–	22	1.7 (1.1-2.7)	–	25	1.6 (1.1-2.5)	–
Active cancer	0	89	11.9 (9.3-15.2)	Ref.	32	8.3 (5.6-12.5)	Ref.	57	15.3 (11.2-21.1)	Ref.
	1	64	12.2 (9.2-16.1)	1.1 (0.8-1.5)	29	10.6 (7.1-16.3)	1.3 (0.8-2.2)	35	13.4 (9.2-19.6)	1.0 (0.6-1.5)
	2	14	22.2 (12.9-38.1)	2.0 (1.1-3.6)	7	22.8 (10.6-49.1)	2.9 (1.3-6.6)	7	21.6 (10.0-46.4)	1.6 (0.7-3.5)

Active cancer: period from six months before a cancer diagnosis until two years after; CI: confidence interval; DVT: deep vein thrombosis; HR: hazard ratio; PE: pulmonary embolism; VTE: venous thromboembolism.

interaction (RERI) was 9.61 (95% CI: -2.38-21.61) and the Rothmans synergy index (RSI) was 1.81 (95% CI: 1.02-3.21). The proportion attributable to interaction (AP) was 0.43 (95% CI: 0.11-0.74). In sub-group analysis, the estimates of biological interaction were stronger for PE (RSI=2.37, 95% CI: 1.05-5.39) than for DVT (RSI=1.46, 95% CI: 0.65-3.27).

Discussion

In the present study, we aimed to investigate the joint effect of the *rs2066865* SNP at *FGG* and active cancer on the risk of VTE in a case-cohort recruited from the general population. Homozygosity at *rs2066865*, occurring in 6.6% of the study population, was associated with an increased risk of VTE. The combination of an *rs2066865* homozygous risk genotype and active cancer showed a synergistic effect on VTE risk (on an additive scale). The effect was particularly strong for PE. The cumulative incidence of VTE increased substantially during the first six months following a cancer diagnosis, especially among patients with two risk alleles at *FGG rs2066865*. Our findings suggest that homozygosity at *FGG rs2066865* may aid to differentiate patients at high and low risk of cancer-related VTE.

Several observational studies have reported an association between homozygous genotype of *rs2066865* and increased risk of VTE in Caucasians.[14-16,21] In a recent meta-analysis including seven observational studies, the odds ratio of VTE was 1.61 for homozygosity at *rs2066865*.[21] Accordingly, in cancer-free subjects, we found that those with two *rs2066865* risk alleles had a 1.7-fold higher VTE risk than those with 0 risk alleles. The risk estimates for DVT and PE were essentially similar in cancer-free subjects.

Even though the role of prothrombotic genotypes in cancer-related VTE have been scarcely studied, previous studies have found that some prothrombotic genotypes (*e.g.* factor V Leiden and prothrombin G20210A) are associated with increased risk of cancer-related VTE.[22,23,30,31] Further, the combined effect of cancer and factor V variants (factor V Leiden and *rs4524*) exceeded the sum of the individual effects, implicating a biological interaction on VTE risk.[22,24] Accordingly, we found that the combination of *FGG* and active cancer yielded a synergistic effect on VTE risk.

In cancer patients, the cumulative incidence curve of VTE was substantially steeper in individuals homozygous for *FGG* during the first six months following the cancer diagnosis. According to the thrombosis potential model,[32] several risk factors need to be present concurrently to exceed the thrombosis potential and facilitate development of a VTE. In the period following a cancer diagnosis, treatment with surgery and/or chemotherapy is typically initiated, and treatment-related complications such as acute infection and immobilization frequently occur. Thus, the accumulation of several treatment-related risk factors, which adds to the background risk in patients with cancer and risk alleles at FGG, may partly explain the substantial increase in VTE incidence the first half year following a cancer diagnosis.

In contrast to cancer-free subjects, we found that the effect of *rs2066865* was stronger for PE than for DVT in cancer patients. This suggests that the *FGG* variant may play a more essential role in the pathogenesis of PE than DVT in cancer patients. The underlying mechanism(s) for the latter observation is unknown, but may imply that *rs2066865* is associated with fragile thrombi, which are prone to embolization and manifest clinically as PE rather than DVT in cancer patients.

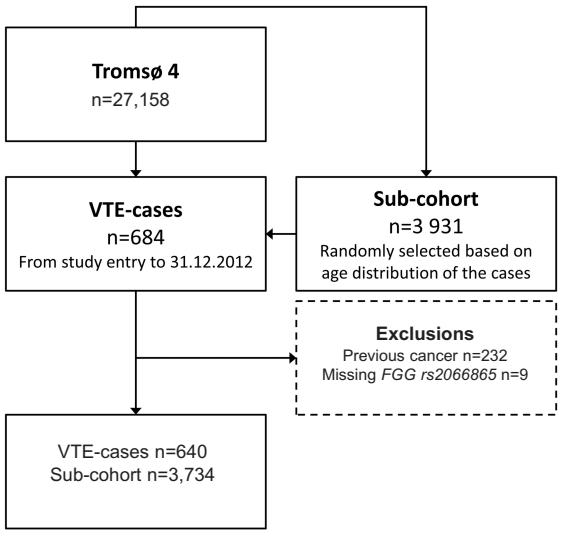

Figure 1. Flow chart for the case-cohort.

The mechanism by which the *rs2066865* affects susceptibility to VTE is not fully elucidated. However, the current hypothesis is that it acts through a phenotype with altered fibrinogen composition and formation. The *rs2066865* SNP tags the *FGG-H2* haplotype. Previous studies have shown that homozygous carriers of the FGG-H2 haplotype had lower levels of γ' fibrinogen and γ' fibrinogen/total fibrinogen concentration[14] without alterations in the total fibrinogen level.[33] The suggested mechanism is that the FGG variant favors formation of the abundant γ-chain isoform (γA) above the minor γ-chain (γ') through alternative splicing of the mRNA of the *FGG*-gene.[14,33] Fibrinogen γ' exhibits an inhibitory activity towards thrombin, due to a high affinity binding site on the γ' chain for thrombin exosite II,[34] which inhibits thrombin-mediated activation of factor VIII,[35] factor V[36] and platelets.[37] Moreover, fibrinogen γ' has been shown to increase the activated protein C (APC) sensitivity.[38] However, studies on the association between low plasma levels of fibrinogen γ' and VTE risk have shown somewhat inconsistent results.[14,20]

Current anticoagulant prophylaxis regimens efficiently prevent first VTE in cancer patients, but at the expense of a substantial risk of major and life-threatening bleedings.[39] Therefore, current international guidelines do not recommend prophylactic anticoagulation to all ambulatory cancer patients.[40,41] Thus, it is vital to recognize patients that are at high risk of cancer associated VTE, in order to identify those who would benefit most from thromboprophylaxis. Prothrombotic genotypes are attractive biomarker candidates, which could be used to distinguish between high and low risk of VTE in cancer patients, since they are fixed and not affected by the clinical status or treatment-related factors. In the present study, 6.4% of cancer

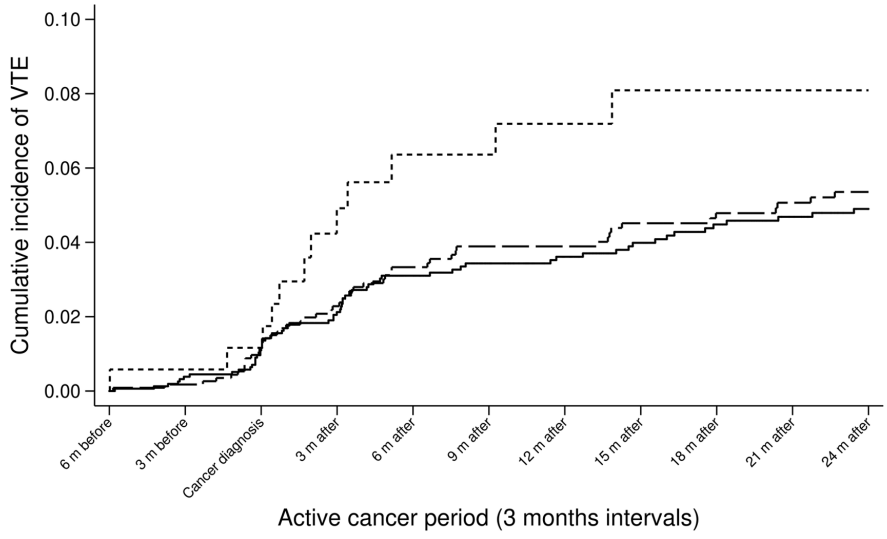

Figure 2. Cumulative incidence of venous thromboembolism in the presence of *FGG rs2066865* risk alleles during the active cancer period. VTE: venous thromboembolism; m: months.

Table 4. Measures of interaction between the homozygous fibrinogen gamma *(FGG)* variant and active cancer on venous thromboembolism.

	Rothmans synergy index (RSI) (95% CI)	Relative excess risk by interaction (RERI) (95% CI)	Proportion due to interaction (AP) (95% CI)
FGG rs2066865			
VTE	1.81 (1.02-3.21)	9.6 (-2.4-21.6)	0.43 (0.11-0.74)
PE	2.37 (1.05-5.39)	13.4 (-4.8-31.7)	0.56 (0.21-0.90)
DVT	1.46 (0.65-3.27)	6.3 (-9.6-22.1)	0.30 (-0.24-0.83)

Rothmans synergy index (RSI) >1 indicates a positive interaction or more than additivity; Relative excess risk by interaction (RERI) >0 indicates a positive interaction or more than additivity; Proportion due to interaction (AP) >0 indicates a positive interaction or more than additivity. VTE: venous thromboembolism; PE: pulmonary embolism; DVT: deep vein thrombosis; CI: confidence interval.

patients with two risk alleles at *FGG rs2066865* developed VTE during the first six months after cancer diagnosis compared to 3.1% of cancer patients without risk alleles. Our findings suggest that FGG may be an attractive gene candidate to pursue in future research on prediction models of VTE risk in cancer patients. We and others have previously reported similar discriminative power of two variants in the *F5* gene (*rs6025* and *rs4524*),[23,24] and a genetic model including nine SNP reported promising predictive capacity on VTE risk in breast cancer.[42] Recently, a new risk prediction model for cancer-related VTE, including clinical characteristics and genetic variants, reported a strong predictive capacity with an area under the curve (AUC) of 0.73 and performed better that the Khorana score (AUC 0.58).[43]

The main strengths of present study are the prospective design, high participation rate and long-term follow-up, making it possible to capture a large quantity of both incident cancer- and VTE-events in the study population. Since all participants live within a single hospital catchment area, the probability of missing outcomes is low. Moreover, both incident VTE-events and cancer diagnoses were systematically validated and objectively confirmed. The study was limited by the lack of statistical power in sub-group analysis (*i.e.* DVT/PE), illustrated by wide CI for our risk estimates. In addition, we did not have access to information on treatment regimens or medical complications among cancer patients. Although there is no reason to believe that the type or intensity of treatment would be influenced by the genetic makeup, such data could have provided further insights into the possible interplay between genes and treatment-related risk factors.

In conclusion, we found that homozygosity at *FGG rs2066865* was associated with an increased risk of VTE, and yielded a synergistic effect on the VTE risk in combination with active cancer, particularly on the risk of PE.

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Paper III

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ORIGINAL ARTICLE

Combined effects of five prothrombotic genotypes and cancer on the risk of a first venous thromboembolic event

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Abstract

Background: The role of combined prothrombotic genotypes in cancer-related venous thromboembolism (VTE) is scarcely studied. We aimed to investigate the impact of a 5-single nucleotide polymorphism (SNP) score on the risk of VTE in patients with and without cancer using a population-based case-cohort.

Methods: Cases with a first VTE (n = 1493) and a subcohort (n = 13 072) were derived from the Tromsø Study (1994-2012) and the Nord-Trøndelag Health Study (1995-2008). Five SNPs previously reported as a risk score were genotyped: ABO (rs8176719), F5 (rs6025), F2 (rs1799963), FGG (rs2066865), and F11 (rs2036914). Hazard ratios (HRs) for VTE were estimated according to cancer status and the number of risk alleles in the 5-SNP score (0-1, 2-3, and ≥4 alleles).

Results: During a median follow-up of 12.3 years, 1496 individuals were diagnosed with cancer, of whom 232 experienced VTE. The VTE risk increased with the number of risk alleles in the 5-SNP score among subjects without and with cancer. In cancer-free subjects, the HR was 2.17 (95% confidence interval [CI] 1.79-2.62) for ≥4 versus 0-1 risk alleles. In cancer patients, the corresponding HR was 1.93 (95% CI 1.28-2.91). The combination of cancer and ≥4 risk alleles yielded a 17-fold (HR 17.1, 95% CI 12.5-23.4) higher risk of VTE compared with cancer-free subjects with 0-1 risk alleles.

Conclusion: The risk of VTE increases with the number of prothrombotic risk alleles in subjects with and without cancer, and the combination of prothrombotic risk alleles and cancer leads to a highly elevated risk of VTE.

KEYWORDS

5-SNP score, cancer, deep vein thrombosis, prothrombotic genotypes, pulmonary embolism, risk, venous thromboembolism

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1 | INTRODUCTION

Venous thromboembolism (VTE), a collective term for deep vein thrombosis (DVT) and pulmonary embolism (PE), is a severe and frequent complication of malignancy, of which the incidence is increasing.[1,2] Cancer is associated with a four- to seven-fold increased risk of VTE,[3-5] and approximately 15% of cancer patients will develop a VTE during the course of malignancy.[6] Cancer patients who develop VTE have shortened life expectancy compared with cancer patients without VTE.[7,8] The clinical consequences of VTE, such as post-thrombotic syndrome, VTE recurrence, and treatment-related bleeding, occur more often in cancer patients than in cancer-free subjects.[7,9] Current guidelines do not recommend routine thromboprophylaxis to all ambulatory cancer patients due to the uncertain benefit-to-harm ratio, which emphasizes the importance of identifying high-risk subjects.[10-12]

Simulation studies have shown that mapping genetic profiles may be useful to detect subjects at high and low risk of disease.[13,14] Genetic profiling may therefore help to identify subjects in need of VTE prophylaxis in high-risk situations, such as cancer, surgery, or prolonged immobilization. Several single nucleotide polymorphisms (SNPs) are associated with the risk of VTE.[15-19] To identify high-risk individuals, de Haan et al created a genetic score based on 31 SNPs previously reported to increase VTE risk.[20] In this score, the SNPs with highest odds ratios of VTE were added one-by-one to finally create a genetic risk score containing five SNPs: rs8176719 (non-O blood type) in ABO, rs6025 (factor V Leiden [FVL]) in F5, rs1799963 (prothrombin G20210A) in F2, rs2066865 in the fibrinogen gamma gene (FGG), and rs2036914 in F11. This 5-SNP score performed similarly to the score of all 31 SNPs,[20] and detected subjects at increased risk of both incident and recurrent VTE.[20-22]

Genetic alterations such as Factor V Leiden,[23,24] ABO rs505922 and rs8176746,[25] ABO rs8176719,[26] and prothrombin G20210A[27-30] have all been found to be associated with VTE risk in cancer. Moreover, the combined effect of cancer and the factor 5 SNPs rs6025 and rs4524 increased VTE risk on a supra-additive scale, indicating a biological interaction.[24] As prothrombotic genotypes only need to be measured once, and are not influenced by disease progression, interventions, or complications, they are attractive candidate biomarkers of VTE risk in cancer patients.

The role of genetics in cancer-related VTE is not yet fully elucidated, and to the best of our knowledge, no previous study has investigated the impact of the 5-SNP score on VTE risk in cancer patients. Therefore, we aimed to investigate the impact of increasing number of risk alleles in the 5-SNP score on the risk of VTE in subjects with and without cancer using a case-cohort recruited from the general population.

Essentials

- The role of prothrombotic SNPs in cancer-related venous thromboembolism (VTE) is scarcely studied.
- We investigated if a 5-SNP score was associated with VTE risk in subjects with and without cancer.
- The risk of VTE increased with increasing number of 5-SNP score risk alleles in both groups.
- The combination of prothrombotic risk alleles and cancer led to a highly elevated risk of VTE.

2 | METHODS

2.1 | Study population

Study participants were derived from the fourth survey of the Tromsø Study, conducted in 1994-1995,[31] and the second survey of the Nord-Trøndelag Health (HUNT) Study, conducted in 1995-1997.[32] The Tromsø Study (Tromsø 4) and the HUNT Study (HUNT2) are Norwegian population-based cohorts of the inhabitants of Tromsø municipality and Nord-Trøndelag County, respectively. A total of 27 158 unique individuals aged ≥25 years participated in Tromsø 4, and 66 140 individuals aged ≥20 years participated in HUNT, yielding attendance rates of 77% (Tromsø 4) and 71% (HUNT2). Detailed descriptions of the studies have been published elsewhere.[31,32]

Participants were followed from the date of inclusion until a verified first VTE diagnosis, migration, death, or end of follow-up (December 31, 2008 in HUNT2 and December 31, 2012 in Tromsø 4). In Tromsø 4, all VTE events were identified by searching the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry at the University Hospital of North Norway (UNN), which is the sole provider of diagnostic radiology and treatment of VTE in the Tromsø area. Trained personnel reviewed the medical records for each potential VTE case, and incident VTE events were included when clinical signs and symptoms of PE or DVT were combined with radiologic confirmation and treatment was initiated (unless contraindications were specified). In HUNT2, VTE events were identified by searching the hospital discharge diagnosis registry and the radiology procedure registry at the two local hospitals in the county (Levanger Hospital and Namsos Hospital) and by searching the discharge diagnosis registry of the tertiary-care center of the region, St. Olav's Hospital in Trondheim. Two physicians reviewed the medical records for each VTE event, and the validation criteria included symptomatic VTE events confirmed by radiologic procedures (ultrasound, venography, computed tomography [CT] scan, or perfusion-ventilation scan) which required treatment. The identification and adjudication process of VTEs in both studies have been previously described in detail.[33,34] Participants with a history of VTE before inclusion in the parent cohorts were excluded.

We created a case-cohort by including all cases with a first lifetime VTE (n = 1493) and a randomly sampled subcohort (n = 13 072) derived from the parent cohorts (Figure 1). Participants not registered as inhabitants of Tromsø or Nord-Trøndelag at study inclusion (n = 3) and subjects with missing information on risk alleles (n = 170) or body mass index (n = 80) were excluded. Further, subjects with a cancer diagnosis prior to or less than 6 months after inclusion (n = 624) were excluded. Eventually, the case-cohort consisted of

13 688 participants, of whom 1362 were VTE cases. Due to the nature of the case-cohort design, in which every person in the cohort, including the cases, has the same probability of being selected to the subcohort, 206 of the subjects randomly selected to the subcohort were also cases. All participants gave their informed written consent to participate and The Regional Committee of Medical Health Research Ethics approved the study.

2.2 | Baseline measurements and genotyping

Baseline information was obtained by physical examination, blood sampling, and self-administrated questionnaires in each study. Body height and weight were measured with participants wearing light clothing and no shoes. Body mass index (BMI) was calculated by dividing the weight in kilograms (kg) by height in meters (m) squared (kg/m^2). Information on history of cardiovascular disease (myocardial infarction, angina, or stroke), diabetes mellitus, and smoking status were obtained from the questionnaires.

DNA was isolated from blood, and the following five SNPs were genotyped: ABO rs8176719 (non-O blood type), F5 rs6025 (Factor V Leiden), F2 rs1799963 (prothrombin G20210A), FGG rs2066865, and F11 rs2036914. In Tromsø 4, the Sequenom platform was used for genotyping rs8176719 (ABO), rs6025 (F5), rs1799963 (F2), and rs2036914 (F11), while rs2066865 (FGG) was genotyped with the TaqMan platform, as previously described.[35] In HUNT2, genotyping was performed using the Illumina HumanCore Exome array.

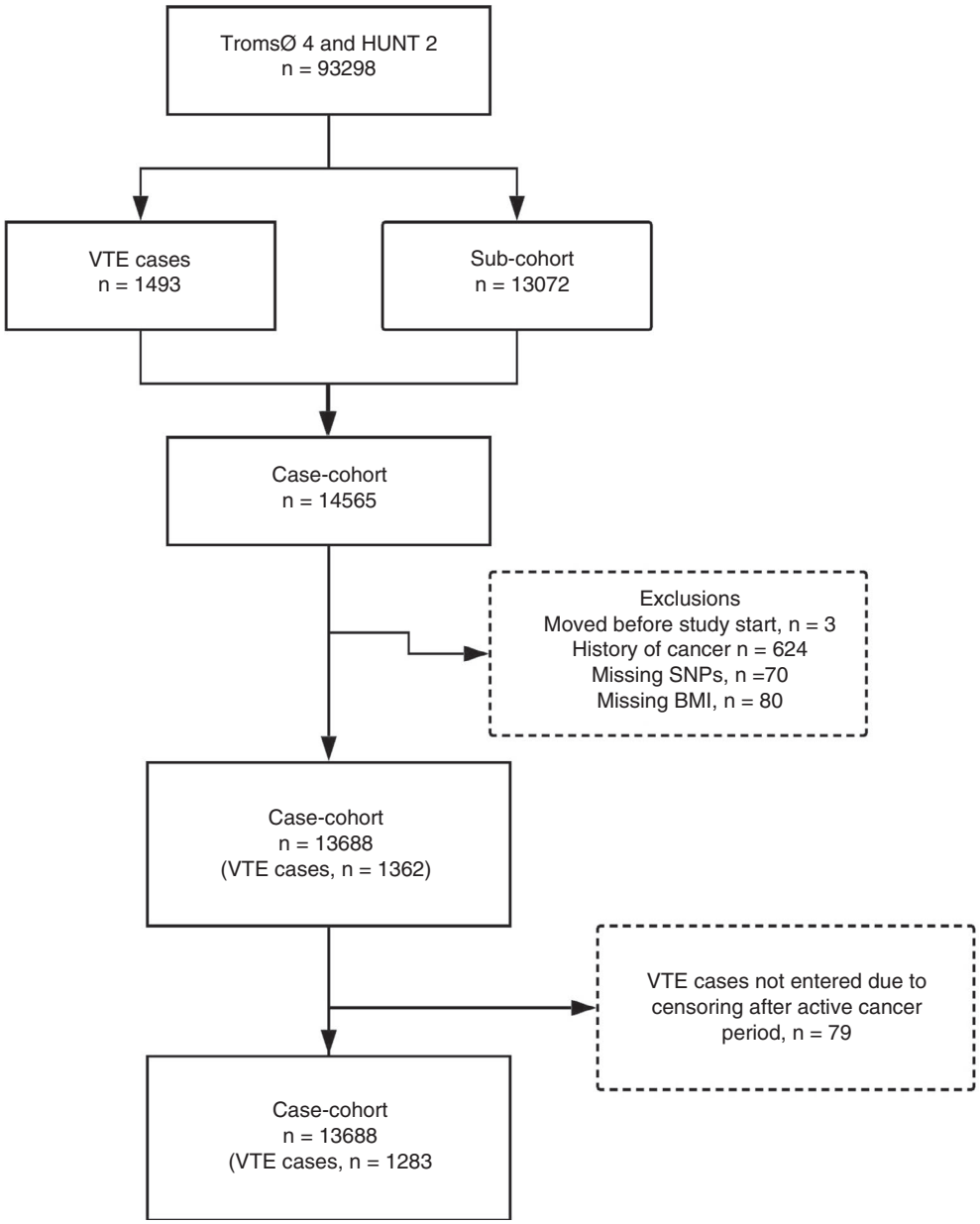

FIGURE 1 Study population. Participants were recruited from the fourth survey of the Tromsø study (1994-2012) and the second survey of the Nord-Trøndelag Health (1995-2008) study. VTE indicates venous thromboembolism; SNPs, single nucleotide polymorphisms

Subjects were considered as carriers of prothrombotic genotypes if one or two risk alleles were present. Hence, we did not differentiate between hetero- and homozygous carriers. Because the minor allele at F11 rs2036914 is associated with reduced VTE risk,[36] the common allele was set as risk allele in our analysis. Zero risk alleles at ABO rs8176719 was defined as O blood type, whereas one or two risk alleles were classified as non-O blood type. The 5-SNP score conceived by de Haan et al was created by summing the number of risk alleles from the five sequenced SNPs.[20]

2.3 | Cancer assessment

Information on cancer diagnosis, primary cancer site (International Classification of Diseases, Revision 7 [ICD-7] codes 140-205), and stage during follow-up, was obtained by linkage to the Cancer Registry of Norway. The Cancer Registry of Norway is considered a complete and valid registry with reported 98.8% completeness and with 94% of the cases being histologically verified.[37] Non-melanoma skin cancers (ICD-7 codes 191.0-191.9) were classified as non-cancer due to the pathophysiology and nature of these cancers.

As temporal proximity to cancer diagnosis is shown to be a strong predictor for VTE risk,[1,24,38] a VTE event was classified as related to active cancer if it occurred within 6 months prior to a cancer diagnosis until 2 years following the cancer diagnosis date. Patients who were not diagnosed with VTE and still alive at the end of the active cancer period were censored at this time, as information regarding cancer progression and remission was not available and extending the active cancer period could result in dilution of the results by including VTE events that were not related to cancer. Consequently, 79 VTE events that occurred beyond the active cancer period were never counted in the analyses. Thus, the total number of VTE events in the analyses were 1283, of which 232 occurred in the active cancer period.

2.4 | Statistical analysis

Statistical analyses were carried out using STATA version 15.0 (Stata Corporation). Cancer was entered as a time-varying covariate and data were split in relation to cancer diagnosis date to distinguish between non-cancer and cancer-exposed periods. Individuals who developed cancer during follow-up contributed with exposure status as cancer-free until 6 months prior to their cancer diagnosis date, and from that point onward exposure status changed to active cancer. Two years after the cancer was diagnosed, the cancer patients who were still alive and had not experienced a VTE event were censored from the analyses. Thus, individuals who developed cancer contributed to both non-exposed and cancer-exposed person-years (PY) at risk during the study period.

Cox proportional hazard regression models were used to estimate the hazard ratios (HRs) with 95% confidence intervals (CIs) for incident VTE according to the individual SNPs or categories of risk alleles (the 5-SNP score categories, ie, 0-1, 2-3, and ≥4 risk alleles) and by cancer status (cancer free and active cancer). In analyses stratified on cancer status, subjects with 0-1 risk alleles were used as reference category. All analyses were adjusted for age, sex, and BMI. The proportional hazard assumption was tested by the use of Schoenfeld residuals and was found not to be violated.

We also investigated the combined effect of cancer and risk allele categories on VTE risk using cancer-free subjects with 0-1 risk alleles as reference category. The presence of a biological interaction between cancer and presence of SNPs was calculated using the relative excess risk attributable to interaction (RERI), the attributable proportion due to interaction (AP), and the synergy index (SI) with corresponding 95% CIs.[39,40] Briefly, the RERI can be interpreted as part of the total effect on outcome (eg, VTE) that is due to interaction (eg, between cancer and prothrombotic SNPs), the AP as the proportion of the combined effect that is attributable to interaction between the two exposures. A RERI and an AP > 0, and a synergy index >1.0 suggest a positive interaction, ie, the combined effect of two exposures is larger than the sum of the two separate effects.[39]

3 | RESULTS

There were 1496 subjects diagnosed with cancer during a median follow-up of 12.3 years. The baseline characteristics of cancer-free subjects and cancer patients with and without VTE are presented in Table 1. In cancer-free subjects, participants who experienced a VTE were older, had more cardiovascular disease, and higher BMI than those without VTE. In cancer patients with VTE, there was a higher proportion of women and smokers and they were of younger age than cancer patients without VTE.

The proportions of non-cancer and cancer patients across increasing number of risk alleles in the 5-SNP score are shown in Figure 2. The total number of risk alleles ranged from zero to seven in cancer-free subjects and from zero to six in cancer patients, with a median of two in both groups.

The risk estimates for VTE according to prothrombotic SNPs in subjects with and without cancer are presented in Table 2. In subjects without cancer, all five SNPs were associated with an increased risk of VTE by the presence of one or more risk allele. The highest VTE risks were found for FVL (rs6025, HR 2.50, 95% CI 2.13-2.95), prothrombin (rs1799963, HR 1.55, 95% CI 1.02-2.36), and ABO (rs8176719, HR 1.47, 95% CI 1.29-1.86). The greatest risk of a cancer-related VTE was seen in subjects with FVL (rs6025, HR 1.89, 95% CI 1.29-2.77), prothrombin (rs1799963, HR 1.39, 95% CI 0.44-4.37), and FGG (rs2066865, HR 1.34, 95% CI 1.03-1.74). Measures quantifying interaction on an additive scale (ie RERI, AP, and synergy index) suggested a positive interaction between cancer and the presence of three of the five prothrombotic SNPs; ABO (rs8176719), FVL (rs6025), and FGG (rs2066865; Table 3).

The risk of VTE increased across categories of increasing risk alleles in the 5-SNP score (0-1, 2-3, ≥4) in subjects with and without

TABLE 1 Baseline characteristics of the study population with and without cancer and VTE

	No cancer		Cancer	
	Subcohort	VTE	Subcohort	VTE
Participants, n[a]	12 637	1051	1264	232
Age, y	50.2 ± 16.3	60.3 ± 15.1	62.0 ± 12.2	60.6 ± 13.4
Male sex	48.3 (5977)	47.8 (502)	53.2 (673)	44.4 (103)
BMI, kg/m^2	26.2 ± 4.1	27.7 ± 4.5	26.8 ± 4.3	26.9 ± 4.2
Cardiovascular disease	7.7 (972)	14.1 (148)	12.3 (156)	12.1 (28)
Smoking	29.8 (3770)	25.0 (263)	32.2 (407)	34.9 (81)
rs8176719 (ABO)[b]	61.4 (7767)	69.9 (735)	60.6 (766)	68.1 (158)
rs6025 (F5)[b]	6.9 (873)	16.3 (171)	7.5 (95)	13.4 (31)
rs1799963 (F2)[b]	1.3(169)	2.1 (22)	1.3 (17)	1.29 (3)
rs2066865 (FGG)[b]	42.3 (5346)	45.9 (482)	40.8 (516)	47.8 (111)
rs2036914 (F11)[b]	78.2 (9879)	82.5 (867)	78.6 (994)	81.0 (188)

Note: Values are in % (n) or mean ± standard deviation.

Abbreviations: BMI, body mass index; VTE, venous thromboembolism.

[a]Participants with cancer contributed to observation periods both in the no cancer and cancer group.

[b]Percentage of participants with ≥1 risk allele.

cancer. In those without cancer, the HR for ≥4 versus 0-1 risk alleles was 2.17 (95% CI 1.79-2.62). In cancer patients, the corresponding HR was 1.93 (95% CI 1.28-2.91). Similar results were found when analyzing DVT and PE separately (Table S1 in supporting information).

In subjects with 0-1 risk alleles, the risk of VTE was nine-fold higher in cancer patients than in those without cancer. Accordingly, there was a synergistic effect between the number of risk alleles and cancer on the relative risk of VTE. Subjects with cancer and ≥4 risk alleles had a 17-fold (HR 17.1, 95% CI 12.5-23.4) higher risk of VTE than cancer-free subjects with ≤1 risk allele. This combined effect was higher than expected on the basis of the individual effects of cancer and ≥4 risk alleles (RERI 6.72 95% CI 1.17-12.26). The AP revealed that 39% of the total VTE events in participants with cancer and ≥4 risk alleles were attributable to the interaction between the two exposures (ie, cancer and ≥4 risk alleles).

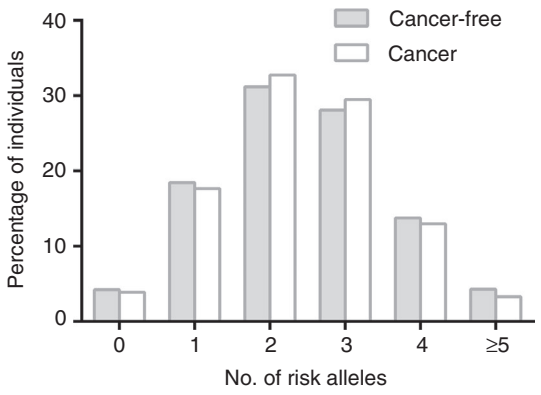

FIGURE 2 Distribution (%) of individuals across number (#) of risk alleles in study participants with and without cancer

4 | DISCUSSION

In this case-cohort study, we investigated the risk of cancer-related VTE by the presence of prothrombotic genotypes, both as individual SNPs and as categories of the 5-SNP score (0-1, 2-3, and ≥4 risk alleles).[20] Moreover, we investigated whether the combination of cancer and prothrombotic genotypes had a biological interaction on VTE risk. For each prothrombotic genotype, the VTE risk increased in both cancer-free subjects and in cancer patients, and particularly for FVL, FGG, and ABO there was a more than additive effect in combination with cancer. When the 5-SNP score was applied, we found a dose-response relationship between the number of risk alleles and VTE risk in subjects with and without cancer. Likewise, the combined effect of cancer and the high-risk category of the genetic score (≥4 risk alleles) yielded a more than additive effect on VTE risk, with an HR of 17 compared with those without cancer in the low-risk category (0-1 risk alleles). The AP revealed that 39% of the VTE events occurring among cancer patients with ≥ 4 risk alleles could be attributed to the interaction between the risk factors.

Our findings on the role of individual prothrombotic genotypes and risk of cancer-related VTE are in line with the previously published studies. Factor V Leiden,[23,24] ABO rs505922 and rs8176746,[25] ABO rs8176719,[26] prothrombin G20210A,[27-30] and FGG rs2066865[41] have all been found to increase the risk of VTE in cancer patients. Moreover, SNPs in the F5 gene (FVL and rs4525) and FGG[41] have been shown to exert a more than additive effect on VTE risk when combined with cancer. Accordingly, we found a positive RERI for the SNPs in ABO, FVL, and FGG, which indicated a biological interaction.

Even though the 5-SNP score has been shown to predict VTE risk in the general population,[20] and in subjects with ischemic stroke,[42] the combined effect of the 5-SNP score and cancer has not been

TABLE 2 Hazard ratios (HR) with 95% confidence intervals (CI) for venous thromboembolism (VTE) by categories of prothrombotic genotypes and cancer

		n	Events	HR (95% CI)[a]	HR (95% CI)[a]
Cancer	rs8176719 (ABO)[b]				
–	–	5210	316	Ref.	Ref.
–	+	8502	735	1.47 (1.29-1.68)	1.48 (1.29-1.69)
+	–	562	74	Ref.	8.68 (6.73-11.20)
+	+	934	158	1.31 (0.99-1.73)	11.07 (9.12-13.44)
Cancer	rs6025 (F5)[b]				
–	–	12 644	880	Ref.	Ref.
–	+	1044	171	2.50 (2.13-2.95)	2.50 (2.12-2.95)
+	–	1370	201	Ref.	8.16 (6.98-9.54)
+	+	126	31	1.89 (1.29-2.77)	15.95 (11.12-22.87)
Cancer	rs1799963 (F2)[b]				
–	–	13 497	1029	Ref.	Ref.
–	+	191	22	1.55 (1.02-2.36)	1.55 (1.02-2.37)
+	–	1476	229	Ref.	7.93 (6.85-9.18)
+	+	20	3	1.39 (0.44-4.37)	9.05 (2.91-28.17)
Cancer	rs2066865 (FGG)[b]				
–	–	7860	569	Ref.	Ref.
–	+	5828	482	1.14 (1.01-1.28)	1.14 (1.01-1.28)
+	–	869	121	Ref.	7.36 (6.03-8.98)
+	+	627	111	1.34 (1.03-1.74)	9.78 (7.96-12.01)
Cancer	rs2036914 (F11)[b]				
–	–	2942	184	Ref.	Ref.
–	+	10 746	867	1.27 (1.08-1.49)	1.27 (1.08-1.49)
+	–	314	44	Ref.	9.21 (6.62-12.81)
+	+	1182	188	1.11 (0.79-1.54)	9.63 (7.84-11.83)
Cancer	5-SNP score[c]				
–	0-1	3106	170	Ref.	Ref.
–	2-3	8111	589	1.33 (1.12-1.57)	1.33 (1.12-1.57)
–	≥4	2471	292	2.17 (1.79-2.62)	2.15 (1.78-2.60)
+	0-1	322	41	Ref.	9.16 (6.51-12.90)
+	2-3	931	139	1.18 (0.83-1.68)	10.3 (8.21-12.9)
+	≥4	243	52	1.93 (1.28-2.91)	17.1 (12.5-23.4)

[a]Adjusted for age, sex, and body mass index.

[b]Positive indicating subjects with one or two risk alleles.

[c]Number of risk alleles.

studied previously. In cancer patients, those with ≥4 risk alleles had an almost two-fold higher VTE-risk than those with 0-1 risk alleles, and the combination of cancer and ≥4 risk alleles yielded a 17-fold increased risk. As 39% of the VTEs occurring among those with cancer and the high-risk category of the genetic risk score could be attributed to the biological interaction, our findings suggest that genetics could be a useful tool for identifying cancer patients at high risk of VTE. However, the predictive performance of the 5-SNP score remains to be determined.

Risk factors for VTE in cancer can be broadly categorized into patient-related, cancer-related, and treatment-related factors. Prothrombotic genotypes are examples of patient-related factors that determine the intrinsic thrombosis potential of a patient and are not influenced by the disease or its progression. The finding that cancer and prothrombotic genotypes have a more than additive effect on VTE risk is especially interesting, as it could indicate that prothrombotic SNPs act through pathophysiological pathways that further increases the procoagulant state of malignancy. The five SNPs are all related to functions of the coagulation system and enhance its performance. Malignant tumors release cell-free DNA, procoagulant factors such as tissue factor, and growth factors that promote the release of neutrophil extracellular traps (NETs) from neutrophils.[43] These are main triggers of the intrinsic and extrinsic pathways of the coagulation system that in combination with

TABLE 3 Measures of interaction on an additive scale between cancer and the individual single-nucleotide polymorphisms (SNPs) or ≥4 risk alleles in the 5-SNP score

	RERI (95% CI)	AP (95% CI)	Synergy index (95% CI)
Individual SNPs (genes)			
rs8176719 (ABO)	1.91 (−0.72-4.54)	0.17 (−0.05-0.39)	1.23 (0.92-1.66)
rs6025 (F5)	6.29 (0.52-12.06)	0.39 (0.17-0.62)	1.73 (1.16-2.58)
rs1799963 (F2)	0.57 (−9.76-10.89)	0.06 (−1.01-1.13)	1.08 (0.30-3.89)
rs2066865 (FGG)	2.28 (0.03-4.53)	0.23 (0.04-0.43)	1.35 (1.01-1.81)
rs2036914 (F11)	0.16 (−2.90-3.21)	0.02 (−0.30-0.33)	1.02 (0.71-1.46)
5-SNP score (≥4 vs ≤1)	6.72 (1.17-12.26)	0.39 (0.16-0.63)	1.71 (1.13-2.61)

Abbreviations: ABO, non-O blood type; AP, proportion attributable to interaction; CI, confidence interval; FGG, fibrinogen gamma gene; RERI, relative excess risk attributable to interaction; SNP, single nucleotide polymorphism.

prothrombotic genotypes will facilitate downstream coagulation activation with subsequent increased risk of thrombus formation. Moreover, acquired resistance to activated protein C is common in cancer patients, and may contribute to further increase the risk in patients with FVL.[44]

For decisions on use of thromboprophylaxis in cancer patients, the risk of VTE needs to be evaluated and weighted against the risk of bleeding. Several risk prediction models for VTE risk have been proposed, such as the Khorana score,[45] the Vienna CATS score,[46] the PROTECHT score,[47] and the CONKO score.[48] However, these risk scores focus mainly on clinical risk factors, and currently, they are not recommended in international guidelines due to unsatisfying performances in validation studies. The TiC-Onco score is the only score that includes genetics,[49] and in this score, eight prothrombotic SNPs were investigated together with clinical variables. The final TiC-Onco score included four SNPs (FVL rs6025, F5 rs4524, F13 rs5985, and SERPINA10 rs2232698). Of note, the TiC-Onco score performed better than the Khorana score in the derivation study,[49] but the performance of these two models has not been compared in a validation study. Nevertheless, the TiC-Onco results supports that prothrombotic genotypes may be promising candidates for risk prediction of VTE in cancer patients.

Major strengths of our study include the large number of genotyped subjects followed for a long period of time, the high attendance rate in the two cohorts, and the thorough outcome assessment. Further, confounding by ethnicity is limited as the study cohorts represent a general Caucasian population. Some limitations of our study need to be addressed. The number of cases was low in some subgroups, particularly for the rare genetic variants, which resulted in limited statistical power. The subgroup results must therefore be interpreted with caution. The effect of genetic variants may vary in different types of cancer, but unfortunately, we did not have power to stratify our analyses on cancer types. Unfortunately, information on cancer treatment modalities was not available, which could have provided additional insight to the relationship between genes and treatment-related risk factors for cancer.

In conclusion, cancer and a high number of prothrombotic genotypes displayed a supra-additive effect on the risk of VTE, indicating a biological interaction between the risk factors. Our findings suggest that the 5-SNP score may be useful for identifying cancer patients at increased risk of VTE.

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CONFLICTS OF INTEREST

There are no conflicts of interest reported by any of the authors.

AUTHOR CONTRIBUTIONS

Conception and design: JBH and SKB; data collection: JBH, SKB, KH, MEG, BB; data analysis and statistical support: HS, KH, SKB; draft of manuscript: HS, JBH, and SKB; revision of manuscript for intellectual content: BP, FRR, KH, KH, MEG, BB. All authors read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Paper IV

Prothrombotic genotypes and risk of venous thromboembolism in occult cancer

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Summary

Background: Studies have reported that the combination of some prothrombotic genotypes and overt cancer yields a synergistic effect on VTE risk. Whether individual prothrombotic genotypes or number of risk alleles in a genetic risk score (GRS) affect VTE risk in occult cancer have not been addressed. The aim of this study was to investigate the joint effect of five prothrombotic genotypes and occult cancer on VTE risk.

Methods: Cases with incident VTE (n=2141) and a subcohort (n=14911) were sampled from the Scandinavian Thrombosis and Cancer Cohort (1993-2012). Five SNPs previously reported in a GRS were genotyped: ABO (rs8176719), F5 (rs6025), F2 (rs1799963), FGG (rs2066865) and F11 (rs2036914). Hazard ratios (HRs) for VTE by individual SNPs and GRS were estimated according to non-cancer and occult cancer (one year preceding a cancer diagnosis) exposure.

Results: During follow-up (median 12.2 years), 1817 subjects developed occult cancer, and of these, 93 experienced a VTE. The VTE risk was 4-fold higher (HR 4.05, 95% CI 3.28-5.00) in subjects with occult cancer compared to those without cancer. Subjects with occult cancer and VTE had a higher proportion of prothrombotic and advanced cancers compared to those without VTE. The VTE risk increased according to individual prothrombotic genotypes and GRS in cancer-free subjects, while no such effect was observed in subjects with occult cancer (HR for ≥4 versus ≤1 risk alleles in GRS: 1.14, 95% CI 0.61-2.11).

Conclusions: Five well-established prothrombotic genotypes, individually or combined, were not associated with increased risk of VTE in individuals with occult cancer.

Keywords: Epidemiology, genetics, neoplasms, polymorphisms single nucleotide, venous thromboembolism

Essentials

- The role of prothrombotic SNPs on venous thromboembolism (VTE) in occult cancer is unknown.
- We studied the role of individual SNPs and a genetic risk score (GRS) on VTE in occult cancer.
- The prothrombotic SNPs and the GRS did not affect VTE risk in occult cancer.
- Subjects with occult cancer and VTE had a higher proportion of prothrombotic and advanced cancers.

1. Introduction

Venous thromboembolism (VTE) is a frequent, severe and often fatal complication of cancer [1, 2]. The incidence of cancer-related VTE is increasing [2], and about 15% of cancer patients develop a symptomatic VTE during the course of their disease [3]. Unprovoked VTE may occur as the first sign of an undetected (i.e. occult) cancer [4-6], and approximately 5% of patients with unprovoked VTE are diagnosed with cancer within the first year following a VTE event [7-12]. VTE patients with occult cancer are more often diagnosed with prothrombotic cancers such as pancreatic, lung, gastrointestinal and hematological cancers [2, 5, 7, 13-15], and more advanced stages (higher degree of regional and distant metastasis) at the time of cancer diagnosis [5, 14, 16].

VTE has a strong hereditary component, and single nucleotide polymorphisms (SNPs) in genes encoding for factor V Leiden (FVL) [17, 18], fibrinogen gamma (FGG) [19], factor 11 (F11) [20], prothrombin (F2) [21-24] and ABO blood group [25, 26] are found to increase the VTE risk in cancer patients. Moreover, the combination of cancer and variations in the F5 (rs6025 and rs4524) and FGG (rs2066865) genes has been shown to increase the VTE risk on a supra-additive scale, indicating a biological interaction between the individual prothrombotic SNPs and active cancer [18, 19]. Similarly, a genetic risk score (GRS) based on five prothrombotic SNPs was associated with VTE risk in overt cancer, and the combination of cancer and a high GRS (≥4 risk alleles) yielded a synergistic effect on VTE risk [27].

The risk of VTE is found to be increased already one year prior to a cancer diagnosis [5, 28]. This could be due to the occult cancer alone, or occult cancer in combination with other patient-related predisposing factors for VTE. It is unknown to what extent individual prothrombotic genotypes and the 5-SNP GRS affect the VTE risk in occult cancer. Therefore, we aimed to investigate the effect of individual prothrombotic genotypes and number of risk alleles in the GRS on the risk of VTE in occult cancer, using a large case-cohort recruited from the general population.

2. Methods

2.1 Study population

We used individual data from The Scandinavian Thrombosis and Cancer (STAC) cohort, which is a large population-based study established to provide comprehensive data to investigate the impact of cancer on VTE risk in the general population [29]. The STAC cohort consists of merged data from three large Scandinavian cohorts with enrollment in 1993-1997: the fourth survey of the Tromsø Study (Tromsø 4, Norway), the second survey of the Nord-Trøndelag health study (HUNT2, Norway) and the Diet, Cancer and Health Study (DCH, Denmark). All three individual cohorts [30-32] and the complete STAC cohort [29] have been previously described in detail. The STAC cohort has a wide age-distribution (19-101 years), long-term follow-up and thorough validation of VTE events and cancer [29]. All subjects with cancer or VTE prior to enrollment were excluded, yielding a study population of 144952 participants. The participants were followed from date of inclusion to date of migration, death, incident VTE or end of follow-up (2007-2012). All first lifetime, symptomatic VTE events in both in- and outpatients included in the STAC cohort were validated by review of medical records, and objectively confirmed by diagnostic tests. The identification and adjudication process of VTE events has previously been published in detail [30, 31, 33]. During follow-up, 2444 VTE events occurred.

We created a case-cohort by including all incident VTE cases in which blood samples were available for genotyping (n=2044) and an age-weighted subcohort (n=14432) randomly sampled from the STAC cohort (Figure 1). We excluded participants with missing values for one of the SNPs studied (n=380) or body mass index (n=83). A total of 372 VTE cases were censored from the analysis as they occurred after the cancer diagnosis date. However, these subjects contributed with person-years prior to censoring. Finally, our case-cohort consisted of 16013 participants of whom 1566 were VTE cases. In case-cohort designs, every person in the cohort, including the cases, has the same chance of being selected to the

subcohort, and thus, 231 of the subjects randomly selected to our sub-cohort were also cases. All participants provided informed written consent, and the respective regional committees for research ethics in Norway and Denmark approved the individual cohort studies and the collaboration study.

2.2 Baseline measurements and genotyping

Baseline information was obtained by physical examination, self-administered questionnaires and non-fasting blood samples for each study. Body height and weight were measured with subjects wearing light clothing and no shoes. Body mass index (BMI) was calculated as weight in kilograms divided by body height in meters (m) squared (kg/m^2). Information regarding history of diabetes mellitus, cardiovascular disease (myocardial infarction, angina or stroke), smoking status, alcohol consumption, physical activity and level of education was obtained by the self-administered questionnaires. Detailed information regarding assessment of baseline variables in each cohort is provided elsewhere [32, 34, 35].

We genotyped the following SNPs: ABO rs8176719 (non-O blood type), F5 rs6025 (Factor V Leiden), F2 rs1799963 (prothrombin G20210A), FGG rs2066865 and F11 rs2036914. These SNPs were chosen because they were previously included in a parsimonious GRS model presented by de Haan *et al.* [36], which performed just as good as a comprehensive model including 31 SNPs. In Tromsø 4, rs1799963 (F2), rs6025 (F5), rs2036914 (F11) and rs8176719 (ABO) were genotyped with the Sequenom platform, and rs2066865 (FGG) with the TaqMan platform, as previously described in detail [37]. In HUNT2, genotyping was performed with the Illumina HumanCore Exome array. In the DCH study, the genotypes were determined using predesigned TaqMan SNP genotyping assays, as described in detail elsewhere [38].

Subjects were defined as carriers of the prothrombotic SNPs when one or two risk alleles were present, with no differentiation between heterozygous (one risk allele) and homozygous (two risk alleles) carriers. Normally, the minor allele is used as risk allele. However, due an inverse association with VTE risk,

the minor allele of the F11 SNP (rs2036914) was used as the common allele [39]. No risk allele at ABO rs8176719 was defined as O blood type. Hence, one or two risk alleles at ABO rs8176719 were classified as non-O blood type. Further, we used the GRS by de Haan *et al.,* which was created by summarizing the number of risk alleles from the five sequenced SNPs [36].

2.3 Cancer assessment

Information on cancer diagnosis, such as location (ICD10 codes C00-96), histological grade (ICO-3) and cancer stage (localized, regional, distant or unknown) was obtained by linkage to the cancer registries of Norway and Denmark where cancer registration is mandatory by law. Reports have found both cancer registries complete and valid, reporting a completeness of 98.8% in Norway and 95-98% in Denmark [40, 41]. The percentage of microscopically confirmed diagnoses in the registries were 94% in Norway and 93% in Denmark, respectively [40, 41]. Subjects with non-melanoma skin cancers (ICD10 C44) and no other cancer diagnosis were regarded as cancer-free, due to the non-metastatic potential of this disease.

Temporal proximity to cancer is shown to be a strong predictor for VTE risk [2, 5, 18]. Studies have found an increased VTE risk the year before a cancer diagnosis, with a seven-fold increased risk six months prior to the cancer diagnosis date [5, 6, 28]. Further, previously undiagnosed cancer is frequent in patients with unprovoked VTE, with a period prevalence of undiagnosed cancer increasing from 6.1% at baseline to 10.0% from the time of VTE diagnosis to 12 months after [6]. Hence, we defined the occult cancer period as one year prior to a cancer diagnosis. Cancer patients who were not diagnosed with VTE and still alive at the end of the defined occult cancer period were censored at the cancer diagnosis date. To test the robustness of our occult cancer variable, we additionally performed two sensitivity analyses where we defined the occult cancer period as 2 years and 6 months prior to cancer diagnosis, respectively.

2.4 Statistical analysis

Statistical analyses were performed with STATA version 16.0 (Stata Corporation, College Station, TX, USA). Occult cancer was entered as a time-varying co-variate. In those who developed cancer during follow-up, the data was split on the date one year before the cancer diagnosis date to differentiate between cancer free and occult cancer. Thus, subjects who developed cancer during follow-up were considered to be *cancer-free* until one year prior to a cancer diagnosis date, and subsequently they were classified as *occult cancer*. When cancer was diagnosed, the cancer patients changed exposure status to *overt cancer* and were censored from the analysis. Accordingly, subjects who developed cancer contributed to both non-exposed and occult cancer-exposed person-years (PY) at risk in our analysis.

Cox proportional hazard regression models were used to estimate hazard ratios (HRs) with 95% confidence intervals (CIs) for VTE according to the different prothrombotic genotypes or categories of risk alleles by the GRS (i.e. 0-1, 2-3, and ≥ 4 risk alleles) in subjects with and without occult cancer. Subjects with 0-1 risk alleles and no cancer were used as reference group. We adjusted all analyses for age, sex and BMI. The proportional hazards assumption was tested by the use of Schoenfeld residuals and was found not to be violated.

To investigate the combined effect of occult cancer and risk alleles on VTE risk, we used the relative excess risk attributable to interaction (RERI), the attributable proportion due to interaction (AP) and the synergy index (SI) with corresponding 95% CIs [42, 43]. RERI can be understood as part of the total effect on an outcome (e.g. VTE) that is attributable to interaction (e.g. the different exposures occult cancer and SNPs), and the AP as the proportion of the combined effect that is attributable to interaction between the two exposures. A RERI > 0, an AP > 0 and a synergy index > 1.0 suggest a positive interaction greater than an additive effect, i.e., the combined effect of occult cancer and SNPs bigger than the sum of the separate effects [42].

3. Results

During a median follow-up of 12.2 years, 1817 subjects developed occult cancer, of whom 93 (5.1%) experienced a VTE event. Overall, the risk of VTE in occult cancer was 4-fold higher (HR 4.05, 95% CI 3.28-5.00) than in cancer-free subjects in analysis adjusted for age, sex and BMI. The baseline characteristics of study participants with and without occult cancer and/or VTE are summarized in Table 1. In cancer-free subjects, those who developed VTE were older, had a higher BMI and a higher proportion were men. In subjects with occult cancer, those suffering a VTE were more often women. In both cancer-free subjects and subjects with occult cancer, the prevalence of prothrombotic genotypes were higher in VTE patients than in those without VTE (Table 1). The distribution of risk alleles of the GRS in cancer-free subjects and subjects with occult cancer was essentially similar (data not shown).

The distribution of cancer sites and stages in subjects with and without VTE in the occult cancer period are presented in Table 2. Among subjects with occult cancer, those who developed VTE were more frequently diagnosed with prothrombotic cancers such as pancreatic-, lung- and hematological cancers compared with those who did not develop VTE. Further, the cancers diagnosed within one year after a VTE were more advanced with a higher proportion of metastasis at the time of cancer diagnosis (Table 2).

HRs for VTE by categories of prothrombotic SNPs and cancer status (cancer-free/occult cancer) are presented in Table 3. In cancer-free subjects, the VTE risk increased with the presence of risk alleles for all prothrombotic genotypes, with the highest risk estimates for F5 (rs6025) (HR 2.68, 95% CI 2.34-3.06), F2 (rs1799963) (HR 1.94, 95% CI 1.41-2.65), and ABO (rs8176719) (HR 1.50, 95% CI 1.34-1-67). In subjects with occult cancer, the VTE risk was not affected by any of the prothrombotic SNPs (Table 3). The risk estimates were essentially unchanged when the definition of the occult cancer period were changed to six months (Supplementary Table 1) and two years (Supplementary Table 2).

The VTE risk in cancer-free subjects increased by the number of risk alleles, displaying a 2.4-fold increased VTE risk (HR 2.39, 95% CI 1.26-1.69) in subjects with more ≥4 risk alleles compared with subjects with 0-1 risk alleles (Table 3 and Figure 2). In subjects with occult cancer, the VTE risk did not increase by the number of risk alleles in the GRS (Table 3 and Figure 2). Similar results were found in sensitivity analyses when the occult cancer period was changed to six months or two years (Supplementary Tables 1 and 2, respectively). Measures of biological interaction (RERI, AP, SI) showed that the combination of occult cancer and presence of prothrombotic genotypes did not yield a more than additive effect on VTE risk (Supplementary Table 3).

4. Discussion

In this large population-based case-cohort study, we investigated the risk of VTE by the presence of prothrombotic genotypes, both as individual SNPs and number of risk alleles in a GRS, in subjects with and without occult cancer. In agreement with previous studies, we confirmed that the VTE risk increased by the individual prothrombotic genotypes and the number risk alleles in the GRS in cancer-free subjects [36]. In contrast, the prothrombotic genotypes were not associated with VTE risk in subjects with occult cancer, and accordingly, the combination of occult cancer and individual risk alleles or number of risk alleles in the GRS, did not yield any supra-additive effect on the VTE risk. Similar findings were observed in sensitivity analyses where the occult cancer period was altered to six months and two years, respectively. Subjects with VTE in the occult cancer period had a higher frequency of prothrombotic cancer types and more advanced cancers at the time of cancer diagnosis. Our findings suggest that the mechanisms related to VTE risk in occult cancer supersede the effect of prothrombotic genotypes.

The mechanisms for VTE in cancer are multifactorial and involve overlapping pathways related to the cancer itself and patient-related factors [44]. Several studies have explored the impact of prothrombotic genotypes on the risk of VTE in patients with overt cancer, and reported an increased risk for SNPs encoding for factor V Leiden, prothrombin mutation and non-O blood type [17-26]. Moreover, we previously showed that in combination with overt cancer, SNPs in F5 (rs6025, rs4524) and FGG (rs2066865), as well as a high number of risk alleles in the GRS (≥ 4 risk alleles), increased the VTE risk on a supra-additive level [18, 19, 27]. Therefore, we hypothesized that prothrombotic genotypes could influence the risk of VTE also in occult cancer. In contrast, we found no effect of the established prothrombotic genotypes on the VTE risk in subjects with occult cancer. This suggests that prothrombotic mechanisms related to the cancer itself are more important than inherent patient-related factors for risk of VTE in the occult cancer period.

Several studies have confirmed the significance of cancer stage, grade and site on VTE risk in overt malignancy, and patients with metastatic cancers, with regional or distant spread, have a higher VTE risk than patients with localized cancers [45]. Moreover, pancreas, brain, lung, ovarian, kidney and stomach cancers, as well as lymphomas, are considered high-risk sites for VTE [45]. In agreement with these findings, we showed that subjects who developed VTE during the occult cancer period had a higher proportion of regional and distant metastasis, as well as a higher proportion of cancers at high-risk sites, when compared with those who did not develop VTE during the occult cancer period. Tissue factor (TF), the main initiator of the coagulation system, is shown to be involved in cancer-progression processes such as tumor growth, angiogenesis and metastasis [46]. Upregulation of TF has been reported in advanced cancer stages [46], as well as in high-risk sites for VTE, including brain, pancreatic, lung, ovarian and colorectal cancers [47, 48]. In addition to TF expression, inflammatory responses with increased levels of circulating proinflammatory cytokines [49-51], inhibition of fibrinolytic activity through expression of plasminogen activator inhibitor-1 (PAI-1) [52-54], and formation of neutrophil extracellular traps (NETs) from neutrophils [55], may substantially contribute to a prothrombotic state in rapidly developing, aggressive, occult cancers. Thus, a massive orchestra of prothrombotic pathways induced by an advancing occult cancer, is likely sufficient to push an individual's thrombotic potential above the threshold for thrombus development, regardless of the presence of inherent prothrombotic risk factors.

The main strengths of our study include the prospective design with participants recruited from the general population, large number of genotyped subjects, long-term follow-up and thorough assessment of both VTE and cancer. In agreement with previous studies, we confirmed that 5% of the VTE events were related to occult cancer [7-12], and similar anatomical sites for cancers [2, 5, 7, 13-15], and degrees of metastatic diseases were found in VTE-patients with occult cancer [5, 14, 16], which supports a high external validity. Some study limitations must be addressed. In some rare genetic variants, the number of cases was low, resulting in limited statistical power. Unfortunately, we did not have sufficient

power to stratify our data by different cancer sites or stages of cancer, as it would be interesting to explore whether the impact of prothrombotic SNPs and combination of SNPs on VTE risk would differ within different sites and stages of occult cancer. In order to be registered with occult cancer, patients had to survive until their cancer diagnosis. Thus, participants with occult cancer who died from a VTE or other causes before a cancer was diagnosed would be misclassified as cancer-free. Such misclassification would likely lead to underestimation of the VTE risk in occult cancer. However, the five prothrombotic SNPs are not expected to increase the death-rate in the general population [56, 57], and thus, we believe that such misclassification, if present, would have negligible impact on our results.

In conclusion, five common prothrombotic genotypes, alone or in combination, were not associated with risk of VTE in occult cancer. Our findings suggest that prothrombotic mechanisms related to rapidly advancing cancers at high-risk sites are prominent for VTE risk in occult cancer and supersede the effect of prothrombotic genotypes.

Author contributions

Conception and design: JB. Hansen and S.K. Brækkan; data collection: JB. Hansen, S.K Brækkan, K. Hindberg, M.E. Gabrielsen, IA. Næss, A. Tjønneland, S.R. Kristensen, M.T. Severinsen; data analysis and statistical support: H. Skille, K. Hindberg, S.K. Brækkan; draft of manuscript: H. Skille, JB. Hansen, and S.K. Brækkan; revision of manuscript for intellectual content: all authors. All authors read and approved the final version of the manuscript.

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Conflicts of interest

The authors report no conflict of interest.

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Tables

Table 1. Baseline characteristics of the study population with and without occult cancer and VTE.

	No cancer		Occult cancer	
	Sub-cohort	**VTE**	**Sub-cohort**	**VTE**
Participants, n*	12723	1473	1724	93
Age, years	49.6±15.4	59.5±12.9	61.1±11.5	61.1±12.1
Male sex	47.5 (6043)	51.5 (759)	52.8 (910)	44.1 (41)
BMI, kg/m^2	26.1±4.1	27.6±4.6	26.7±4.3	27.0±4.4
rs8176719 (ABO)†	61.0 (7760)	70.0 (1031)	62.4 (1075)	64.5 (60)
rs6025 (F5)†	6.8 (868)	17.8 (262)	8.2 (142)	10.8 (10)
rs1799963 (F2)†	1.4 (172)	2.7 (40)	1.5 (25)	1.1 (1)
rs2066865 (FGG)†	42.5 (5413)	47.0 (692)	43.0 (741)	46.2 (43)
rs2036914 (F11)†	77.7 (9891)	82.6 (1216)	78.8 (1359)	78.5 (73)

Values are in % (n) or mean ± standard deviation. BMI, body mass index.

* Subjects with cancer contributed to observation periods in both the no cancer and occult c cancer group.

† Percentage of participants with ≥1 risk allele

Table 2. Distribution of cancer sites and stages in subjects with occult cancer, defined as one year prior to cancer diagnosis, with and without venous thromboembolism (VTE).

	No VTE (n=1724)	**VTE (n=93)**
Cancer site	Number (%)	Number (%)
Colorectal	272 (15.8)	8 (8.6)
Pancreatic	54 (3.1)	6 (6.4)
Lung	205 (11.9)	18 (19.4)
Breast	198 (11.5)	2 (2.2)
Gynecological	101 (5.9)	4 (4.3)
Prostate	224 (13.0)	10 (10.8)
Urological	150 (8.7)	8 (8.6)
Central nervous system	69 (4.0)	1 (1.1)
Hematological/Lymphoma	138 (8.0)	11 (11.8)
Upper GI	90 (5.2)	7 (7.5)
Malignant melanoma	66 (3.8)	0 (0)
Other*	157 (9.1)	18 (19.4)
Cancer stage	Number (%)	Number (%)
Localized	473 (27.4)	10 (10.8)
Regional lymph nodes	431 (25.0)	14 (15.1)
Distant metastasis	292 (16.9)	34 (36.7)
Metastasis diagnosed, unknown where	408 (23.7)	25 (26.9)
Not staged	120	10

*Cancers of ear, nose and throat, eye, endocrine, heart, sarcomas, connective tissue and unknown site

Table 3. Hazard ratios (HR) with 95% confidence intervals (CI) for venous thromboembolism (VTE) by categories of single nucleotide polymorphisms (SNPs) and occult cancer defined as the occurrence of a cancer diagnosis within one year after VTE diagnosis.

		Events	HR (95% CI)*	HR (95% CI)*
Cancer	**rs8176719 (ABO)†**			
-	-	442	Ref.	Ref.
-	+	1031	1.50 (1.34-1.67)	1.50 (1.34-1.67)
+	-	33	Ref.	5.01 (3.51-7.14)
+	+	60	1.10 (0.72-1.69)	5.43 (4.14-7.12)
Cancer	**rs6025 (F5)†**			
-	-	1211	Ref.	Ref.
-	+	262	2.68 (2.34-3.06)	2.68 (2.34-3.06)
+	-	83	Ref.	4.45 (3.56-5.57)
+	+	10	1.26 (0.64-2.45)	5.60 (3.00-10.44)
Cancer	**rs1799963 (F2)†**			
-	-	1433	Ref.	Ref.
-	+	40	1.94 (1.41-2.65)	1.94 (1.42-2.66)
+	-	92	Ref.	4.11 (3.33-5.09)
+	+	1	0.82 (0.11-6.03)	3.10 (0.44-22.05)
Cancer	**rs2066865 (FGG)†**			
-	-	781	Ref.	Ref.
-	+	692	1.18 (1.06-1.30)	1.17 (1.06-1.30)
+	-	50	Ref.	4.08 (3.06-5.44)
+	+	43	1.10 (0.73-1.67)	4.72 (3-47-6.42)
Cancer	**rs2036914 (F11)†**			
-	-	257	Ref.	Ref.
-	+	1216	1.31 (1.14-1.50)	1.31 (1.14-1.49)
+	-	20	Ref.	5.33 (3.38-8.40)
+	+	73	0.95 (0.58-1.57)	4.94 (3.81-6.42)
Cancer	**Genetic risk score‡**			
-	**0-1**	224	Ref.	Ref.
-	**2-3**	836	1.46 (1.26-1.69)	1.46 (1.26-1.69)
-	**≥4**	413	2.39 (2.03-2.82)	2.39 (2.03-2.81)
+	**0-1**	21	Ref.	6.75 (4.31-10.56)
+	**2-3**	52	0.84 (0.50-1.39)	5.44 (4.02-7.37)
+	**≥4**	20	1.14 (0.61-2.11)	8.04 (5.09-12.72)

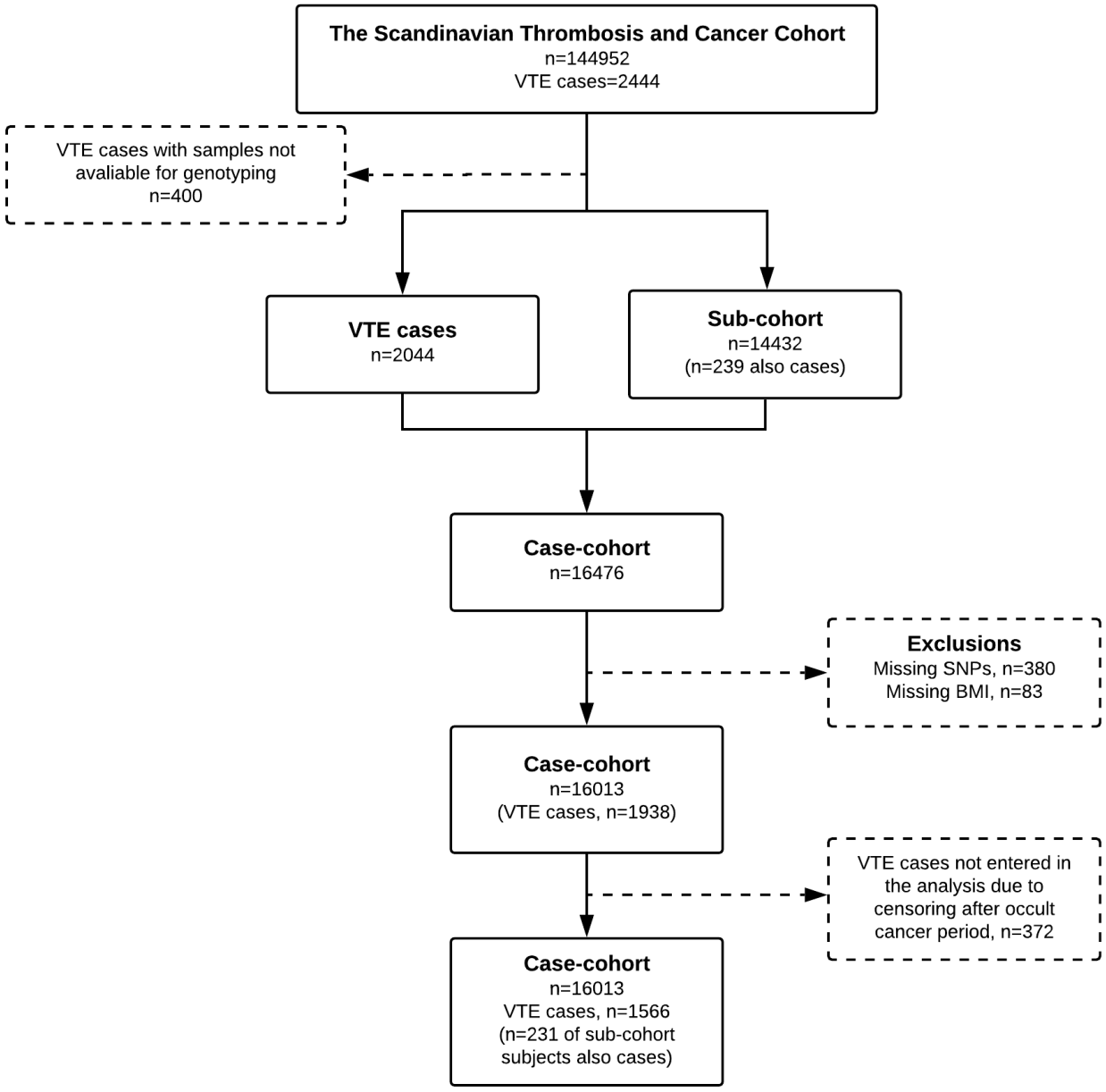
* Adjusted for age, sex and body mass index (BMI)

† Positive indicating subjects with one or two risk alleles

‡ Number of risk alleles

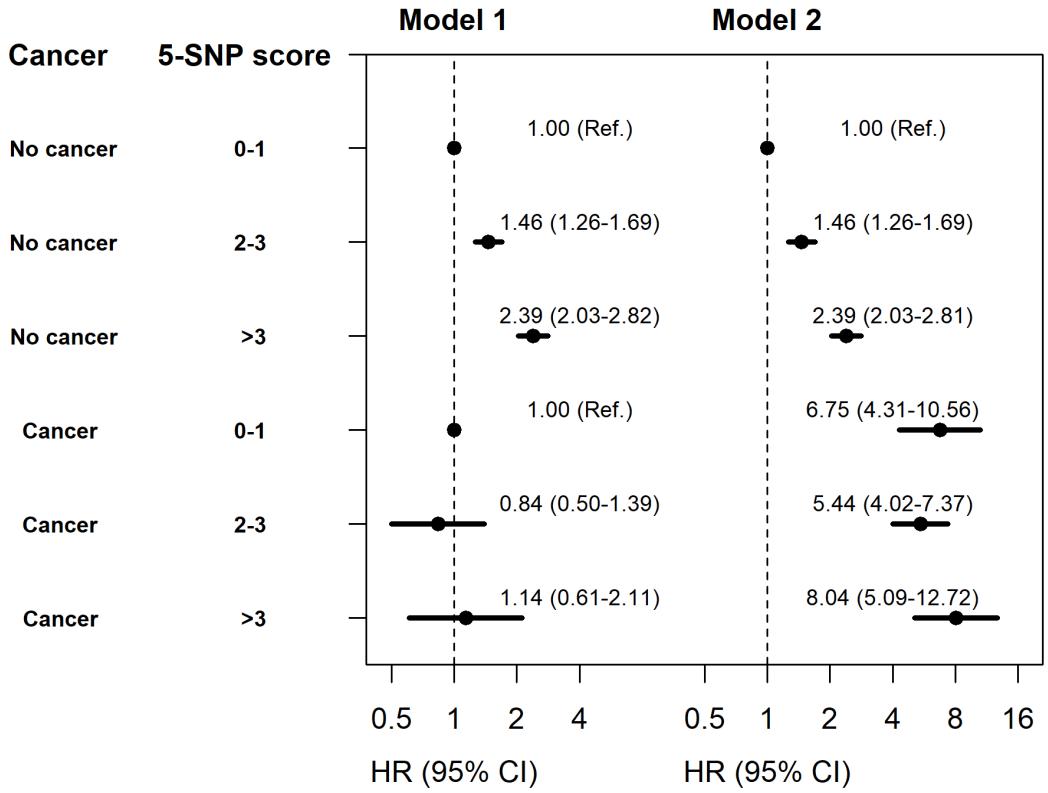
Figures

Figure 1. Study population. Participants were recruited from The Scandinavian Thrombosis and Cancer Cohort (1993-1997).



VTE indicates venous thromboembolism; SNPs, single nucleotide polymorphisms.

Figure 2. Hazard ratios (HR) with 95% confidence intervals (CI) for venous thromboembolism (VTE) by categories of single nucleotide polymorphisms (SNPs) and occult cancer defined as the occurrence of a cancer diagnosis within one year after VTE diagnosis.



Supplementary

Supplementary Table 1. Hazard ratios (HR) with 95% confidence intervals (CI) for venous thromboembolism (VTE) by categories of single nucleotide polymorphisms (SNPs) and occult cancer defined as six months prior to cancer diagnosis.

		Events	HR (95% CI)*	HR (95% CI)*
Cancer	**rs8176719 (ABO)†**			
-	-	447	Ref.	Ref.
-	+	1044	1.50 (1.34-1.67)	1.50 (1.34-1.67)
+	-	28	Ref.	8.22 (5.60-12.05)
+	+	47	1.05 (0.65-1.68)	8.41 (6.22-11.37)
Cancer	**rs6025 (F5)†**			
-	-	1228	Ref.	Ref.
-	+	263	2.65 (2.31-3.03)	2.65 (2.31-3.03)
+	-	66	Ref.	6.94 (5.41-8.90)
+	+	9	1.45 (0.71-2.97)	9.74 (5.05-18.76)
Cancer	**rs1799963 (F2)†**			
-	-	1451	Ref.	Ref.
-	+	40	1.91 (1.40-2.62)	1.92 (1.40-2.62)
+	-	74	Ref.	6.49 (5.13-8.21)
+	+	1	1.13 (0.15-8.38)	5.85 (0.82-41.67)
Cancer	**rs2066865 (FGG)†**			
-	-	789	Ref.	Ref.
-	+	702	1.18 (1.07-1.31)	1.18 (1.07-1.31)
+	-	42	Ref.	6.71 (4.91-9.17)
+	+	33	1.04 (0.65-1.66)	7.15 (5.03-10.13)
Cancer	**rs2036914 (F11)†**			
-	-	264	Ref.	Ref.
-	+	1227	1.28 (1.12-1.47)	1.28 (1.12-1.46)
+	-	13	Ref.	6.60 (3.78-11.54)
+	+	62	1.27 (0.69-2.32)	8.13 (6.16-10.74)
Cancer	**Genetic risk score‡**			
-	**0-1**	231	Ref.	Ref.
-	**2-3**	841	1.42 (1.23-1.64)	1.42 (1.23-1.64)
-	**≥4**	419	2.35 (2.00-2.76)	2.35 (2.00-2.76)
+	**0-1**	14	Ref.	8.59 (5.00-14.74)
+	**2-3**	47	1.15 (0.63-2.12)	9.44 (6.89-12.94)
+	**≥4**	14	1.25 (0.59-2.65)	11.01 (6.41-18.90)

* Adjusted for age, sex and body mass index (BMI)

† Positive indicating subjects with one or two risk alleles

‡ Number of risk alleles

Supplementary Table 2. Hazard ratios (HR) with 95% confidence intervals (CI) for venous thromboembolism (VTE) by categories of single nucleotide polymorphisms (SNPs) and occult cancer defined as two years prior to cancer diagnosis.

		Events	HR (95% CI)*	HR (95% CI)*
Cancer	**rs8176719 (ABO)†**			
-	-	431	Ref.	Ref.
-	+	1020	1.52 (1.36-1.70)	1.52 (1.36-1.70)
+	-	44	Ref.	3.49 (2.56-4.77)
+	+	71	0.97 (0.67-1.42)	3.35 (2.60-4.31)
Cancer	**rs6025 (F5)†**			
-	-	1195	Ref.	Ref.
-	+	256	2.66 (2.32-3.04)	2.66 (2.33-3.05)
+	-	99	Ref.	2.75 (2.24-3.38)
+	+	16	1.57 (0.92-2.69)	4.52 (2.75-7.40)
Cancer	**rs1799963 (F2)†**			
-	-	1412	Ref.	Ref.
-	+	39	1.91 (1.39-2.63)	1.92 (1.40-2.64)
+	-	113	Ref.	2.61 (2.15-3.17)
+	+	2	1.37 (0.34-5.64)	3.28 (0.82-13.13)
Cancer	**rs2066865 (FGG)†**			
-	-	770	Ref.	Ref.
-	+	681	1.17 (1.06-1.30)	1.17 (1.06-1.30)
+	-	61	Ref.	2.59 (1.99-3.36)
+	+	54	1.12 (0.78-1.63)	3.03 (2.30-4.00)
Cancer	**rs2036914 (F11)†**			
-	-	256	Ref.	Ref.
-	+	1195	1.29 (1.13-1.48)	1.29 (1.13-1.48)
+	-	21	Ref.	2.86 (1.83-4.46)
+	+	94	1.18 (0.73-1.90)	3.26 (2.57-4.13)
Cancer	**Genetic risk score‡**			
-	**0-1**	219	Ref.	Ref.
-	**2-3**	826	1.48 (1.27-1.71)	1.48 (1.27-1.72)
-	**≥4**	406	2.41 (2.04-2.84)	2.40 (2.04-2.83)
+	**0-1**	26	Ref.	4.36 (2.90-6.55)
+	**2-3**	62	0.80 (0.51-1.27)	3.40 (2.56-4.52)
+	**≥4**	27	1.23 (0.72-2.12)	5.56 (3.72-8.30)

* Adjusted for age, sex and body mass index (BMI)

† Positive indicating subjects with one or two risk alleles

‡ Number of risk alleles

Supplementary Table 3. Measures of interaction on an additive scale between occult cancer and the individual single-nucleotide polymorphisms (SNPs) or ≥4 risk alleles in the genetic risk score.

	RERI (95% CI)	AP (95% CI)	Synergy index (95% CI)
Individual SNPs (genes)			
rs8176719 (ABO)	-0.07 (-2.27-2.12)	-0.01 (-0.42-0.39)	0.98 (0.60-1.61)
rs6025 (F5)	-0.53 (-4.15-3.09)	-0.10 (-0.80-0.61)	0.90 (0.41-1.95)
rs1799963 (F2)	-1.96 (-8.12-4.21)	-0.63 (-3.85-2.59)	0.52 (0.03-9.47)
rs2066865 (FGG)	0.47 (-1.35-2.28)	0.10 (-0.26-0.46)	1.14 (0.68-1.92)
rs2036914 (F11)	-0.69 (-3.29-1.92)	-0.14 (-0.68-0.40)	0.85 (0.48-1.52)
Genetic risk score (≥4 vs ≤1)	-0.06 (-4.67-4.54)	-0.01 (-0.58-0.56)	0.99 (0.52-1.89)

AP, proportion attributable to interaction; CI, confidence interval; RERI, relative excess risk attributable to interaction.

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