Faculty of Health Sciences, Department of Clinical Medicine

The Role of Plasma Extracellular Vesicles and Procoagulant Phospholipid Activity in Venous Thromboembolism

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A dissertation for the degree of Philosophiae Doctor, July 2021
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Tromsø, July 2021
Summary

Venous thromboembolism (VTE) is the formation of a blood clot in, most commonly, the deep veins of the lower extremities and the pulmonary circulation. VTE is a prevalent disease associated with severe short- and long-term complications. Negatively charged procoagulant phospholipids (PPL), and phosphatidylserine (PS) in particular, are vital to efficient coagulation activation, and found expressed on the surface of extracellular vesicles (EVs) and activated platelets.

The overall aim of the present thesis was to develop an easily available and reproducible FXa-dependent clotting assay to measure PPL activity in plasma, and further use the assay to investigate the association between plasma PPL activity and the risk of VTE.

In paper I, we investigate the impact of several pre-analytical conditions on EV concentration and size measured by Nanoparticle Tracking Analysis (NTA) and scanning electron microscopy (SEM). In paper II, we developed a modified FXa-dependent clotting assay by substituting the chemically phospholipid depleted plasma with PPL-depleted plasma obtained by ultracentrifugation. In paper III, we used our modified PPL assay to investigate the association between PPL clotting time (PPL\text{CT}) and the risk of incident VTE in a nested case-control study derived from a population based cohort (the Tromsø study). Previous studies have suggest that statin treatment reduced the risk of recurrent VTE. In paper IV, we investigated the impact of statin treatment (rosuvastatin) on PPL activity, using the modified PPL assay and plasma samples from the STAtins Reduce Thrombophilia trial.

The impact of pre-analytical conditions (i.e. anticoagulants, centrifugation protocols, and fasting status) on EV measurements was demonstrated, and the obstacle of post-prandial lipoproteins interfering with NTA analysis was particularly highlighted. We found that the modified PPL assay displayed similar sensitivity and reproducibility compared to commercial assays based on chemically phospholipid-depleted plasma. We observed an inverse association between plasma PPL\text{CT}, assessed by the modified assay, and the risk of future VTE in a population-based nested case-control study. Additionally, rosuvastatin treatment aused a substantial decrease in plasma PPL activity in subjects with a history of VTE. The development of the modified PPL assay enabled us to perform high-quality measurements in large-scale studies. The inverse association between PPL\text{CT} and VTE risk supports an important role of plasma PPL in the pathogenesis of VTE and may partly explain the reduced risk of VTE recurrence observed by statin treatment.
Sammendrag
Venøs tromboembolisme (VTE) er en fellesbetegnelse for blodpropp som dannes i de dype venene i underekstremitetene eller i lungekretsøpet. VTE er en vanlig sykdom med alvorlige kort- og langtidskomplikasjoner. Negativt ladede prokoagulante fosfolipider (PPL), og da spesielt fosfatidylserin, er avgjørende for en effektiv aktivering av koagulasjonskaskaden, og finnes uttrykt på overflaten av ekstracellulære vesikler (EVs) og aktiverte blodplater.

Det overordnede målet med denne avhandlingen var å utvikle et lett tilgjengelig og reproducerbart FXa-avhengig koagulasjonsassay for å kunne måle PPL-aktiviteten i plasmaprøver. Vi brukte så assayet til å undersøke sammenhengen mellom PPL-aktivitet i plasma og risikoen for VTE.

I artikkel I undersøkte vi effekten av ulike pre-analytiske faktorer på konsentrasjonen og størrelsen av EVs, målt ved hjelp av Nanoparticle Tracking Analysis (NTA) og skanning elektronmikroskopi (SEM). I artikkel II utviklet vi et modifisert FXa-avhengig koagulasjonsassay ved å erstatte plasma hvor PPL var kjemisk fjernet med plasma hvor vi brukte ultrasentrifugering for å oppnå samme effekt. I artikkel III brukte vi det modifiserte PPL assayet for å undersøke sammenhengen mellom PPL-koagulasjonstid (PPL_CT) og risikoen for førstegangs VTE i en nøstet kasus-kontroll studie avledet fra en populasjonsbasert kohortestudie (Tromsøundersøkelsen). Tidligere studier har vist at statinbehandling reduserte risikoen for residiv av VTE. I artikkel IV undersøkte vi effekten av statinbehandling (rosuvastatin) på PPL-aktiviteten, målt ved hjelp av det modifiserte PPL assayet i plasmaprøver fra studien STAtins Reduce Thrombophilia.

Effekten av pre-analytiske faktorer (dvs. antikoagulanter, sentrifugeringsprotokoller og fastestatus) på EV-målinger ble demonstrert, og utfordringen med at postprandiale lipoproteiner forstyrret NTA-analysene ble fremhevet spesifikt. Vi fant at det modifiserte PPL assayet viste lik sensitivitet og reproduserbarhet som kommersielle assay hvor fosfolipider fra plasma er fjernet kjemisk. Vi observerte en invers sammenheng mellom PPL_CT, målt i det modifiserte PPL assayet, og risikoen for fremtidig VTE. I tillegg viste vi at rosvuvastatinbehandlingen gav en betydelig reduksjon i PPL-aktiviteten i plasma hos personer med tidligere VTE. Utviklingen av det modifiserte PPL assayet gjorde det mulig for oss å utføre målinger av høy kvalitet i stor skala. Den inverse sammenhengen mellom PPL_CT og VTE-risiko underbygger en viktig rolle for plasma PPL i sykdomsutviklingen av VTE og kan delvis forklare den reduserte risikoen for residiv av VTE under statinbehandling.
List of papers

I. **Impact of preanalytical conditions on plasma concentration and size distribution of extracellular vesicles using Nanoparticle Tracking Analysis**
Simin Jamaly, Cathrine Ramberg, Randi Olsen, Nadezhda Latysheva, Paul Webster, Timofey Sovershaev, Sigrid K. Brækkan, and John-Bjarne Hansen
*Scientific Reports 2018 Nov 21 8(1):17216*

II. **A modified clot-based assay to measure negatively charged procoagulant phospholipids**
Cathrine Ramberg, Simin Jamaly, Nadezhda Latysheva, Line Wilsgård, Timofey Sovershaev, Omri Snir, and John-Bjarne Hansen
*Scientific Reports 2021 April 29 11(1):9341*

III. **Plasma Procoagulant Phospholipid Clotting Time is Inversely Associated with Future Risk of Incident Venous Thromboembolism**
Cathrine Ramberg, Line Wilsgaard, Nadezhda Latysheva, Sigrid K. Brækkan, Kristian Hindberg, Timofey Sovershaev, Omri Snir, and John-Bjarne Hansen
*Pending revision in Research and Practice in Thrombosis and Haemostasis*

IV. **Rosuvastatin treatment decreases plasma procoagulant phospholipid activity after a VTE: A randomized controlled trial**
*Pending revision in Journal of Thrombosis and Haemostasis*
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACCP</td>
<td>American College of Chest Physicians</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>AT</td>
<td>Antithrombin</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAT</td>
<td>Calibrated automated thrombogram</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>COCs</td>
<td>Combined oral contraceptives</td>
</tr>
<tr>
<td>CTAD</td>
<td>Sodium Citrate Theophylline Adenosine Dipyridamole</td>
</tr>
<tr>
<td>CTEPH</td>
<td>Chronic thromboembolic pulmonary hypertension</td>
</tr>
<tr>
<td>DOAC</td>
<td>Direct oral anticoagulant</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep vein thrombosis</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EVs</td>
<td>Extracellular vesicles</td>
</tr>
<tr>
<td>F</td>
<td>Factor</td>
</tr>
<tr>
<td>FVL</td>
<td>Factor V Leiden</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association studies</td>
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<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
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<tr>
<td>LMWH</td>
<td>Low molecular weight heparin</td>
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<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
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<tr>
<td>NTA</td>
<td>Nanoparticle tracking analysis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>PFP</td>
<td>Platelet-free plasma</td>
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<tr>
<td>PPL</td>
<td>Procoagulant phospholipids</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>PPL&lt;sub&gt;CT&lt;/sub&gt;</td>
<td>Procoagulant phospholipid clotting time</td>
</tr>
<tr>
<td>PPLDP</td>
<td>Procoagulant phospholipid depleted plasma</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTS</td>
<td>Post-thrombotic syndrome</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
</tr>
<tr>
<td>UNN</td>
<td>University Hospital of North Norway</td>
</tr>
<tr>
<td>VKA</td>
<td>Vitamin K antagonist</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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</table>
1. Introduction
Venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), is the formation of a blood clot in, most commonly, the deep veins of the lower extremities. The clot prevents the return of blood from the legs to the heart and cause pain, swelling and redness in the affected limb. Traditionally, a PE has been seen as a complication of a DVT, where a part of the thrombus breaks off and travels with the bloodstream through the heart to the lungs. In later years, research has found that a PE might also arise de novo in the lungs or originate from a right intracardiac thrombi in patients with atrial fibrillation. VTE is effectively treated with anticoagulation, but at the cost of increased risk of potentially lethal major bleeding events. VTE is considered a multicausal disease, where the sum of an individual’s risk factors at a particular point in time might exceed the thrombosis threshold and cause an event. A number of acquired and inherited risk factors for VTE have been identified. However, only a few of the currently known risk factors are modifiable and related to lifestyle. A VTE event is classified as provoked if it occurs in the presence of known risk factors (transient or persistent), while an unprovoked event occurs in the absence of known triggers. The classification of an event influences both the prognostics as well as the treatment strategy.

Extracellular vesicles (EVs), including exosomes, microvesicles and apoptotic bodies, are bilayered vesicles, which are either released from or bud off a parental cell membrane. EVs have been extensively studied in recent years and found to be associated with several disease states. The proposed role of EVs in VTE has mainly been thought to be caused by the surface expression of tissue factor (TF), a well-known trigger of the coagulations system. In contrast to TFs vital role in activating coagulation, elevated levels of TF+EVs are only found in certain disease states such as severe cancers or disseminated intravascular coagulation (DIC). Negatively charged procoagulant phospholipids (PPL), and particularly phosphatidylserine (PS), are exposed on the EV surface as a consequence of the budding process. They are vital to coagulation activation, and the mere presence of negatively charged phospholipids increases the activity of the extrinsic tenase complex (TF-FVII) by several orders of magnitude. Given the importance of PPL to coagulation, we sought out to modify a clot-based FXa dependent assay, creating a sensitive and reproducible method for measuring PPL clotting time (PPL_CT). We addressed several pre-analytical challenges when working with plasma EVs, like the choice of anticoagulant, plasma preparation and fasting.
status. Further, we investigated the association between procoagulant phospholipids, and the risk of incident and recurrent VTE.

1.1 Epidemiology of VTE

VTE is considered the third most common cardiovascular disease, following myocardial infarction and ischemic stroke. The annual incidence rate of VTE for people of European ancestry is estimated to 1-2 per 1000 person years. The annual incidence has remained unchanged or even increased over time, in contrast to the decreasing incidences of myocardial infarction and ischemic stroke.

VTE is primarily a disease of older age and the incidence increases exponentially with age for both sexes. The reported incidences of VTE for men and women separately range from studies reporting an overall higher risk in women, to a higher risk in men to no difference between the sexes. However, when considering sex and age groups together, it appears that in the younger population (< 50 years) women have a higher risk of VTE than men, most probably due to female reproductive risk factors. Though, in the middle aged (50 to 70 years) the risk was reported higher for men than women. Still, the life-time risk of VTE for men and women individually is not known. The incidence rate of VTE differs with regards to patient ethnicity. The African-American population has the highest incidence rate of a first time VTE, followed by the Caucasian, Hispanic and Asian/Pacific Islander populations, respectively.

VTE most commonly presents as DVT, which accounts for approximately two-thirds of all events, while one-third of patients experience a PE. However, recent studies have challenged this ratio where an increase in PE events were observed, while the number of DVT events remained unchanged or even decreased over the same period of time, most probably explained by improved diagnostic tools as well as growing awareness of the disease. Approximately 30% of patients will experience a recurrent event within 10 years. The risk of recurrence is highest the first 6 to 12 months after the initial VTE event. DVT patients are at higher risk of a recurrent event than PE patients, with a 1.4-fold higher hazard ratio. Recurrent events are more likely to occur at the same site as the initial event, hence patients experiencing a first DVT are more likely to develop a recurrent DVT, than a PE.
Similarly, a first unprovoked event more likely recurs as an unprovoked event, while a provoked first event is as likely to recur as an unprovoked as a provoked event.

In addition to the risk of recurrence, VTE can lead to severe short- and long-term complications such as the post-thrombotic syndrome (PTS), the post PE- syndrome including its most extreme manifestation chronic thromboembolic pulmonary hypertension (CTEPH), and death. PTS is a chronic complication of DVT where the venous valves are damaged by insufficient thrombus resolution, leading to venous hypertension, structural changes in the vessel wall, and impaired venous return. The clinical signs of PTS include chronic pain, swelling, edema, skin changes, and heaviness of the affected leg. PTS develops in 20 to 50% of DVT patients, with 5% developing severe ulcers within a 10-year period. Risk factors for developing PTS include obesity, female sex, proximal DVT and varicose veins. The health burden of PTS is substantial, both in terms of cost to the healthcare system as well as the reduced quality of life of the patients.

Long-term follow-up studies have consistently reported that 50% of patients, after an acute PE event, suffer from functional limitations and/or decreased quality of life. The concept of “post-PE syndrome” was suggested as a collective term encompassing all complications of PE, where CTEPH is the most extreme manifestation of the syndrome. CTEPH affects 0.4 to 4% of PE patients and is a serious complication which may ultimately result in right ventricular failure. CTEPH leads to occlusive vascular remodeling and obstruction of pulmonary arteries, as well as increased resistance in the pulmonary circulation due to incomplete thromboembolic resolution after a PE event. CTEPH patients typically suffer from exertional dyspnea, and with disease progression leading to further limitations of cardiac output, exertion-related presyncope, frank syncope, and exertional chest pain may develop. As the clinical presentation of CTEPH is often nonspecific and subtle, and survival without intervention is poor, correct and early diagnosis is of high importance.

The 30-day case-fatality rate for all VTE was reported to be from 6 to 11 %, while the 1-year case-fatality rate ranged from 21 to 24%. PE is the most fatal manifestation of VTE, and presents an almost 2.5-fold higher 30-day case fatality rate compared to DVT. A PE
event is regarded as an independent predictor of reduced survival for up to three months post event. It has been reported that 25% of PE cases present with sudden death. Still, the highest fatality rates are found in cancer-related VTE with a 1-year case fatality rate reported to range from 63% to 88%.

1.2 Pathophysiology of VTE
In 1856 the German scientist Rudolph Virchow proposed three components, later named Virchow’s triad, which he found essential in thrombus formation. The triad is comprised of changes in blood flow (stasis), changes in the composition of blood (hypercoagulability) and vessel wall injury (Figure 1). The triad is still used today to explain the pathophysiology of VTE, and most known risk factors for the disease can be classified under one or more of the three components of the triad.

![Figure 1. Illustration of Virchow’s triad with risk factors for VTE categorized by the triad component; stasis, vessel wall injury and hypercoagulability.](image-url)
Autopsy and phlebography studies have suggested that non-trauma related venous thrombi typically originate in the deep recess of the valvular sinuses in the presence of an intact endothelium. The observation of intact endothelium advocates for a TF driven fibrin generation and deposition as the initiating event. As blood crosses the leaflets of the venous valves, some blood begins a vortical flow (Figure 2). As a consequence, blood cells and blood components are trapped in the deepest recesses of the valvular sinuses, where a severely hypoxic environment develops. Local hypoxia leads to a proinflammatory state where leukocytes and platelets are activated and release procoagulant EVs. It has been shown that the number of venous valves positively correlates with the risk of VTE, and that a limb with more valves has a higher risk of DVT. As we age, veins lose their compliance and the venous valves stiffen from fibrosis, which further results in disrupted blood flow. Such physical changes in the veins likely contribute to the increased incidence of VTE with increasing age.

Figure 2. The pathophysiology of venous thrombosis. Blood is trapped in the valve pockets by a counter rotating vortex at the base of the sinus, creating a hypoxic and proinflammatory milieu. Blood cells, as well as the endothelium, are activated and produce procoagulant extracellular vesicles. EVs= Extracellular vesicles, Plt= Platelet, Mc =Monocyte

The evidence for a relationship between stasis and thrombosis is substantial. Contrast media used for venography was found to linger in the veins of the elderly for up to 60 minutes after
the procedure, supporting the presence of stasis in the venous valves. Observations of VTE after long-haul flights, increased incidence of DVT in a paralyzed leg compared to the normal leg, increasing risk of DVT with the length of immobilization of a patient, as well as a decrease in risk of DVT in hospitalized patients when they begin to walk again or use pneumatic leg compression, all strengthen the role of stasis in the development of VTE.

Hypercoagulability is the abnormal tendency for blood to clot and can either be due to inherited genetic factors or caused by external factors. An inherited hypercoagulable state can be caused by single nucleotide polymorphisms, such as Factor V Leiden and prothrombin mutation G20210A, and deficiencies in natural anticoagulants such as protein S, protein C or antithrombin (AT). External factors that cause a hypercoagulable state include oral contraceptive use, hormone replacement therapy, pregnancy and obesity.

An undamaged endothelium expresses a range of anticoagulants like tissue factor pathway inhibitor (TFPI), heparin-like proteoglycans, thrombomodulin, and endothelial protein C receptor. Upon activation, the endothelium will downregulate the expression of its anticoagulant properties, while the procoagulant properties are upregulated. In response to vessel wall injury, TF as well as adhesive molecules like P-selectin, E-selectin and vWF are exposed initiating the extrinsic coagulation pathway and recruit platelets and leukocytes to the site of injury. Hypoxia and inflammatory mediators have been suggested as causes of endothelial dysfunction in relation to VTE. However, the role of the third component of the triad, vessel injury, in venous thrombosis is debated, and most often associated with surgery or trauma related thrombosis. In a study from 1974, Sevitt investigated 50 small thrombi from femoral valve pockets without finding any significant evidence of preceding intimal damage in the vessel wall. This indicates that “endothelial dysfunction” would possibly be a more fitting term.

1.3 Risk factors for VTE
A risk factor is defined as any attribute, characteristic or exposure of an individual that increases the chance of developing a disease. As VTE is a multicausal disease, a combination of risk factors is required for disease development. The risk factors for VTE can further be divided into categories based on whether they are acquired or inherited. The complex
relationship between an individual’s risk factors is illustrated by the thrombosis potential model (Figure 3)\textsuperscript{6}. The model describes how the combination of two or more risk factors at a particular point in time exceeds a threshold and results in a VTE event. Figure 3 exemplifies the effect of different types of risk factors on the thrombosis threshold. An inherited risk factor, like FVL, is constant over time. While age, an acquired risk factor, will increase an individual’s risk over time. The combination of risk factors is the individual’s baseline potential. If a provoking factor, like immobilization, cancer or pregnancy, occurs it could potentially lead to a combined effect that exceeds the thrombosis threshold and cause an event. The baseline risk of an individual is higher after a first event, and about 30\% of patients will experience a recurrent event within 10 years. The risk of recurrence is highest the first 6 to 12 months after the initial VTE event\textsuperscript{40}. The presence of a residual thrombus after a first DVT event is considered an independent risk factor for recurrence\textsuperscript{65}. Potential mechanisms leading to the higher baseline risk after a first event are alterations in the vessel wall, impaired venous outflow, or the presence of additional risk factors.

Figure 3. The thrombosis potential model. Factor V Leiden (FVL) (blue) exemplifies a hereditary risk factor, while age (green) represents an acquired risk factor that increases with time. The orange line illustrates an individual’s baseline potential, with provoking factors (red bars) at different time points in life. The thrombosis threshold is illustrated by the dotted line (grey). When the combination of the baseline potential together with a provoking factor
exceeds the thrombosis threshold, a VTE event occurs, and potentially reoccurs. (Adapted from Rosendaal, Lancet 1999 6)

1.3.1 Hereditary risk factors
VTE has a strong genetic component with an overall heritability of 50-60% as estimated by twin and family studies 66,67. The functional and clinical importance of the genetic risk factors has been unraveled over time through case reports, observations and knock-out mouse studies on abnormal levels of coagulation factors, regulators and anticoagulants 68,69. The methodological advances in science, such as high-throughput micro-array based genotyping and genome-wide association studies (GWAS), have contributed greatly in both confirming previous gene findings as well as discovering novel genes and loci associated with the risk of VTE 70. Although several gene variants have been proposed, only 16 of the genes have been robustly associated with VTE risk 71. More recent GWAS studies and meta-analysis have discovered additional gene variants and loci associated with VTE, however the strength of the associations and effect sizes found might suggest that the most important common variants have already been discovered 72-74.

The thrombotic tendency caused by inherited thrombophilias can either be by mutations leading to the loss of anticoagulant function or by gained procoagulant function. Inherited loss-of-function thrombophilias include antithrombin, protein S and protein C deficiencies. They are all natural inhibitors of the coagulation cascade, and the loss of function is often quite severe. AT deficiency is associated with a 10-50 fold increased risk of VTE but rarely occurs in the general population (0.02%) although it can be caused by more than 200 different mutations 68,71. Protein C and protein S deficiencies are also rare in the population (<1%). These deficiencies are associated with an 8-10 fold higher risk of VTE and several mutations have been reported 71,75.

Inherited gain-of-function thrombophilias are more prevalent in the general population but often less severe in effect. The non-O blood group, with a prevalence of approximately 60%, is the most common gain-of-function mutation affecting VTE risk 76-78. The risk of VTE is 1.5-2 fold higher for individuals with blood groups A1 and B compared to blood group O 76,79. Although the increase in VTE risk is small, the prevalence of the non-O blood group in the
general population makes this an important inherited risk factor for VTE. The factor V Leiden mutation (FVL) is caused by a single point mutation (G to A substitution) in the factor V gene causing APC resistance. The FVL variant varies among ethnicities, with the highest prevalence of approximately 5% found in the Caucasian population. The risk of VTE for heterozygous carriers of FVL is 2-5 fold increased, while homozygous carriers have a 10-80 fold increased risk, compared with non-carriers. The prothrombin G20210A mutation is similarly more prevalent in the Caucasian population with a prevalence of 1-3%, and carriers have a 3-4 fold increased risk of VTE compared to non-carriers. The prothrombin mutation leads to a hypercoagulable state through increased levels of prothrombin, and consequently enhanced thrombin generation.

1.3.2 Acquired risk factors
A number of acquired risk factors for VTE have been identified through epidemiological studies. High and advancing age is one of the strongest risk factors for VTE, and about 70-90% of VTE events in the population can be ascribed to aging. The risk increases exponentially after the age of 50, while at ≥ 85 years of age the risk of VTE is 80 fold higher, compared to individuals aged 20-30 years. The proposed mechanisms behind the observed association are increased levels of procoagulant factors in blood, degenerative and functional changes to the vessels and valves, and general frailty and immobility due to illness, infection and comorbidities. The loss of muscle mass following inactivity also contributes to reduced venous return and stasis in the lower extremities.

Obesity, defined as body mass index (BMI) above 30 kg/m², is associated with a 2-3 fold higher risk of VTE, compared with normal weight individuals. Furthermore, there is a dose-dependent relationship between increasing BMI and increasing VTE risk. Weight gain increases the risk for VTE, particularly for already obese individuals where a 4-fold increase in risk is seen, compared to obese individuals maintaining a stable weight. Although BMI is the most commonly used anthropometric measure of obesity, waist circumference has been shown to be a more precise measure for detecting and predicting the risk of VTE in obese individuals. The proposed mechanisms behind the association are venous stasis due to intra-abdominal pressure, changes in blood components leading to a procoagulant state accompanied by decreased fibrinolytic activity and chronic low-grade inflammation. Recent
Mendelian randomization studies imply a causal relationship between obesity and VTE \(^{92-94}\). As the prevalence of obesity increases worldwide \(^{95}\), it is an important risk factor to consider when evaluating VTE risk as it is one of the few modifiable lifestyle risk factors currently known. Additionally, studies on the synergistic effects of obesity in combination with risk factors like prothrombotic genotypes (FVL, prothrombin mutation G20210A, non-O blood group), and oral contraceptive use on VTE risk have been reported \(^{96-99}\). The joint effect of obesity and FVL and prothrombin mutation G20210A increased the risk of VTE 6-8 fold, compared to normal weight individuals without the genetic predispositions \(^{96}\). Similarly, the joint effect of obesity and oral contraceptive use imposed a 24-fold higher risk of VTE compared to normal weight women who did not use oral contraceptives \(^{96}\).

**Body Height** is a risk factor for VTE observed particularly in men \(^{33,100}\). A prospective cohort study reported a 34% increased risk of VTE per 10 cm increase in body height in men, and a 13% increase in risk in women \(^{100}\). The link between tall stature and VTE could be explained by the fact that an increase in height will subsequently also increase the vessel area at risk, the hydrostatic pressure, as well as the number of venous valves in the legs \(^{101,102}\). Height has been found to be more strongly associated with DVT than PE, supporting the proposed mechanisms behind the observed association \(^{102}\). A synergistic effect of body height and obesity on VTE risk has been reported \(^{103}\). Men who were tall (\(\geq 182\) cm) and obese had a 5-fold higher risk of VTE than short (\(\leq 172\) cm) and normal weight men \(^{103}\). For women, the combination of tall stature and obesity increased the risk of VTE 3-fold, compared to short, normal weight women \(^{103}\).

**Cancer** imposes a major risk for VTE, and about 20-30% of all incident VTE events are associated with cancer \(^{27,104,105}\). Overall cancer increases the risk of VTE 4-7 fold compared to the general population, but the risk varies considerably according to both cancer type and treatment regime \(^{106,107}\). The cancer types associated with the highest risk of VTE include pancreatic, ovarian, brain, hematological and lung cancers \(^{104,108}\). The risk of VTE in cancer patients is found to be the highest 6 months before cancer diagnosis and up to 12 months after diagnosis \(^{104,109}\). The potential explanations for the association between VTE and cancer can be grouped into treatment-, patient- and cancer-related factors. The treatment related factors include surgery, chemotherapy, radiotherapy, central venous catheters, blood transfusion and
erythropoiesis-stimulating agents. Patient-related factors include increasing age, a previous history of VTE, comorbidities, obesity and immobilization. Cancer type, cancer stage and time since cancer diagnosis are cancer-related factors. The impact of the tumor itself may play a greater role on the risk of VTE than the treatment related risk factors, as it has been shown that the risk of VTE was similar six months prior to and after a cancer diagnosis, when taking competing risk by death into account. Tumor growth can lead to a physical obstruction in the vessels and result in stasis. Additionally, tumor cells are known to enhance the procoagulant potential by activating coagulation through TF positive extracellular vesicles (TF+EVs), interacting directly with platelets and endothelial cells, influencing the release of proinflammatory cytokines and dysregulating the fibrinolytic system. For instance, in pancreatic cancer a hypercoagulable state is caused by the release of TF+ tumor-derived EVs into the circulation, which in turn triggers a VTE event. Lung cancer has been found associated with leukocytosis, which may enhance the risk of VTE through neutrophils and the generation of neutrophil extracellular traps (NETs).

Hospitalization, current or recent, is estimated to account for more than 50% of all VTE events, due to factors like immobilization, infection, surgery and fractures. Hospitalization-related VTE events were found to be the leading cause of disability-adjusted life-years lost in low and middle income countries, and second in high income countries worldwide emphasizing the global burden of VTE. Major surgery, that is surgery under general anesthesia exceeding 30 minutes, is one of the most important hospital-related risk factors for VTE, accounting for about 20% of the VTE events in the general population. Major surgery in general is associated with a 4-22 fold increased risk of VTE, but the risk estimates vary according to the type of surgery. Orthopedic surgery, and particularly total hip arthroplasty, as well as major vascular surgery and neurosurgery are procedures with particularly high risk for VTE. Patients suffering major trauma are at a 12-fold increased risk of VTE, and it has been estimated that as many as 58% of trauma patients may suffer from DVT in the absence of thromboprophylaxis. Similarly, acute medical conditions, like myocardial infarction, ischemic stroke, heart failure, infections and respiratory diseases are all associated with increased risk of VTE. Immobilization is a risk factor often accompanying hospitalization in the form of confinement to bed or wheelchair, bed rest for more than three days, plaster casts or paralysis and presents with an approximately 2-fold
increased risk for developing VTE \textsuperscript{118}. Additionally, immobilization as a risk factor for VTE can also be in the form of long-haul air travel \textsuperscript{119}.

**Pregnancy** is associated with a 4-5 fold higher risk of VTE compared to non-pregnant women of similar age. The risk increases further to 20-fold in the postpartum period, compared to non-pregnant women \textsuperscript{120,121}. Pregnancy alters the hemostatic system to a more procoagulant state, characterized by an increase in several coagulation factors (FVII, FVIII, and FX) and a decrease in the natural anticoagulant protein S, leading to acquired APC resistance \textsuperscript{122}. The alterations to the hemostatic system favoring a more procoagulant state during pregnancy are thought to be an important measure in minimizing potentially lethal blood loss during child birth. The risk of VTE during pregnancy is also increased due to mechanical changes caused by the expanding uterus leading to stasis by the increased intra-abdominal pressure and compression of the vena cava \textsuperscript{123}.

The use of **exogenous hormone supplements**, that is combined oral contraceptives (COC) or hormone replacement therapy, increases the risk of VTE by causing alterations to coagulation and fibrinolysis leading to an overall more procoagulant state \textsuperscript{124,125}. The risk of VTE increases 3-4 fold for COC users, and 2-4 fold for those undergoing hormone replacement therapy, compared to non-users. For both types of hormone supplements the increase in risk is most profound in the first months of use \textsuperscript{124,125}. The combined effect of COC use and the genetic risk factor FVL has been shown to account for a 35-fold higher risk of VTE, compared to non-users without FVL \textsuperscript{126}. Similarly, it has been reported that other genetic risk factors (non-O blood group, SNP rs2289252 in FXI), as well as environmental factors (smoking, high BMI) additionally increase the risk of VTE in women using COC \textsuperscript{127,128}.

1.4 Treatment of VTE
Anticoagulant treatment is associated with a 90\% risk reduction for recurrent events and all-cause mortality following two weeks treatment compared to no treatment \textsuperscript{5}. VTE treatment can be divided into the three following phases; acute (first 5-10 days), long-term (first 3 months) and extended (beyond 3 months). Generally, all DVT and PE patients are treated for 3 months with anticoagulation. However, decisions regarding extended treatment need to carefully balance the risk of recurrence against the risk of major bleeding \textsuperscript{129}. Vitamin K
antagonists (VKA), low molecular weight heparin (LMWH) and unfractionated heparin were for a long time the treatment options of choice, but in recent years additional options are available in the form of direct oral anticoagulants (DOACs). In 2014 the first DOAC, the direct thrombin inhibitor Dabigatran, was approved for the treatment of VTE in the United States. Shortly after, the first direct factor Xa inhibitors, namely Rivaroxaban, Apixaban and Edoxaban were launched and included in the 2016 American College of Chest Physicians’ (ACCP) guidelines. Several randomized controlled trials (RCTs) have compared the use of DOACs to LMWH and Warfarin and concluded that they are non-inferior when it comes to efficacy in preventing recurrent events and VTE-related deaths. However, the RCTs report diverging results regarding the risk of major bleeding ranging from statistically significant lowered risk to no difference between the study drugs. To date, there is no treatment or prophylactic option that effectively reduces VTE without the accompanying risk of major bleeding.

1.5 The coagulation system
Blood is a liquid that circulates in the vasculature under pressure. In case of injury to the vasculature it is important to minimize blood loss by rapidly converting liquid blood into a gel-like clot to serve as a plug. Blood consists of a cell portion including erythrocytes, leukocytes and platelets, as well as a plasma portion containing soluble proteins which act together to form a fibrin clot. Hemostasis is the normal process where the clotting cascade prevents blood loss following vascular damage. It can further be divided into primary hemostasis covering platelet activation, aggregation and adhesion at the site of injury, and secondary hemostasis covering the activation of the coagulation factors, the formation of fibrin and plug stabilization. Thrombosis, on the other hand, is the formation of a blood clot caused by coagulation triggered inside the lumen of a blood vessel, often in the absence of tissue damage.

In 1964 two research groups proposed at about the same time a cascade or waterfall model for blood coagulation. The models were based on a series of steps in which one coagulation factor would lead to the activation of the following factor, and so on, resulting in the generation of thrombin and the formation of a fibrin clot. Initially, all coagulation factors were suggested to be proenzymes which upon activation would be converted to an
active enzyme. However, this concept has later been modified after some of the coagulation factors, like FVa and FVIIIa, were found to serve as cofactors for other coagulation factors and not hold enzymatic activity individually. The coagulation system is comprised of the intrinsic and extrinsic pathways that merge into the common pathway with FX activation to FXa. FXa activates prothrombin to thrombin, which culminates in the formation of cross-linked fibrin (Figure 4).

**Figure 4.** Simplified overview of the intrinsic (blue), extrinsic (orange), and common pathway (red) of the coagulation system. (Adapted from Mackman N., J Clin Invest 2012)

The **extrinsic pathway** is also known as the tissue factor pathway. TF is an integral membrane protein expressed by specific cells within the vessel wall and surrounding the blood vessels, like pericytes, vascular smooth cells, and adventitial fibroblasts. TF is also expressed by monocytes under pathological conditions. TF deficiency in humans has never been discovered, and mouse models show that TF deficiency is incompatible with life.
underscoring the fact that TF is essential to hemostasis. TF initiates the coagulation cascade by binding with high affinity to the circulating coagulation factor FVII and the trace amounts (~1%) of circulating activated FVII(a). The inactive zymogen FVII is rapidly converted to FVIIa after complexing with TF. Negatively charged phospholipids, like phosphatidylserine expressed on the surface of damaged cells, activated platelets and microvesicles are crucial for the assembly of both the extrinsic and the intrinsic tenase complex. The activity of the extrinsic tenase complex increases by several orders of magnitude in the presence of negatively charged surfaces. In the absence of an appropriate surface, most coagulation enzymes show low activity with their substrate within a biologically relevant time frame. The TF:FVIIa complex activates its substrates FIX and FX to FIXa and FXa, respectively. Thrombin is subsequently formed in small amounts leading to the activation of cofactors FV and FVIII. In order for the coagulation cascade to propagate further, both FIXa and FXa need an appropriate surface in order to assemble together with their individual cofactors, FVIIIa with FIXa, and FVa with FXa. The prothrombinase complex, FXa-FVa, activates prothrombin to thrombin. With the formation of large amounts of thrombin fibrinogen is cleaved to fibrin monomers. The monomers polymerize spontaneously and FXIIIa cross-links the fibrin chains in order to stabilize the clot.

The intrinsic pathway of the coagulation system is activated by FXII coming in contact with negatively charged artificial surfaces. For in vivo activation of the intrinsic pathway, several potential activators are proposed like platelet-derived polyphosphates and extracellular nucleic acids. The activation of FXII leads to the formation of small amounts of FXIIa. A positive feedback loop is initiated by FXIIa activating prekallikrein (PK) to kallikrein, which again activates FXII. FXIIa further activates FXI to FXIa. FXIa activates FIX to FIXa, which together with cofactor FVIIIa forms the intrinsic tenase complex. This activates FX to FXa in the common pathway, leading to the generation of thrombin and ultimately the formation of fibrin. Interestingly, neither mice nor humans deficient in FXII suffer from defects in hemostasis, suggesting that the intrinsic pathway does not contribute significantly to hemostasis. However, several studies have suggested a role for FXII and the intrinsic pathway in thrombosis, and for FXII and FXI as potential therapeutic targets.
Excessive clotting is harmful to the host, hence coagulation must be tightly regulated through the natural anticoagulants TFPI, AT and protein C. TFPI directly inhibits FXa, and therefore the activity of the prothrombinase (FXa/FVa) complex. TFPI also inhibits the TF/FVIIa complex. TFPI is the only endogenous protein that effectively inhibits these complexes under physiological conditions. No human deficient in TFPI has ever been reported, emphasizing the importance of TFPI in hemostasis. Observations from mouse studies show that TFPI deficiency leads to death in utero, strongly suggesting that TFPI deficiency is not compatible with life. Antithrombin, another natural anticoagulant, primarily inhibits FXa and thrombin in the common pathway, as well as the TF/FVIIa complex in the presence of heparin. Heparin and heparin-like glycosaminoglycans enhance the anticoagulant activity of AT by 100 to 1000-fold. APC bound to its cofactor protein S, inhibits FVa and FVIIIa. Defects in the regulation of coagulation can lead to either bleeding events or thrombosis. As previously discussed, deficiencies in AT, protein C and protein S are established genetic risk factors for the development of VTE.
1.6 Extracellular vesicles
Our knowledge on EVs has grown immensely during the last decades from the first observations of “thromboplastic substances”\textsuperscript{162} and “platelet dust”\textsuperscript{163} to the vast knowledge of EVs that we hold today. As early as 1946 Chargaff and West published their observations of “thromboplastic substances” in blood affecting coagulation. These substances were sedimentable by high speed centrifugation, and the sedimentation of these substances resulted in prolonged plasma clotting time\textsuperscript{162}. In 1967 Peter Wolf published his findings on what he named “platelet dust”. The platelet dust could be separated by ultracentrifugation, was rich in phospholipids and described to hold coagulant properties like Platelet Factor 3\textsuperscript{163}. With time and advances in technology and methodology numerous papers have been published on the topic of EVs\textsuperscript{164}.

EVs are small vesicles that are released from a parental cell either by direct budding from the plasma membrane or by fusion of multivesicular bodies with the plasma membrane releasing small vesicles into the extracellular space (Figure 5). EVs are enclosed by a phospholipid bilayer membrane, which distinguishes them from other membrane vesicles like lipoproteins\textsuperscript{9,23}. From an evolutionary perspective, the secretion of bi-layered membrane vesicles seems to be a common and conserved process. The release of EVs has been reported for both eukaryotic as well as prokaryotic cells. In humans, EVs have been isolated and studied in most bodily fluids like blood, urine, breast milk, saliva, amniotic fluid and semen\textsuperscript{9,23,165}. Several different names have been proposed for the vesicles, and for a long time a common consensus was lacking. This gave rise to a vast nomenclature either referring to their size (using the prefixes micro or nano for microparticles, microvesicles, nanoparticles, nanovesicles), to a proposed function (calcifying matrix vesicles), the cell or tissue they derived from (prostosomes, oncosomes), or by their presence outside of a cell (using the prefixes exo or ecto for exosomes, ectosomes)\textsuperscript{165}. However, the most commonly used subgroups are exosomes, microvesicles (MVs) and apoptotic bodies. Exosomes are the smallest of the EVs ranging from 30 to 100 nm and are released into the extracellular space by multivesicular bodies fusing with the plasma membrane. Microvesicles range in size from 100 to 1000 nm and bud directly from the plasma membrane. Apoptotic bodies are the largest of the released vesicles and have been described to range from 1000 to 5000 nm. These bud off the plasma membrane of apoptotic cells\textsuperscript{9,23}. 
Figure 5. Overview of the different types of membrane vesicles released by eukaryotic cells. Microvesicles and apoptotic bodies are released by direct budding from the plasma membrane (PM), while exosomes are released by fusion of internal multivesicular bodies (MVB) with the PM. (Used and modified with permission of Annual Reviews, Inc., from Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicle. Colombo, M., Raposo, G., and Théry C. Annu Rev Cell Dev Biol, 2014. 30: p. 255-89; permission conveyed through Copyright Clearance Center, Inc.)

EVs are important as they are able to transfer information from one cell to another, and influence the recipient cell function. EVs can transfer messages in the form of proteins, lipids, nucleic acids (mRNA, microRNA) and sugars. The phospholipid bilayer membrane surrounding the vesicle protects the message molecule and allows for the vesicle to travel with the biological fluid to sites remote to the parental cell. The release of EVs is a natural process, however most of the attention in research has been focused on the role of EVs in disease states. Pathological mechanisms known to trigger EV release include inflammation, shear stress, inducers of apoptosis, as well as activation of the coagulation system and the complement system.
1.6.1 Formation and uptake of EVs
The cytoplasmic membrane of eukaryotic cells has an asymmetric distribution of phospholipids, where negatively charged phospholipids like PS are located in the inner leaflet of the membrane of resting cells (Figure 6). There are three important enzymes that maintain the lipid asymmetry of the membrane: flippase, floppase and scramblase 166,169. The formation of EVs is initiated by an increase in the cytosolic concentration of calcium ions, which in turn activates scramblase. This results in a loss of membrane phospholipid asymmetry by increased scramblase activity together with the activation of floppase and the inactivation of flippase. PS is subsequently translocated to the outer leaflet of the membrane and exposed to the surroundings. Calpain, a calcium-dependent proteolytic enzyme is also activated, and causes a calcium dependent degradation of various proteins, allowing for the outward budding of MV from the membrane 166,169. The importance of proper enzymatic maintenance of the lipid asymmetry of the membrane is emphasized by the rare bleeding disorder Scott syndrome. Scott syndrome is characterized by a dramatic impairment of the procoagulant activity of stimulated platelets due to a functional defect in the surface exposure of anionic phospholipids 170. Scramblase is found to be defective and thereby unable to translocate PS from the inner to the outer leaflet of the membrane of activated platelets in addition to causing impaired membrane vesiculation.166,170.

Figure 6. The cytoplasmic membrane of eukaryotic cells has an asymmetric distribution of phospholipids with negatively charged phospholipids, shown in green, mainly located in the
inner leaflet of the membrane of resting cells. During EV formation the negatively charged phospholipids are translocated to the outer leaflet of the membrane and exposed to the surroundings. (Modified from Schindler S. M., Microparticles: A New Perspective in Central Nervous System Disorders, BioMed Res Int 2014 171. Printed with permission through the Creative Commons Attribution License: https://creativecommons.org/licenses/by/4.0/).

The biogenesis of exosomes starts with the inward budding of the endosomal membranes generating intraluminal vesicles (ILV) within large multivesicular bodies (MVBs) (Figure 5). Cytosolic components are engulfed within the ILV, while transmembrane proteins are incorporated into the invaginating membrane 172. Intracellular MVB can either be subjected to proteosomal degradation in the lysosomes, or fuse with the plasma membrane. Upon fusion with the plasma membrane, the MVBs release their contents (ILV) into the extracellular space. The intraluminal vesicles are referred to as exosomes as they are released from the MVB and into the extracellular space 172,173.

Several mechanisms has been proposed for EV uptake into cells, including phagocytosis, clathrin- and caveolin-mediated endocytosis, macropinocytosis and plasma or endosomal membrane fusion 174. EV uptake by the target cell may depend on the type of recipient cell and its physiological state, as well as ligand-receptor recognition by the EV and the target cell 173. Different mechanisms for EV internalization has been described, with clathrin-dependent endocytosis or phagocytosis in neurons, caveolin-mediated endocytosis in epithelial cells and cholesterol and lipid raft dependent endocytosis in tumors 173. In addition, EVs can exert their functional effect on the target cell through direct receptor-ligand interaction 173. The level of EVs in circulation reflects the balance between generation and clearance. Studies differ with regard to the reported half-life of EVs 23. A study conducted in rabbits reported that biotin-labelled platelet-derived EVs which were reintroduced into the animal were cleared within 10 minutes from the circulation 175. In rats, red blood cell derived EVs which were labelled and injected into the animal, were found to be 91% cleared from circulation within 30 minutes 176. A similar clearance of 90% within half an hour was also reported for EVs derived from splenocytes 177, and B16 melanoma cells 178 in mouse models. The biodistribution of EVs varies with the cellular origin of the EVs. Melanoma-derived EVs were mainly taken up by lungs and spleen 178. However, red blood cell derived EVs were mainly taken up by the liver.
(44.9%) followed by bone (22.5%), skin (9.7%), muscle (5.8%), spleen (3.4%), kidney (2.7%) and lung (1.8%) 176.

1.6.2 EVs and coagulation
EVs are procoagulant due to their surface expression of procoagulant proteins such as TF and negatively charged phospholipids (mainly phosphatidylserine, PS), which explains their role in coagulation activation. In contrast to TF’s vital role in activating coagulation, elevated levels of TF+EVs are only found in certain disease states such as certain cancers or disseminated intravascular coagulation (DIC) 19-22. During EV formation the phospholipid asymmetry in the cell surface membrane is distorted and PS is exposed on the surface of the EV. PS facilitates the assembly of coagulation factors FVII, IX and X, as well as prothrombin on the EV surface, and it has been shown that the cleavage of factor X by soluble TF-VIIa showed a more than 100-fold enhanced reaction rate in the presence of phospholipids 144,179. PS binds to the γ-carboxyglutamic acid (GLA) domains located at the N-terminal of coagulation factors FVII, IX, X and prothrombin. PS binds in a reversible and calcium-dependent manner to the GLA domains 180. The impact of PS+EVs on coagulation can be assessed directly in plasma using a PPL activity clotting assay.

1.6.3 EVs and VTE
Several studies have investigated the association between plasma levels of EVs and VTE. While some report elevated plasma levels of EVs 181,182, others focus on the expression of particular antigens 11,19, the procoagulant activity 20, or a combination of the above 21,181,182. However, the relationship between EVs and VTE is to date still best understood in cancer patients 19-22.

There are limited original papers on the relationship between EV procoagulant activity as measured by clotting assays, and VTE. However, in a cross-sectional study including 100 patients referred to the Emergency Department under suspicion of VTE, plasma clotting time was assessed by the STA Procoag PPL assay. It was found that PPL clotting time did not discriminate between patients with (n=31) and without VTE 183. Still, the lack of discriminatory diagnostic power by the PPL assay may have been diluted by other conditions associated with shortened clotting time among patients without VTE, and therefore do not
exclude the potential of plasma clotting time as a potential predictive biomarker of VTE. Still, circumstantial evidence supports an association between procoagulant plasma clotting time and future risk of VTE. First, the procoagulant clotting time is inversely associated with annexin V+ EVs \(^{184,185}\) and the plasma levels of EVs are associated with VTE risk in most \(^{181}\), but not all studies \(^{186}\). Second, in a cross-sectional study including 100 samples from healthy individuals and patients with obstructive sleep apnea, plasma clotting time presented strong and inverse correlations to parameters of thrombin generation. This was measured using the Calibrated Automated Thrombogram method with the addition of minimal amounts of phospholipids and TF (1 pM) to trigger thrombin generation \(^{185}\). Several studies have shown that parameters of the thrombogram are associated with incident \(^{187-189}\) and recurrent \(^{190}\) VTE. Third, carriers of rare (e.g. deficiencies of antithrombin, protein C and S) \(^{191}\) and common (e.g. factor V Leiden and the prothrombin mutation G20210A) \(^{192,193}\) prothrombotic genotypes had significantly shorter plasma clotting time than non-carriers, providing indirect evidence for lower risk of VTE with prolonged plasma clotting time. However, most of the current studies are cross-sectional or retrospective, making it difficult to deduce whether EVs play a causal role of the disease or are merely a consequence of it. In addition, methodological challenges such as reverse causation and selection bias may hamper the interpretation of the results.
2. Aims of the thesis

The overall aim of the present thesis was to develop an easily available and reproducible FXa-dependent clotting assay to measure plasma procoagulant phospholipid (PPL) activity, and investigate the association between plasma PPL activity and the risk of VTE.

The specific aims were:

I. To investigate the impact of plasma preparation, assessed by freezing plasma before (PPP) or after (PFP) a second high-speed centrifugation, various anticoagulants in commercial blood collection tubes (Citrate, EDTA, CTAD, and Heparin), and fasting status on plasma concentration and size distribution of EVs using Nanoparticle Tracking Analysis (NTA) and scanning electron microscopy (SEM).

II. To develop a modified PPL-dependent clotting assay, capable of measuring the PPL activity in human plasma and cell supernatants of in vitro experiments, by removing PPL from plasma by sequential centrifugation, including final ultracentrifugation.

III. To investigate the association between plasma PPL clotting time and the risk of incident VTE in a nested case-control study derived from the general population.

IV. To investigate the impact of rosuvastatin treatment on plasma PPL activity in individuals with a previous history of VTE in a randomized controlled trial, and explore the effect of rosuvastatin treatment on total- and platelet-derived EV counts using a sensitive flow cytometer.
3. Methods
3.1 Study populations
For the papers included in the current thesis, different study populations were used including healthy volunteers (paper I and II), a nested case-control study derived from a population-based cohort (paper III) and a randomized controlled trial (paper IV). The two latter populations will be further addressed in this section.

3.1.1. The Tromsø study
The Tromsø study is a single-center population-based cohort study with repeated health surveys on the inhabitants of the municipality of Tromsø 194. Tromsø is the largest city in northern Norway with a population of about 77,000 inhabitants. The first Tromsø study was conducted in 1974 to investigate why there was such high mortality from cardiovascular disease among young men in Norway, and particularly in the northern part of the country. The University of Tromsø was fundamental in the creation, as well as the continuation of the health surveys, resulting in numerous research findings made available through publications and doctoral thesis. Overall, more than 45,000 unique individuals, aged 25-97 years, have participated in one or more of the Tromsø studies. The participation rates for the studies have been high, ranging from 65-85% 194. To date seven surveys have been completed, with an eighth survey planned for 2024-25.

The fourth survey of the Tromsø study (Tromsø 4) was conducted in 1994-95. All inhabitants of the municipality of Tromsø aged 25 years or older were invited to participate in the study. Tromsø 4 is the largest of the studies with 27,158 participating individuals, and a participation rate of 77%. The participants were followed from the date of inclusion in 1994-95 and until migration from the municipality, death or end of follow-up (September 1, 2007). Baseline information was collected through self-administered questionnaires, blood sampling, and physical examinations. The questionnaires assessed aspects of the participant’s general health, lifestyle, diet, medication use, and the occurrence of particular disease states in the immediate family (arterial cardiovascular diseases and cancer). All participants had their height (to the nearest cm) and weight (to the nearest 0.5 kg) measured, wearing light clothing and no shoes. Body mass index (BMI) was calculated using weight in kilograms divided by height in meters squared (kg/m²). Non-fasting blood samples were collected by venipuncture of an antecubital vein, with minimal stasis, into blood collection tubes containing ethylenediaminetetraacetic
acid (K$_3$-EDTA 40 µL, 0.37 mol/L per tube) (Becton Dickinson, Meylan Cedex, France). Cell counts were performed using a Coulter Counter (Coulter Electronics, Luton, UK). Platelet poor plasma (PPP) was prepared by centrifugation at 3000 x g for 10 minutes at room temperature. Plasma aliquots were transferred to 1 mL cryovials (Greiner Laboratechnik, Nürtringen, Germany) and stored at -80°C until analysis. The Regional Committee for Medical and Health Research Ethics, North Norway (REC Nord) approved the study, and all participants provided informed written consent.

**Identification and validation of the VTE events**

All incident VTE events occurring among the participants from inclusion and until the end of follow-up were identified by searching the hospital discharge diagnosis registry, autopsy registry and the radiology procedure registry at the University Hospital of North Norway (UNN). UNN is the sole provider of all hospital-based and outpatient medical care in the region, including all relevant diagnostic radiology and treatment of VTE. International Classification of Diseases (ICD), revision 9 (ISC-9), codes 325, 415.1, 451, 452, 453, 671.3, 671.4 and 671.9 were used to identify VTE events occurring from 1994 to 1998, while revision 10 (ICD-10) codes I26, I80, I81, I82, I67.6, O22.3, O22.5, O87.1, and O87.3 were used for the time period 1998 to 2007. After the identification of events, trained personnel reviewed the medical records for every potential VTE case for validation. VTE events from the hospital discharge diagnosis registry and the radiology procedure registry were recorded and found valid only if all of the following criteria were met:

1. Clinical signs and symptoms of DVT or PE, or both, were present
2. The event was objectively confirmed by a diagnostic procedure (compression ultrasound, venography, computed tomography, perfusion-ventilation scan, pulmonary angiography, or autopsy)
3. A physician noted the diagnosis of DVT or PE in the patient’s medical record
4. Treatment was initiated (anticoagulant medication, thrombolysis, vascular surgery) unless contraindications were specified

VTE cases from the autopsy registry were recorded if VTE was indicated as the cause of death, or was noted as a significant contributing condition to death in the autopsy report.
The confirmed VTE events were further classified as a DVT or PE, and in the case of both present, the event was classified as a PE. A VTE was classified as provoked if one of the following risk factors were found in the medical records up to 8 weeks preceding diagnosis; major surgery, trauma, active cancer, acute medical conditions like acute myocardial infarction, ischemic stroke or major infectious disease, immobilization defined as bed confinement exceeding 3 days, wheelchair confinement or long haul travel exceeding 4 hours within two weeks before VTE event, or other factors specified in the medical records to be provoking, like plaster cast or intravascular catheter. For unprovoked events, none of the above mentioned risk factors were present.

**Paper III** is based on a nested case control study derived from the 4th survey of the Tromsø study. There were 462 individuals who experienced a VTE event during the follow-up period (1994-2007). For each case, two age- and sex-matched controls, who were alive at the index date of the corresponding VTE-case were randomly sampled from the source cohort (n=924). In total, 349 (140 cases and 209 controls) lacked plasma samples and 67 (26 VTE cases and 41 controls) had plasma samples of insufficient quality (e.g. due to hemolysis). Hence, our study population consisted of 296 subjects with incident VTE and 674 age- and sex-matched controls. We measured the PPL clotting time in all samples using our modified FXa-dependent clotting assay as described in detail previously. Information on age, sex, BMI, previous cardiovascular disease and cancer were obtained from the Tromsø study.

### 3.1.2 The Statins Reduce Thrombophilia (START) Trial
STAtins Reduce Thrombophilia, or the START trial (NCT01613794), is a multicenter, randomized, controlled, open label clinical trial aimed to investigate the impact of rosvustatin treatment on the coagulation profile of individuals with a previous history of VTE. The study has been described in detail elsewhere. In brief, participants were recruited from three Dutch anticoagulation clinics (Leiden, Hooíddorp, and Rotterdam) which monitor anticoagulant treatment of VTE patients within a geographical area. Subjects with confirmed initial or recurrent symptomatic proximal DVT or PE allowed to stop oral anticoagulation treatment by their treating physician and aged 18 years or older, were invited to participate. Exclusion criteria were the following: individuals already using statins or other lipid lowering drugs, or if contraindications for 20 mg/day rosvustatin use were present,
based on information provided by the instruction leaflet of the drug manufacturer. Participants were randomly assigned to either 20 mg/day of rosuvastatin or no study medication for the 28-day study duration. The random allocation sequence was implemented by a central telephone, and the sequence was concealed until interventions were assigned. Adherence to the study protocol was assessed in two ways. First, participants in the treatment group took the first tablet of rosuvastatin in the presence of an investigator. Second, compliance to treatment was assessed by measurements of total cholesterol levels at baseline and at study end in all participants. The START trial was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands, and all study participants gave written informed consent prior to participation.

The study baseline was set as the last regular visit of the participants to the anticoagulation clinic. All participants were screened on acquired risk factors for VTE through a questionnaire, in addition to being tested on kidney and liver function. The participants had stopped using their vitamin K antagonist one month prior to baseline blood draw to allow a wear off of the anticoagulant drugs. Non-fasting blood samples were collected in Vacutainer tubes containing 3.2% sodium citrate (Becton Dickinson, Meylan Cedex, France) at baseline and at study end (i.e. 28 days later). Samples were centrifuged at 2,500 x g for 15 minutes at 18°C and platelet poor plasma (PPP) was stored at -80°C until analysis.

**In paper IV** we measured the PPL clotting time in plasma samples from 125 participants who received rosuvastatin treatment and 120 participants from the control group. Measurements were performed using our modified FXa-dependent clotting assay previously described. In addition, we measured total and platelet-derived microvesicle counts using a sensitive flow cytometer (CytoFLEX, Beckman Coulter) in a selection of the trial participants (40 from the treatment group and 20 from the control group).
3.2 Methods used for EV measurements

3.2.1 Isolation of EVs

EVs can be isolated from their source fluid by several methods, such as ultracentrifugation (UC), size-exclusion chromatography, ultrfiltration, sucrose-gradient UC, density-gradient UC, or by a combination of the above. Precipitating agents like PEG and other commercial reagents have been suggested as rapid methods to use in order to enrich EVs from a biological fluid. However, these reagents also co-precipitate contaminant like lipoproteins.

Choosing the method of EV isolation is a process of considering the pros and cons of each method while keeping in mind the source of the biological fluid, the input volume, the downstream application (required degree of purity), and the number of samples to be processed. For the work in the current thesis, the biological fluids used have been human blood and blood plasma. EVs have either been measured directly in plasma (PPL activity and CAT assay) or isolated by ultracentrifugation (20,000 x g for 30 minutes) and resuspended in buffer for subsequent analysis (NTA, flow cytometry, and electron microscopy).

Ultracentrifugation is considered the gold standard for EV isolation. Blood is first centrifuged by one or more low-speed centrifugations to remove cells and cell debris and to generate plasma. Larger EVs (microvesicles) are most commonly pelleted from plasma by centrifugation speeds in the range of 10,000-20,000 x g for approximately 30 minutes. Smaller EVs (exosomes) are pelleted at a higher speed (100,000-120,000 x g) for a longer duration of time (from 1 hour and longer). Ultracentrifugation as a method cannot be used to absolutely discriminate between EV sizes, since sedimentation also will depend on the density and cargo of a vesicle, as well as the distance it needs to travel to be pelleted. To overcome the latter obstacle, it has been recommended to dilute biological fluids with PBS in order to alter the viscosity of the sample fluid and thereby enhance the sedimentation of EVs. Ultracentrifugation has been described to induce aggregation of EVs as a result of the high speed applied. The method will also to some extent co-isolate contaminants from the source fluid. However, it is a relatively quick and easy isolation procedure with the possibility of high throughput of samples, and it requires minimal sample handling. The importance of reporting all aspects of EV isolation, in order to ensure reproducibility of study findings procedures has been stressed. The physical separation of vesicles not only depends on the g force applied, but also the type of rotor used (fixed angle, swinging bucket), the pelleting efficiency (rotor k-factor) and sample viscosity.
3.2.2 Quantification and size distribution of EVs

Nanoparticle tracking analysis (NTA) is an advanced technique allowing for direct and real-time visualization and analysis of vesicles in solution. The technique combines the light scattering properties of vesicles with their Brownian motion in order to determine the size distribution and concentration. The technique is unique in that each particle is individually tracked and analyzed. In brief, the sample of interest, in this case vesicles in suspension, is inserted into a sample chamber. The vesicles are then visualized using a conventional optical microscope connected to a video camera. The sample is recorded by the camera for a set period of time, typically 60s, and for a number of repeats, and video files are generated. The software uses the videos generated to track individual vesicles frame-by-frame as they move in the sample chamber throughout the length of the video. The size is determined using the mean squared displacement to calculate their theoretical hydrodynamic diameter using the Stokes Einstein equation. NTA also estimates the concentration of vesicles in the sample.

The NanoSight (Malvern, UK) instruments are the most commonly used NTA instruments in the EV field. According to the manufacturer they offer a detection range of approximately 10-1000 nm. The detection range depends on the refractive index of the particles or vesicles in question. Analyzing particles at the lowest end of this range is possible only for particles composed of materials with high refractive index, for example gold and silver, while biological vesicles have a lower refractive index. The upper size limit is restricted by the limited Brownian motion of large particles. The viscosity of the solvent also influences the movement of particles, and it too plays a part in determining the upper size limit for a specific system. All the models of the NanoSight instruments are based on the same principle of detection, but the more advanced models also offer measurements in fluorescence mode (NS300 and NS500) and measurements of single particle zeta potential (NS500).

The advantages of NTA are that it is a rapid method that provides information on both the size and count of individual vesicles in a sample. The method discriminates well between size populations in heterogeneous samples, which biological samples most often are. As NTA measures vesicles in suspension, it does not require major handling or processing of a sample prior to analysis. This limits potential artifacts, like shrinkage due to fixation. As for
disadvantages, NTA is unable to identify the phenotype of the vesicles measured. The later models of NanoSight (NS300 and NS500) introduced a fluorescence mode allowing for the detection of labelled vesicles. However, labelling for NTA require fluorophores that are bright, photostable and small in size, so it does not add substantially to the size of the EV itself. Another disadvantage of the method is that NTA is not able to discriminate between true EVs and other spherical vesicles or particles in a sample, for example lipoproteins or contamination of chemical particles from the reagents used. Therefore, an absolute requirement for obtaining correct measurements using NanoSight is the removal of contaminating particles from buffers, reagents and culture media used.

There are several publications evaluating the performance of the NanoSight instruments, as well as publication comparing their performance to other established methods like flow cytometry and electron microscopy. NanoSight was shown to be a sensitive instrument able to accurately determine the size distribution of both monodisperse and polydisperse samples. The coefficients of variation (CV) for NTA have been reported for intra-day variation to range from 1-7% for size measurements, and 5-12% for concentration, while the inter-day variations ranged from 1-9% for size and 5-18% for concentration measurements.

NanoSight NS300 (Malvern, UK) was used in paper I to evaluate the effect of various pre-analytical conditions on the concentration and size distribution of EVs isolated from human plasma by ultracentrifugation. Samples of isolated EVs were diluted (50–100×) in particle-free Dulbecco’s phosphate buffered saline without CaCl₂ and MgCl₂ (Sigma-Aldrich, St. Louis, MO, USA), and manually inserted into the sample chamber using a 1 ml syringe. The samples were recorded at ambient temperature with automatic temperature monitoring. Three separate dilutions of the samples were used for analysis. Each dilution was recorded 3 times for 60 seconds each at camera level 16 (highest magnification of the microscope). The sample was refreshed between the captures, and the sample chamber was cleaned between each sample. The nine resulting videos were analyzed with NTA software version 3.0 at detection threshold 5, and the mean values for concentration and size distribution were calculated and compared.
Flow Cytometry is an established and commonly used method for the simultaneous measuring of multiple physical characteristics of a single cell, like size and granularity. In addition, fluorescent labelling of specific antigens using antibodies provides additional information on subpopulations in a sample. The main principle of flow cytometry is the light scattering and fluorescence emission generated by a cell or vesicle. The main components of a flow cytometer are fluidics, optics, detectors and a computer. The fluidics are responsible for transporting the sample through the instrument. The sample in question (i.e. cells or vesicles in solution) is diluted by sheath fluid in the instrument, and the cells or vesicles are made to align in a single file fashion by pressure differences when passing through the laser beam in the flow chamber. The optical system consists of a laser, lenses and collection optics. The laser beam is directed at the fluid stream with a single line of cells or vesicles, and light is scattered as the laser light strikes the cell or vesicle. Two types of light scatter occur, forward scatter (FSC) and side scatter (SSC). FSC is used as a measure of the cell/vesicle size, while SSC is a measure of cell granularity or internal complexity. In flow cytometers there is a variety of laser configurations able to excite a number of different fluorophores. The collection optics are lenses designed to collect the light emitted from the interaction between the laser beam and the cell/vesicle, as well as a system of optical mirrors and filters (long pass, short pass or band pass filters) that separate and direct specified wavelengths to the designated optical detectors. The light signals are converted into voltages by photodetectors and then into digital signals for computer processing.

As EVs are only a fraction of the size of cells, analyzing EVs using conventional flow cytometers is rather challenging. Conventional flow cytometers have been reported to have a lower detection limit of approximately 300-500 nm for polystyrene beads. However, biological vesicles have a lower refractive index than beads, and the main population of EVs are below the detection limit of conventional flow cytometers. Specialized flow cytometers, on the other hand, have been reported to be able to detect 100 nm beads, corresponding to a biological vesicle of about 160-220 nm. Flow cytometry is a relatively easy method and the conventional instruments are commonly found in research laboratories making it an accessible method. Flow cytometry is useful as it provides information on multiple aspects of individual vesicles in solution. However, a major disadvantage is that specialized instruments are required for the optimal use of flow cytometry in EV research.
In paper IV we used the sensitive flow cytometer CytoFLEX (Beckman Coulter, Indianapolis, USA) to determine the concentration of total EVs and platelet-derived EVs in 60 plasma samples (40 from treatment group, 20 from control group) from the START trial. EVs were isolated from plasma by ultracentrifugation at 20,000 x g for 30 minutes, and stained for phosphatidylserine with FITC-labeled bovine lactadherin (Haematologic Technologies, Vermont, USA) and the platelet marker CD41 with APC-H7 clone HIP8 (Biolegend, San Diego, USA). EV pellets were incubated with either antibody or isotype control mixture for 20 min at 4°C in the dark. Samples were washed with 1 ml pre-filtered DPBS and centrifuged at 20,000 x g for 30 minutes at 4°C. The EV pellet was resuspended in Dulbecco's phosphate-buffered saline without Ca²⁺/Mg²⁺ (Thermo Fisher Scientific). The EV gate was set using Rosetta calibration beads (Exometry, Amsterdam, The Netherlands), and data analysis were performed using the software CytExpert 2.0 (Beckman Coulter, Indianapolis, USA). EVs were defined according to size and lactadherin-positive staining. The total number of EVs was calculated from the number of detected lactadherin-positive events in a sample, and further converted to EV number per microliter plasma (EV/µl) using the original volume of analyzed plasma (150 µl).

3.2.3 Measurements of EV activity
The activity of EVs, or their contribution to coagulation, can be measured using different assays. For the work in the current thesis, emphasis has been given to EV procoagulant activity driven by negatively charged phospholipids, measured using a modified FXa-dependent clotting assay.

Procoagulant phospholipid (PPL) activity clotting assays are based on the ability of procoagulant phospholipids to accelerate the conversion of prothrombin to thrombin with subsequent fibrin formation. An equal volume of sample plasma is mixed with PPL-free plasma provided as a reagent by the assay, and the assay read-out is seconds of clotting time. The reaction is triggered by the addition of FXa in excess together with calcium. This eliminates the influence of coagulation factors upstream of FXa, leaving the PPL provided by the sample as the rate-limiting factor. Hence, procoagulant plasma clotting time should be considered as the potential to facilitate coagulation activation when blood is exposed to triggers of the coagulation system. Clotting assays offer a complex reaction as they measure the PPL activity of plasma and not only captured PS-positive EVs, and they measure a
physiological end-point. In addition, clotting assays do not rely on a particular instrument as they can be performed on a variety of routine coagulation analyzers.

There are currently two main clotting assays commercially available, the STA-Procoag-PPL assay from Diagnostica Stago and the XACT assay from Haematex. The two assays use the same principal for testing the PPL activity of a plasma sample. For both assays, the phospholipid-free plasma provided as a reagent is chemically treated with phospholipase in order to remove PPL. For the XACT assay, they specify that this is achieved by the use of snake phospholipase \(^{217}\), while it is not specified for the Stago assay [Diagnostica Stago, Asnières sur Seine Cedex, France]. Additionally, the origin of PPL-depleted plasma differs between the assays. While porcine plasma is used in the XACT assay, the Stago assay uses human plasma \(^{216,217}\). The reasoning for using plasma from a different species was to try to overcome the effect of lupus anticoagulant on PPL measurements. The coagulation factors in the porcine plasma will not be inactivated by lupus anticoagulant, and therefore the assay sensitivity would remain the same \(^{218}\). Both the Stago and the XACT assay use FXa of bovine origin \(^{216,218}\). The XACT assay initially reported adjustment of the FXa concentration to give a clotting time of 100 seconds \(^{218}\), but later standardized this to a concentration of 0.01 U/ml \(^{217}\). The Stago assay similarly provides a bovine FXa solution of the same concentration [Diagnostica Stago, Asnières sur Seine Cedex, France]. The two PPL assays differ with regard to the use of a phospholipid calibrator. The XACT assay solves this problem by the inclusion of a synthetic PPL calibrator, while the Stago assay leaves it up to the users to create a reference range and standards for the clotting time \(^{218}\) [Diagnostica Stago, Asnières sur Seine Cedex, France]. The benefit of including a standardized phospholipid calibrator is that the clotting times measured can then be converted into a unit of phospholipids.

A way of measuring the degree of variability of an assay is by the coefficient of variability (CV). The Stago assay reports intra-assay CVs of 0.3% and 0.6% for the two samples tested, and inter-assay CVs of 1.3% and 2.1% [Diagnostica Stago, Asnières sur Seine Cedex, France]. Slightly higher CVs were reported by van Dreden and colleagues in the XACT assay, with intra-assay CVs of 3.3% and 3.1% for normal pooled plasma and patient plasma, respectively, and inter-assay CVs of 3.9% and 4.2% \(^{217}\).
The commercial assays are sensitive, easy to use and portray low CVs. However, when running larger series of samples, using the commercial assays becomes quite costly. Given the importance of PS and PS+EVs in coagulation activation, there is a need for a cost-effective assay with the possibility of between-laboratory comparisons of data. We therefore set out to modify a FXa-dependent clotting assay. We assumed that the vast majority of procoagulant phospholipids in plasma were EV-bound, and we examined whether sequential centrifugation, including final ultracentrifugation, might substitute phospholipase treatment for the production of a suitable assay plasma. We additionally chose to include a standardized phospholipid reagent (UPTT, BioData Corporation, Horsham, Pennsylvania, USA) to allow for clotting times to be converted into a standardized unit of phospholipids.

In **paper II** we developed a modified FXa-dependent clotting assay by substituting chemically phospholipid depleted plasma with PPL depleted plasma obtained by ultracentrifugation. We tested several aspects of the assay performance, like the influence of individual coagulation factors, postprandial lipoproteins and plasma centrifugation protocols. The modified PPL assay was compared to the commercial PPL assay STA-Procoag-PPL from Stago Diagnostica. The two PPL assays displayed similar sensitivity to exogenously added standardized phospholipids, and the PPL activity measured by the modified assay strongly correlates with the results from the commercial assay. The intra-day and between-days coefficients of variation ranged from 2–4% depending on the PPL activity in the sample. The modified PPL assay was insensitive to postprandial lipoprotein levels in plasma, as well as to TF+EVs isolated from stimulated whole blood.

In **paper III and IV**, we used the modified PPL assay to measure the PPL activity of plasma samples from a nested case-control study derived from the general population (paper III) and a randomized controlled trial (paper IV). In brief, 25 μl sample plasma was mixed with 25 μl phospholipid-depleted plasma (PPLDP). PPLDP was generated from pooled citrated PFP (n = 18), centrifuged at 100,000 x g for 60 minutes at 16°C (Beckman Optima LE-80K Ultracentrifuge, rotor SW40TI, Beckman Coulter, Indianapolis, Indiana, USA), aliquoted and stored at -80°C until use. The mixture of sample and PPLDP was incubated for 2 minutes at 37°C, before the reaction was initiated by the addition of 100 μl pre-warmed factor Xa reagent containing bovine Factor Xa (0.1 U/ml) in 15 mM calcium chloride, 100 mM sodium
chloride and 20 mM HEPES buffer (pH 7.0). Clotting tests were carried out in duplicates on a StarT4 instrument from Diagnostica Stago (Asnières sur Seine Cedex, France) and measured in seconds of clotting time or converted into phospholipid activity in mU/ml by the use of a UPTT calibrator.

**The Calibrated Automated Thrombogram (CAT)** is a method to measure thrombin generation in clotting plasma, either with or without platelets present in the sample (PRP, PPP or PFP). The method has previously been described by Hemker et al. 219,220. The CAT assay is a general physiologic function test of the thrombotic hemostatic system, and can be used to measure both thrombotic and bleeding tendencies. The CAT assay can detect deficiencies in all coagulation factors (except FXIII), as well as the effect of anticoagulant treatments (VKA, heparin and heparin-like, direct inhibitors). The assay CVs for endogenous thrombin potential (ETP) has been described to range from 15-19% for inter-individual variability, and from 4.5-7.4% for intra-individual variability, depending on the sample type (PPP or PRP, respectively) 219.

The general principle of the CAT assay is that a plasma sample, or plasma diluted with buffer, is mixed with trigger(s), e.g. TF and phospholipids, in the wells of a 96-well plate. The concentration of TF and phospholipids can be altered according to the objective of the experiment. For each plasma sample used, additional wells are needed where a thrombin calibrator is added to plasma. The thrombin calibrator is used to correct for the inner filter effect, donor-to-donor variability in the color of plasma, substrate depletion and instrumental differences. The plate is then incubated in the instrument at 37°C for 10 minutes. The reaction is initiated by the addition of calcium and a fluorogenic substrate dispensed into the wells by the instrument. Thrombin activity is calculated as a function of time by comparing the fluorescent signal from the sample well to that of a known stable concentration of thrombin activity in a parallel non-clotting sample (the calibrator well). A computer software will then calculate thrombin generation parameters like lag time (LG) (min), the time to peak (tPeak) (min), the peak of thrombin generation (peak) (nM), the area under the thrombin generation curve (nM*min) and endogenous thrombin potential (ETP) (Figure 7) 219,220.
**Figure 7.** Overview of the most commonly used thrombin generation parameters; lag time (min), the time to peak (tPeak) (min), the peak of thrombin generation (Peak) (nM), and the area under the thrombin generation curve, that is the endogenous thrombin potential (nM*min) (ETP). (Adapted from Hemker, H.C., Pathophysiolog Haemost Thromb 2003 and 2002 219,220).

In **paper III** we used the CAT assay to measure thrombin generation of plasma samples. Thrombin generation was measured in a Fluoroscan Ascent Fluorometer (Thermolabsystems OY, Vantaa, Finland). Fluorescence intensity was detected at wavelengths of 355 nm (excitation filter) and 460 nm (emission filter). Forty µl of plasma was mixed with 40 µl Hepes buffer (20 mM Hepes and 140 mM NaCl) and pipetted into the wells of round bottom 96-well microtiter plates (Immuno1, Lab Consult, Lillestrøm, Norway). Ten µl of TF solution (final concentration of 3 pM) (Innovin, Bade Behring) and 10 µl of a standardized phospholipid in solution (diluted 1:20) (UPTT, BioData Corporation, Horsham, Pennsylvania, USA.) were added as triggers. Both TF and UPTT were diluted to the stated concentrations in Hepes buffer. The plasma samples measured were a combination of pooled citrated PFP and
phospholipid depleted plasma (PPLDP) added in ratios of 100:0, 80:20, 60:40, 40:60, 20:80, 10:90, and 0:100, respectively. For each experiment, a fresh mixture of 2.5 mM fluorogenic substrate (Z-Gly-Gly-Arg-AMC from Bachem, Bubendorf, Switzerland), 0.1 M CaCl2, 20 mM Heps (Sigma Aldrich, St Louis, USA) and 60 mg/ml BSA (A-7030, Sigma Aldrich) with pH 7.35 was prepared. Each dilution of PFP/PPLDP was assigned its own calibrator (Thrombinoscope BV, Maastricht, The Netherlands). The computer software calculated lag time (LG) (min), the time to peak (TTP) (min), the peak of thrombin generation (PEK) (nM) and the area under the thrombin generation curve (nM*min) and endogenous thrombin potential (ETP). Plasma samples were run in duplicates and each experiment was repeated three times.

3.2.4 Electron microscopy

Transmission electron microscopy (TEM) is used to image, phenotype and count EVs. TEM is able to detect and characterize individual EVs. TEM is highly sensitive with an imaging resolution down to 1 nm\textsuperscript{209}. Thus, using immuno-gold labelling for TEM, proteins on the EV surface can be detected. TEM is a useful method to visualize and confirm the presence of biological vesicles with a bi-layered membrane after an EV isolation technique has been applied. However, electron microscopy is a labor-intensive method and does not allow for high-throughput of samples. EV sample preparation requires chemical fixation and dehydration, which potentially alter the morphology of the EVs. Additionally, limited and non-uniform adhesion of vesicles to the grid surface may affect the read-out on size distribution\textsuperscript{214,221}.

Scanning electron microscopy (SEM) is also used to characterize individual EVs. SEM uses an electron laser beam to scan the surface of a vesicle and generates information on its topography. Similarly to TEM, EV samples are processed before analysis by fixation and dehydration. Immobilized samples are spray-coated with a thin layer of conductive material like cobber or gold before imaging, which might affect the surface structure of EVs\textsuperscript{222}.

In paper I we used both TEM and SEM to investigate EVs isolated from human plasma by ultracentrifugation. For TEM, EVs were isolated by ultracentrifugation, resuspended in buffer and fixed in formaldehyde. The EVs were adsorbed onto an epoxy resin substrate containing
colloidal gold particles. The substrate was prepared by adsorbing 15 nm gold particles (Department cell biology, University of Utrecht, the Netherlands) on formvar/carbon coated copper specimen grids and then the gold-coated grids were embedded in a thin layer of epoxy resin between two layers of Aclar film and polymerized at 60 °C for 48 hours. The EV suspension was placed on the gold-loaded, epoxy-embedded specimen grids and in 1% glutaraldehyde, postfixated in 1% OsO4 and stained with 1% aqueous uranyl acetate. The EVs on the epoxy-embedded grids were dehydrated in a graded series of ethanol, infiltrated in an Epon Equivalent (AGAR 100, DDSA, MNA and DMP-30, Agar Scientific, UK) and polymerized at 60 °C for 48 hrs. Ultrathin sections of the embedded EVs were prepared using Ultracut S ultramicrotome (Lieca Microsystems, Vienna, Austria) and a Diatome diamond knife (Diatome, Biel, Switzerland). Images using a JEOL JEM 1010 transmission electron microscope (Tokyo, Japan) were acquired with a Morada camera system (Olympus Soft Imageing System, Münster, Germany).

For immune-gold labelling the EVs were fixed with 1% buffered glutaraldehyde and adsorbed onto carbon-formvar coated specimen grids before immunolabelling. Unspecific labelling was blocked on 0.1% cold water fish skin gelatin (CWFSG) (Sigma G-7765) and 1.5% bovine serum albumin. Samples were incubated with anti-annexin V (Anx5) antibody (abcam, Cat# ab14196), diluted in Anx5 binding buffer (BD Pharmingen, Cat#556454) and protein A-gold (University of Utrecht, The Netherlands). All immune-reagents were diluted in CWFSG and the grids washed in PBS between each step. The grids were finally fixed in 1% glutaraldehyde, washed in distilled water and dried in 1.8% Methylcellulose containing 0.3% uranyl acetate.

For SEM analysis, isolated EVs were negatively stained on formvar/carbon coated copper grids. The grids floated on sample drops for 30 minutes, and were treated with 1% glutaraldehyde, washed in PBS and ddH2O and contrasted/dried with the addition of 1.8% methyl cellulose and 0.3% uranyl acetate. EV size measurements were performed using the iTEM software (Olympus Soft Imaging Solutions, Münster, Germany) by measuring the diameter of at least 200 EV from the SEM pictures. The start and end of every diameter was set manually and the diameter was calculated by the program. Grids were mounted on a
specimen holder and coated with gold/palladium before SEM examination. The images were obtained using a Zeiss Merlin VP compact scanning microscope.

3.3 Statistical analysis
All statistical analyses were conducted using either SPSS for Windows (SPSS Inc., Chicago, Illinois, USA), GraphPad Prism for Windows (GraphPad Software, San Diego, California, USA) or R for Windows (The R Foundation for Statistical Computing c/o Institute for Statistics and Mathematics, Vienna, Austria).

In paper I, median values and interquartile ranges for continuous data (EV concentrations) were presented, as data was not normally distributed. To test for differences in EV concentrations between anticoagulants we used Friedman’s test for non-parametric and dependent continuous data. Bar graphs were used to display (i) EV concentrations according to size categories of EVs (<100 nm, 100–199 nm, 200–299 nm, 300–1000 nm), and (ii) mean sizes of EVs measured by NTA and SEM in the different anticoagulants. The correlation between triglycerides and EV concentrations as well as VLDL and EV concentrations was calculated using Pearson’s correlation coefficient. All analyses were performed using IBM SPSS Statistics version 22.

In paper II all statistical analyses were performed in Graph Pad Prism 9.0. ANOVA was used to test for differences in performance between the modified PPL assay and the STA-Procoag-PPL assay.

In paper III statistical analyses were performed using R (Version 4.0.4). Unconditional logistic regression models were used to estimate odds ratios for VTE with 95% confidence intervals with plasma procoagulant phospholipid clotting time (PPL_CT) used as a continuous variable, discretized to quartiles and dichotomized according to PPL_CT ≤ 25th percentile versus PPL_CT > 95th percentile. The analyses were adjusted for age, sex and BMI. The PPL_CT quartile cut offs were determined using the control group.
As the follow-up time in the source cohort was long (more than 12 years for many persons), the results based on baseline PPL\textsubscript{CT} measurements could be influenced by regression dilution bias. To investigate this, we performed analyses where we restricted the maximum time from blood sampling in Tromsø 4 to the VTE events, while keeping all controls in the analyses. The logistic regression analyses on time restrictions were set to require at least 10 VTE events, and odds ratios were generated at every 0.1 year increase and plotted as a function of time from blood sampling to VTE.

In paper IV statistical analysis were performed using R (Version 4.0.3). Descriptive statistics were used to describe the baseline difference between the intervention and the control group. For the results tables, the treatment and non-treatment group, as well as subgroups, were compared using two-sample t-tests with equal variance assumed and standard multivariate linear regression models adjusting for age and sex. Pearson’s correlation coefficient was used to estimate correlations.
4. Main results
4.1 Paper I: Impact of preanalytical conditions on plasma concentration and size distribution of extracellular vesicles using Nanoparticle Tracking Analysis

EVs are most often subjected to several pre-analytical handling steps before analysis. Optimal pre-analytical handling is therefore crucial to ensure valid measurements of parameters such as plasma concentration and size distribution of EVs.

In this study we aimed to investigate the impact of plasma preparation, various anticoagulants, including citrate, EDTA, CTAD, and heparin, as well as fasting status on the concentration and size distribution of EVs measured by Nanoparticle Tracking Analysis and scanning electron microscopy.

Blood was drawn from 10 healthy volunteers to investigate the impact of plasma preparation and anticoagulants. We also used plasma samples from 40 individuals from a previous population-based study to investigate the impact of postprandial lipidemia. Transmission electron microscopy was used to confirm the presence of EVs. The plasma concentrations of EVs were measured by NTA after isolation by high-speed centrifugation. The size distributions of the plasma EVs were determined using NTA and SEM.

TEM confirmed the presence of EVs after isolation by high-speed centrifugation from plasma. TEM and SEM-analyses showed that the EVs kept a spherical morphology after high-speed centrifugation. The plasma concentrations and size distributions of EVs were essentially similar for the various anticoagulants investigated. We found no statistical difference in either size or concentration of EVs measured by NTA, when plasma was prepared as PPP or PFP before freezing. Plasma levels of EVs were not significantly altered in response to a high-fat meal, but the mean sizes of VLDL particles were increased and interfered with EV measurements.

Isolation of EVs from plasma by high-speed centrifugation yielded similar concentrations and size distributions of EVs for the four anticoagulants tested. We found no statistical difference in concentration nor size of EVs when plasma was prepared as PPP or PFP before freezing. Plasma VLDL particles interfered with EV measurements particularly under postprandial conditions due to an increase in the median particle diameter of VLDLs.
4.2 Paper II: A modified clot-based assay to measure negatively charged procoagulant phospholipids

The interest in PPL activity has increased during recent years, mainly due to the increased understanding of the role of EVs in thrombosis and hemostasis. There are currently two commercial PPL-dependent clotting assays available, the STA-Procoag-PPL assay from Diagnostica Stago (Asnières sur Seine Cedex, France) and the XACT assay from Haematex (Hornsby, NSW, Australia). Both assays use chemical phospholipase treatment to deplete phospholipids from the reagent plasma.

The purpose of our study was to modify the PPL assay by substituting the chemically phospholipid depleted plasma with PPL depleted plasma obtained by ultracentrifugation This in order to get readily access to a sensitive and reliable assay to measure PPL activity in human plasma and cell supernatants.

The performance of the assay was tested, including the influence of individual coagulation factors and postprandial lipoproteins and compared to a commercial PPL assay (STA-Procoag- PPL assay). The two PPL assays displayed similar sensitivity to exogenously added standardized phospholipids. The PPL activity measured by the modified assay strongly correlates with the results from the commercial assay. The intraday- and between-days coefficients of variation ranged from 2–4% depending on the PPL activity in the sample. The modified PPL assay was insensitive to postprandial lipoprotein levels in plasma, as well as to TF+EVs from stimulated whole blood.

Our findings showed that the modified assay performed equally to the comparator, and was insensitive to postprandial lipoproteins and TF+ EVs. In addition, we introduced a standardized PPL reagent (UPTT) which allowed for clotting times to be converted into a standardized unit of phospholipids. These modifications led to the establishment of an accessible and convenient in-house assay.
Several observational studies have reported elevated plasma levels of EVs in VTE. Negatively charged procoagulant phospholipids, and phosphatidylserine in particular, are expressed on the surface membrane of EVs and are vital to coagulation. However, no previous study has investigated the association between plasma PPL\textsubscript{CT} and future risk of VTE.

In this study we aimed to investigate the association between plasma PPL\textsubscript{CT} and the risk of incident VTE in a nested case-control study.

We conducted a nested case-control study using 296 VTE patients and 674 age- and sex-matched controls derived from a general population cohort (The Tromsø Study 1994-2007). PPL\textsubscript{CT} was measured in platelet-free plasma using our modified factor Xa-dependent clotting assay (Paper II). Logistic regression was used to estimate odds ratio (OR) with 95% confidence intervals (CI) for VTE with PPL\textsubscript{CT} modelled as a continuous variable, across quartiles and in dichotomized analyses.

There was a weak inverse association between plasma PPL\textsubscript{CT} and risk of VTE per one standard deviation increase of PPL\textsubscript{CT} (OR 0.93, 95% CI 0.80-1.07) and when comparing those with PPL\textsubscript{CT} in the highest quartile (OR 0.89, 95% CI 0.60-1.30) with those in the lowest quartile. The inverse association was stronger when the analyses were restricted to samples taken shortly before the event. Subjects with PPL\textsubscript{CT} > 95th percentile had substantially lowered OR for VTE (OR 0.35, 95% CI 0.13-0.81). The risk estimates by categories of plasma PPL\textsubscript{CT} were similar for deep vein thrombosis and pulmonary embolism.

In conclusion, our results indicate an inverse association between plasma PPL\textsubscript{CT} and the risk of future VTE. Our findings suggest that high plasma PPL\textsubscript{CT} is associated with reduced risk of VTE. The results were strongly influenced by regression dilution bias.
4.4 Paper IV: Rosuvastatin treatment decreases plasma procoagulant phospholipid activity after a VTE: A randomized controlled trial

VTE is a frequent cardiovascular disease with severe complications, including recurrence and death. There is a great need for preventive treatment options against recurrence as anticoagulation is accompanied by increased bleeding risk. Statins are reported to reduce the risk of incident and recurrent VTE, but the mechanisms are elusive. Procoagulant phospholipids, and phosphatidylserine in particular, are crucial for efficient coagulation activation, but no studies have investigated the effect of statin treatment on plasma PPL activity.

In this study we aimed to investigate the impact of rosuvastatin treatment on plasma PPL activity and levels of EVs in subjects with a history of VTE.

Participants of the STAtins Reduce Thrombophilia (START) trial (NCT01613794) were randomized to either 20 mg/day of rosuvastatin treatment or no treatment for 28-days. Plasma sample were collected at baseline and study end. The PPL activity was measured in samples from 245 participants using our FXa-dependent clotting assay (Paper II). The levels of total and platelet-derived EVs were measured in a selection of the participants using a sensitive flow cytometer.

Rosuvastatin treatment yielded an overall 22% (95% CI -38.2 to -5.8) reduction in PPL activity, and 37% (95% CI -62.9 to -11.2) reduction in PPL activity in participants with a history of pulmonary embolism. The effect of rosuvastatin on plasma PPL activity was not explained by changes in total cholesterol nor change in plasma levels of total- or platelet-derived EVs measured by flow cytometry.

In conclusion, rosuvastatin treatment caused a substantial decrease in plasma PPL activity, suggesting that a PPL-dependent attenuation of coagulation activation may contribute to a reduced risk of VTE recurrence following statin treatment.
5. General discussion
5.1 Methodological considerations

5.1.1 Study design

**Cohort studies** follow a defined population from inclusion (baseline) and until the outcome of interest or other censoring events occur (death, end of study). Participants are classified according to the status of exposure(s), allowing for differences in outcome to be investigated based on exposure status (non-exposed vs exposed) during follow-up. With a cohort design one can estimate both absolute risk and relative risk. Cohort studies are useful tools to investigate common diseases, while other study designs are more suitable for rare outcomes. Cohort studies are generally comprised of large participant pools and follow the participants for a long time. A major strength of the cohort study design is the clear temporal sequence between exposure and outcome, which is the most important prerequisite for determining causality. However, according to the Bradford Hill criteria for causation, additional criteria should also be considered including experimental evidence, the strength of the observed association, consistency across studies, biological gradient (dose-response) and biological plausibility. Challenges with this study design include selection bias, confounders and bias by differential loss to follow-up (missing data).

**Case-control studies** have a retrospective design. A group with the outcome of interest (case) is compared to a group without the outcome (control). The selected controls should be representative of the population from which the cases were drawn. The information on exposure status is registered for both groups, and odds ratios are estimated for the proportion of cases with an exposure compared to the proportion of controls with the same exposure. This determines the relative importance of the exposure with respect to the presence or absence of the outcome. Since the cases included in a study are chosen based on the fact that they have the outcome of interest, case-control studies are cost-effective and require a smaller sample size than for example cohort studies. The case-control study design allows for multiple exposures to be investigated, while the outcome of interest is limited. It can also be applied to investigate rare outcomes. Challenges with case-control studies include selection bias, recall bias and confounding. An additional potential challenge with case-control studies is reverse causality. Reverse causality is a temporal bias where the outcome of interest causes alterations in the exposure of interest. The observed association would then be the opposite of the hypothesized causal relationship.
Nested case-control studies encompass subjects sampled, or “nested”, from within a cohort study, hence the name. Random sampling of controls from the cohort strengthens the probability of the controls being representative for the general population. Nested case-control studies are both cost- and time-effective, as well as maintaining the temporal sequence of exposure-outcome. In addition, this study design allows for better control of confounding (age, sex) by the matching of cases and controls. A limitation with nested case-control studies is that we are unable to estimate absolute risk.

In paper III we used a nested case-control study derived from a prospective population-based cohort study. In the Tromsø study participants were followed until a VTE event, death, migration from the municipality, withdrawal from the study, or end of study. The exposure statuses were recorded and blood samples were drawn at inclusion in the study. For the nested case-control study, two age- and sex-matched controls, alive at the index date of the VTE event were sampled from the cohort for each case included.

Randomized controlled trials (RCTs) are considered the gold standard of study designs when it comes to determining causal relationships, as they provide experimental evidence, an important Bradford Hill criteria for causation. In a RCT the participants are randomly assigned to groups (control or intervention), followed for a set period of time and compared with each other according to the preset study outcomes. RCTs allow for the manipulation of exposures in a controlled environment. Proper participant randomization is crucial in order to ensure that all covariates, apart from the exposure, are randomly distributed among the study groups, thus minimize confounding. When controlling for confounding and bias, we allow for the estimation of true associations between the exposure and the outcome. RCTs have a high internal validity, but the strict inclusion criteria might reduce their external validity. Challenges with RCTs are that they are resource demanding, time consuming, and may not always be possible to conduct or ethically acceptable.

In paper IV we used a randomized controlled study design. The STAtins Reduce Thrombophilia (START) trial, is a multicenter, randomized, controlled, open label clinical trial where the impact of rosuvastatin treatment on the coagulation profile of individuals with
a previous history of VTE was investigated. Participants were randomly assigned to either 20 mg/day of rosvastatin treatment or no study treatment for the 28-day study period. Participants were randomized by using a random allocation sequence implemented by a central telephone. The sequence was concealed until interventions were assigned. Adherence to the study protocol was assessed in two ways. First, participants in the treatment group took the first tablet of rosvastatin in the presence of an investigator. Second, compliance to treatment was assessed by measurements of total cholesterol levels at baseline and at study end in all participants. The START trial was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands, and all study participants gave written informed consent prior to participation.

5.1.2 Bias
Bias is systematic error(s) in the design or execution of a study leading to incorrect estimates of a true association between exposure and outcome. Several systematic errors have been described, however most of the biases relate to the selection of participants (selection bias), measurements performed and the classification of exposure and outcome (information bias), or the presence of confounding factors.

Selection bias is a result of any errors in selecting the study participants and/or from factors affecting the study participation. Selection bias affects the relationship between the included participants and the non-participants or non-responders with regards to exposure and disease status. As we often do not know the exposure-disease status of the non-participants, selection bias can most often only be hypothesized. Selection bias can further be divided into non-response bias, incidence-prevalence bias, loss to follow-up bias, confounding by indication bias and volunteer bias. Cohort studies are less likely to be affected by selection bias as all of the participants are included in the study before the outcome of interest occur. Still, cohort studies are prone to non-response bias, implying that the participants of the study differ from the non-participants. Individuals who choose to participate in cohort studies are generally thought to be healthier than non-participants. This is because it is unlikely that individuals who are immobilized or institutionalized due to disease or illness of any kind would attend health surveys. Cohort studies are also prone to the loss to follow-up bias. If the losses during follow-up are random, it will not affect the estimated associations. However,
differential loss to follow-up, where the exposure affects the probability of completing the study, will result in study bias.227.

The 4th survey of the Tromsø study had a high attendance rate of 77% of the invited population (aged 25 years and older). Generally, the participation rates for epidemiological studies have declined during the last decades.228 This was similarly observed for the Tromsø study, with participation rates ranging from 81%-85% for the first three of the surveys (1974-1987) and declining to 65%-77% for the last four studies (1994-2016).194 We know that the non-participants of the Tromsø study tended to be male, unmarried, and younger than 35 years old or older than 80 years.194 The profile of the non-participants are in line with previous findings that those more likely to not participate in epidemiological studies were male, unmarried, at extremes of age (younger or older), have a lower level of education and be of lower socioeconomic status.228 As the incidence of VTE is low in the younger population, and the participation rate of Tromsø 4 is quite high, we presume that the selection bias as well as the non-responder bias would be low in our study.

In the START trial participants were randomly assigned to the treatment group or the control group. Generally, randomization is implemented to reduce the effect of bias in a study. However, in our study the distribution of age and sex were unevenly distributed among the study groups. The control group had more men (69.2% vs 53.6%) and the participants in this group were slightly older than the treatment group (59 years vs 57 years). However, adjusting for age and sex in statistical analysis did not alter the results, indicating that the impact of selection bias is low. In addition, participants were compared to themselves (baseline vs study-end samples) for analyses. As participants were recruited from an outpatient setting, it limits the risk of confounding diseases at randomization, limiting selection bias in our study. For each study group, a low number of participants were excluded after randomization, six in the treatment group and four in the control group. However, none of the participants were lost to follow-up. Two participants in the treatment group was excluded due to not starting the study drug.

**Information bias** occurs during data collection and is the result of the systematic tendency to erroneously place participants in different exposure and outcome categories. The accuracy of any exposure-outcome relationship depends on the performance of the diagnostic tests used.
A common source of misclassification in clinical practice is the inaccuracy of diagnostic tests. Misclassification bias is the most important type of information bias, and can be divided into differential or non-differential misclassification. Non-differential misclassification is when the probability of misclassifying the exposure is not related to the outcome (disease), or the other way around. The ability of the diagnostic test to determine exposure status is the same for cases and controls, and they are both equally likely to be misclassified according to disease status. In the case of differential misclassification, the performance of the diagnostic test for the exposure identification differs between cases and controls. Recall bias is an example of differential misclassification where the cases are more likely to remember relevant exposures compared to the controls.

In *paper III*, information on the exposure status was obtained from blood samples which were drawn at study inclusion in 1994-95 and stored at -80°C until analysis. Information on the participants from the Tromsø study were derived from self-administered questionnaires. As blood samples and the questionnaires (exposure information) were collected before the outcome (VTE event) occurred, potential misclassification in our study is likely to be unrelated to the outcome (non-differential). Non-differential misclassification tends to lead to an underestimation of an association. Potential sources of information bias include measurements obtained by the Tromsø study personnel, our laboratory measurements and instrument errors. The PPL assay is a sensitive method to determine PPL clotting time of a sample. The assay was found to display low intra- and inter-series coefficients of variations (CV) ranging from 2.8% to 4.1%. In addition, the technician carrying out the PPL assay measurements was blinded to the identity and case-control status of the samples in order to reduce the potential information bias. The outcome of our study, VTE events, are also prone to information bias. All events were identified by searching the hospital discharge diagnosis registry, autopsy registry and the radiology procedure registry at the University Hospital of North Norway (UNN). The events were thoroughly validated by multiple criteria such as presence of symptoms, objective confirmation by diagnostic procedures, and the initiation of treatment. The strict criteria implemented for an event to be found valid and included in the study will reduce the probability of false positive cases, and reduce study bias. Potential misclassification in our study would most likely be non-differential.
In paper IV, using the START trial, we measured the exposure (PPL activity) in plasma samples collected at baseline and study-end. As mentioned in the section above, the PPL assay is a sensitive method which displays low intra- and inter-day CVs. The technicians conducting the laboratory analyses were blinded to sample treatment. In the trial neither the patients nor the physicians were blinded to study treatment. However, it is unlikely that knowledge of the treatment would affect the laboratory outcomes (PPL clotting time). For the statistical analysis performed, the participants are compared to themselves (baseline vs study-end).

**Regression dilution bias** is a type of information bias affecting longitudinal studies assessing the association between the baseline measurements of a continuous modifiable variable and the risk of an outcome \(^{227}\). The exposure status of modifiable risk factors might change during follow-up. If not accounted for it may introduce regression dilution bias to a study resulting in an underestimation of the true association between the exposure and outcome \(^{229}\).

In paper III, the follow-up time in the source cohort was more than 12 years for several of the participants (from 1994 to September 1\(^{st}\) 2007). Because of the long follow-up time in our study, we chose to investigate the potential impact of regression dilution bias. Statistical analyses were performed where we restricted the maximum time from blood sampling in Tromsø 4 to the VTE events, still keeping all controls in the analyses. The logistic regression analyses on time restrictions were set to require at least 10 VTE events, and odds ratios were generated at every 0.1 year increase and plotted as a function of time from blood sampling to VTE. We do observe regression dilution bias in our study, and found that the inverse association between high plasma PPL\(_{CT}\) and VTE was stronger with shortened time between the blood sampling and the VTE events. Our exposure (PPL\(_{CT}\)) was subject to variation over the long follow-up time, making our results prone to underestimation of the true association with the outcome (VTE).
5.1.3 Validity

Validity can be divided into internal or external validity. The **internal validity** of a study is the extent to which the observed results in fact reflect the true association within the study population investigated. **External validity**, or generalizability of a study, describes to what extent the study findings can be applied to subjects beyond the investigated study population. External validity depends on internal validity, but internal validity does not necessarily result in external validity \(^ {226}\). Bias will reduce the validity of a study. Population-based cohort studies generally have high external validity, while RCTs have low external validity, but a high internal validity. For extrapolation of the findings from RCTs the inclusion criteria should be as general as the study allows, while at the same time maintaining scientific precision \(^ {225}\).

In paper III the mean age of the VTE cases was 68 years, and the proportion of men and women was approximately the same (47% male, 53% women). Both mean age at VTE and the sex distribution in our study were comparable to reports from a pooled Scandinavian cohort (Norway and Sweden)\(^ {230}\). Similarly, the proportions of DVT (58.8%) and PE (41.2%) in our study, have also been reported by others \(^ {230}\). The VTE cases in our study are seemingly representative of VTE cases in the general population, strengthening the external validity of our study. In our nested case-control study two controls, matched for age, sex and time, were chosen for each VTE case. The controls were sampled from the same parent cohort (Tromsø 4). However, with knowledge of that the non-participants of the Tromsø study tended to be male, unmarried, and younger than 35 years or older than 80 years, our study generalizability will be decreased for individuals belonging to these subgroups. In addition, the inhabitants of the municipality of Tromsø, hence the invited population, are predominantly Caucasian, with a Sami minority \(^ {194}\). As we know that the incidence of VTE differs with regard to ethnicity, the generalizability of our findings to other ethnicities is not known. High internal validity in our study is supported by the high participation rate (77%) in the cohort study, as well as the strict criteria for the validation of the included VTE events. In addition, the PPL assay is a sensitive method to determine PPL clotting time, and it displays low intra- and inter-series coefficients of variations (CV) ranging from 2.8% to 4.1% \(^ {195}\). The technician carrying out the PPL assay measurements was blinded to the case-control status of the samples, strengthening internal validity of our study.
In *paper IV*, our study design (RCT) suggests high internal validity, but often holds a low external validity. The participants in our study were recruited from three different anticoagulation clinics in the Netherlands (Leiden, Hoofddorp, and Rotterdam). Participants were included in the trial if they were 18 years or older, had an initial or recurrent confirmed symptomatic proximal DVT or PE, and were allowed to stop oral anticoagulant treatment by their treating physician. The exclusion criteria were: individuals already using statins or lipid lowering drugs or any contraindications for using 20 mg/day of rosuvastatin for the duration of the study.196. Strict inclusion criteria ensure that the study participants are as similar as possible before randomization to reduce study bias and increase internal validity. In addition, the study participants were compared to themselves for statistical analysis. However, the strict inclusion criteria affect the degree of generalizability of our study. The included participants from both study groups were slightly younger than the mean age of VTE found in studies derived from the general population (57 and 59 years versus 68 years). Of the 245 participants included in our study the majority were male (61%), and 75% of the participants had cardiovascular risk factors. The participants included were allowed to stop anticoagulant treatment, possibly distinguishing them from other VTE patients. Still, a wide age range (19 to 83 years of age) was observed in the participants.

5.1.4 Confounding
A confounder is a variable that is both associated with the exposure and the outcome, but is not an intermediate variable in the causal pathway between the exposure and outcome.231. If a confounder is unevenly distributed across the exposure status, the association will be distorted. The presence of confounding may strengthen, weaken or even reverse the studied association. The type of study design will impact the degree of potential confounding in a study. For RCTs the participants are randomly assigned to the study groups which reduces the risk of confounding by (ideally) causing an even distribution of confounders in the treatment and control group. However, associations found in RCTs may be confounded if exposures, by random, are unevenly distributed between the groups. In *paper IV*, we observed that the age and sex distribution were unevenly distributed among the study groups. We therefore adjusted the statistical analysis for age and sex, but the adjustments did not alter the observed effect. In addition, the participants of the trial were recruited from an outpatient setting, limiting the risk of confounding by other diseases. Furthermore, participants were compared with themselves for analysis, reducing the effect of potential confounding.
The presence of confounding is a concern for observational studies since the non-random allocation to a study group may result in an uneven distribution of confounding factors among the groups. Strategies to overcome confounding include multivariable regression analysis, matching and stratification. Pairwise matching is a way of controlling for confounding at an early stage of a study. In a case-control study participants can be matched according to status of the known confounder, for example smoking status. Stratification is a way of controlling for confounding after the study has been completed. The confounding variable can be divided into subgroups for analysis. However, stratification will reduce the statistical power.

Multivariable regression analysis is a way of estimating an association between exposure and outcome, while controlling for one or more confounding variables. In paper III, the cases and controls were matched on age and sex. For analysis, PPL activity was stratified into four groups (quartiles) of clotting time, and the logistic regression analysis was run as crude analysis as well as analysis adjusted for age, sex and BMI. It is important to acknowledge that despite applying various methods for dealing with confounding factors in our study, residual confounding may still affect our results. Residual confounding occurs when unrecognized (unknown or unmeasured) confounders exist, which in turn could potentially impact the observed associations. Residual confounding is recognized as a challenge in observational studies. The associations described in our study could potentially be affected by unrecognized confounding variables.
5.2 Discussion of the main results

5.2.1 The impact of preanalytical conditions on plasma concentration and size distribution of EVs

In paper I we assessed the impact of preanalytical conditions, such as centrifugation steps for plasma preparation and EV isolation, the choice of anticoagulant, and fasting status on plasma concentration and size distribution of EVs. As a proof of concept, we used TEM (negative staining and ultrathin sectioning) to confirm the presence of bilayer membrane vesicles (i.e. EVs) after isolation from plasma by ultracentrifugation. A bilayer membrane was demonstrated in the majority of vesicles from the isolated EV pellets, confirming the presence of EVs. As suggested in the statement paper from the International Society for Extracellular Vesicles (ISEV), we chose to apply several different methods, including NTA, SEM and TEM, for investigating the presence of EVs, their concentration and size. EV isolation by ultracentrifugation has been described to induce aggregation and alter the shape of the EVs. However, in our study we did not observe EV aggregation or great variation in the morphology of the EVs, assessed by TEM and SEM. The main EV population was spherical, and the EV population investigated by electron microscopy was relatively pure, given that the presence of monolayer vesicles (lipoproteins) was a rare finding. However, knowing that the EV sample preparation for TEM and SEM require chemical fixation and dehydration, the observed size and morphology should be interpreted with that in mind. We therefore applied a second method for size determination of the isolated EV population. Interestingly, we found similar size distributions of plasma EVs measured by NTA and SEM. Both methods found the majority of EVs to be below 200 nm in diameter, and the presence of larger vesicles (>300 nm) were rarely observed. The mean size of the EVs were slightly smaller using SEM compared to NTA, and the size distribution measured by SEM was shifted towards smaller EVs (<199 nm). NTA and electron microscopy have previously been found to provide similar size measurements, where the differences observed could be explained by differences in the lower detection threshold (minimum detectable size).

In our study we observed that the mean diameter of EVs was between 80-90 nm, which was similar to findings in other studies. In our study, the majority of the EVs isolated by ultracentrifugation were in the smaller size categories (<100 nm: 76.4% to 78.2%). Only a small fraction of EVs were larger than 300 nm (300–1000nm: 0.7% to 1.3%). The majority of
the observed EVs were below 300 nm. The discrepancies observed for EV concentrations measured by flow cytometry and NTA may partly be explained by the fact that the lower detection limit of conventional flow cytometers is approximately 300-500 nm.

We observed that the plasma concentration and size distribution of EVs were similar in plasma isolated from blood with various anticoagulants (citrate, EDTA, heparin and CTAD). Neither the total plasma concentration, nor the concentrations of EV subgroups defined by size (<100 nm, 100–199 nm, 200–299 nm and 300–1000 nm) differed significantly between the anticoagulants. However, although not statistically significant, blood anticoagulated with sodium citrate showed the lowest plasma concentration of EVs across all size categories, whereas heparinized plasma yielded an almost 2-fold higher concentration of large EVs (300–1000 nm) compared to the other anticoagulants. The impact of different anticoagulants on plasma concentration of EVs has previously been investigated by several studies. It was similarly found that heparin gave a higher concentration of EVs compared to citrate or CTAD. We observed that EDTA displayed the highest plasma concentrations of EVs, supporting the notion that EDTA promotes the formation of ex vivo microvesicles possibly due to platelet activation. Citrate, a weaker calcium chelator than EDTA, displayed the lowest median concentrations of EVs across all size categories. Calcium chelators, such as citrate and EDTA, make the ex vivo calcium unavailable to the coagulation system and prevents leukocyte and platelet degranulation. Calcium plays an important role in the phospholipid re-modelling of the membrane during EV formation. Therefore, calcium chelating anticoagulants are believed to prevent vesiculation, to a certain extent. However, differences in EV concentration are observed with regard to the type of chelator used, suggesting that other mechanisms are involved. EDTA is known to additionally affect platelets by inducing a P-selectin dependent platelet activation process, dissociate the platelet integrin αIIbβ3 complex, and result in pseudo-thrombocytopenia and platelet aggregates on blood smears. The use of heparin for EV research was reported as not recommended due to its impact on platelet activation and aggregation. As heparin does not chelate free calcium it will not prevent EV generation by platelets after blood collection. Our findings of a lower EV concentration in samples with citrate may indicate that in vitro vesiculation potentially occurs to a larger extent with the other anticoagulants, and supports the recommended use of citrate as anticoagulant for studies on EVs.
The impact of freezing PPP compared to PFP on concentration and size distribution of plasma-derived EVs was also evaluated in our study. Lacroix and colleagues showed that the choice of centrifugation protocol for the preparation of plasma impacted the levels of EVs, measured by flow cytometry. The impact of residual platelets and platelet debris poses the risk of generating artifactual platelet-derived EVs. It has been shown that if platelets are insufficiently depleted before freezing, the EV count is affected after thawing. Double centrifugation prior to freezing has been shown to decrease the number of annexin V positive EVs, as well as platelet-derived EVs when measured by flow cytometry. In addition, freeze-thaw cycles are known to alter the number of platelet derived EVs. However, in our study, no statistical differences in total concentration and size distribution of EVs between plasmas prepared as PPP and PFP before freezing were observed for any of the anticoagulants used (citrate, EDTA, CTAD and heparin), measured by NTA.

NTA as a method does not allow for the distinction between EVs and other particles within the same size range in plasma. Triglyceride-rich lipoproteins (chylomicrons, chylomicron remnants and very-low-density lipoproteins) are in molar excess, but of similar size to cell-derived EVs, and therefore detectable by light scattering. Previous studies showed that the plasma concentration of EVs measured by NTA was strongly correlated to the plasma concentration of triglycerides, and that the concentration of EVs declined by more than 98% when only vesicles labelled with a cell tracker dye were counted. It is important to avoid the interference of triglyceride-rich lipoproteins on NTA measurements of EVs. As previously described in the methods section, ultracentrifugation as a method cannot be used to absolutely discriminate between EV sizes, since sedimentation also will depend on the density and cargo of a vesicle, as well as the distance it needs to travel to be pelleted. It has therefore been recommended to dilute biological fluids with PBS in order to alter the viscosity of the sample fluid and thereby enhance the sedimentation of EVs. In our study we diluted PFP in Dulbecco’s phosphate buffered saline without CaCl₂ and MgCl₂ when we isolated EVs by ultracentrifugation. This was done in order to facilitate a better separation of the plasma constituents. In our study, the median concentrations of EVs isolated from plasma varied between 1.6–2.0×10¹⁰/mL with an interquartile range from 1.3 to 2.7×10¹⁰/mL, which is in line with the results based on specific labelling of EVs by a cell tracker dye.
We observed that the ingestion of a standardized high-fat meal was accompanied by a significant increase in serum triglycerides which peaked at 4 hours and returned to baseline levels within 8 hours after the meal. However, the postprandial lipidemia was not accompanied by significant changes in the concentration of EVs in plasma determined by NTA. Similarly, the plasma concentration of VLDL particles did not change from the fasting to the postprandial state, but the median diameter of VLDL particles increased from 42 ± 6 nm in fasting plasma to 55 ± 9 nm in postprandial plasma samples. Serum triglycerides and the concentration of VLDL particles in plasma collected 4 hours after ingestion of the meal showed a strong correlation with the plasma concentration of EVs and explained 59–66% of the variation in plasma EVs. These findings suggest that the particle count, measured by NTA, was influenced by VLDL particles under postprandial conditions. Still, our findings provide several lines of evidence for at least partial separation of EVs from triglyceride-rich lipoproteins by high-speed centrifugation of plasma. First, the concentration of triglycerides in the plasma supernatant remained unchanged after high-speed centrifugation, suggesting that VLDL-particles in fasting blood samples were not pelleted and mainly remained floating. Second, even though serum triglycerides and the concentration of VLDL particles in plasma from fasting individuals displayed a moderate correlation with the plasma concentration of EVs, it only explained 13–19% of the variation in plasma EVs, suggesting that the particle count, measured by NTA, was not dominated by VLDL particles. Third, we developed a novel procedure to section the EVs, and could thereby clearly visualize the bilayer phospholipid membrane which distinguishes EVs from lipoproteins. Fourth, the results of our TEM analysis confirmed that the majority of isolated vesicles in our samples were in fact EVs (characterized by the bilayer phospholipid membrane) and not lipoproteins or protein complexes.
5.2.2. A modified clot-based assay to measure negatively charged procoagulant phospholipids

In paper II, we thoroughly validated a modified and easy to use PPL assay for the measurement of procoagulant phospholipids in test specimens (plasma samples or isolated EVs) and compared its performance with the commercial STA-Procoag-PPL assay from Stago Diagnostica. Our primary modification included preparation of PPL-depleted plasma by ultracentrifugation to remove EVs, which are assumed to be the main source of negatively charged phospholipids in plasma. This modification allowed for the establishment of an accessible in-house assay with comparable performance to the commercial assay. The addition of a standardized phospholipid reagent (UPTT) allows for clotting times to be converted into a standardized unit of phospholipids.

There are currently two main clotting assays commercially available, the STA-Procoag-PPL assay from Diagnostica Stago and the XACT assay from Haematex, as previously mentioned in the methods section. Both assays are well established in research as reliable tools for assessing PPL activity in human plasma, and they have been used as tools for measuring the PPL activity in disease states\(^{191,244-246}\), for the determination of pre-analytical parameters affecting EVs\(^{234}\), and quality control of cell storage\(^{247}\). The clotting assay, as a method, is favorable to use since it offers the measurement of PPL activity directly in plasma, compared to the antibody mediated capture based chromogenic assays. Clotting assays offer the measurement of a complex reaction with a physiological end-point, and can be performed on a variety of routine coagulation analyzers. However, for large scale application of clotting assay, the commercial options become quite costly.

The commercial assays are based on the ability of procoagulant phospholipids to accelerate the conversion of prothrombin to thrombin with subsequent fibrin formation. Experimentally, equal volumes of sample plasma are mixed with PPL depleted plasma, provided as a reagent by the assay. The reaction is triggered by the addition of FXa in excess, together with calcium, and clotting time is measured. In both of the commercial assays, the phospholipid depleted plasma provided as a reagent is chemically treated with phospholipase in order to remove PPL. However, assuming that the vast majority of procoagulant phospholipids in plasma are EV-bound, we examined whether ultracentrifugation of plasma might substitute phospholipase treatment for the production of the PPL depleted assay plasma. As a proof of
concept, we subjected plasma to sequential centrifugation steps, with increasing g power and time, which resulted in prolongation of the PPL_C. The notion of a procoagulant property of plasma, removable by centrifugation is in line with the observations of “thromboplastic substance” by Chargaff and West in 1946162, and “platelet dust” by Wolf in 1967163. The removal of EVs by ultracentrifugation, hence the reduced PPL activity, did not affect the ability of the depleted plasma to clot when subjected to standard coagulation test (activated partial thromboplastin time, aPTT and prothrombin time, PT). Moreover, the clotting time of the PPL-depleted plasma we generated by ultracentrifugation was comparable to the depleted plasma provided by the commercial assay, supporting its use in the modified assay. As plasma depleted of PPL is an essential reagent in the modified assay, the importance of not introducing assay variations is critical. We demonstrated that three independent batches of PPL-depleted plasma prepared from the same donors performed similarly, supporting the use of ultracentrifugation as a method for PPL depletion.

The performance of our modified assay was compared to the Stago assay using both dilutions of a standardized phospholipid reagent (UPTT) and samples from ten individuals. Our study provides evidence for comparable performance of the modified assay with regard to sensitivity and measurement of levels of PPL in plasma samples. Our modified PPL assay displayed minor variation in the assay performance. CVs obtained using the standards from the Stago STA-Procoag-PPL or in-house pooled PFP range from 2.8 to 4.1%, well within recommended acceptable limits for within-day and between-day variability. Similar results were shown by van Dreden and colleagues in the XACT assay, with intra-assay CVs of 3.3% and 3.1% for normal pooled plasma and patient plasma, respectively, and inter-assay CVs of 3.9% and 4.2%. We showed that the PPL activity measured in the modified assay was largely dependent on PS in the test sample by blocking with lactadherin. The abundance of PS on the EV surface is often used to characterize EVs by exploiting the ability of annexin A5 or lactadherin to bind PS on the outer leaflet of the membrane. Lactadherin has been demonstrated to be an effective anticoagulant blocking the activity of PS and inhibiting the procoagulant activity of blood cells, endothelial cells and EVs by 80%248. In our experiment, PPL_C was prolonged with increasing concentrations of lactadherin to the extent that it was no longer measurable in the assay. Aung et al. similarly showed that pre-treatment of packed red blood cell supernatants with lactadherin prolonged clotting times, using the XACT assay247.
The effect of pre-analytical parameters such as the plasma centrifugation protocol and the impact of postprandial lipemia on PPL activity was investigated in this study. It was previously reported by Lacroix and colleagues that the choice of centrifugation protocol for the preparation of PFP influenced the PPL$_{CT}$ after a freeze-thaw cycle. Similarly, we observed that a common protocol for PFP preparation ($3000 \times g$ for 10 minutes followed by $13,500 \times g$ for 2 minutes) yielded a 24% shorter PPL$_{CT}$ than PFP prepared as recommended by ISTH ($2,500 \times g$ for 15 minutes twice). Hence, the plasma centrifugation protocol should be taken into consideration when comparing plasma PPL$_{CT}$ between studies. An additional pre-analytical challenge for population-based studies is the availability of fasting blood samples as the PPL activity in plasma may be affected by plasma levels of triglyceride-rich lipoproteins. We therefore tested the PPL activity in plasma isolated before and four hours after a high fat meal on the modified PPL assay. We observed that the PPL$_{CT}$ was unchanged in fasting and postprandial plasma, while an increase in triglycerides was observed. This suggests that PPL$_{CT}$ is independent of postprandial lipemia and that there is no need to use fasting blood samples to obtain reliable PPL activity in plasma samples. Our findings are supported by Silveira et al. who reported no effect of postprandial lipemia on the overall PPL measures, using the STA-Procoag PPL assay. It was also shown by Mørk and colleagues that even a non-standardized meal and a shorter time interval between the ingestion of the meal and blood draw (75 min) resulted in no change in PPL between fasting and postprandial samples measured by the Stago assay.

The inter-individual variability of coagulation factors in the test samples may affect the degree of activation measured by the modified assay. We know that procoagulant phospholipids, with PS in particular, affect the activity of both the intrinsic and extrinsic tenase and prothrombinase complexes, as well as the activation of FXI by thrombin. We therefore tested a wide range of FVIIa and FVa concentrations, where high concentrations of coagulation factors proved to shorten the clotting time in the modified PPL assay. However, these effects occurred only at supra-physiological concentrations significantly higher than those observed in vivo. While TF is thought to be a major procoagulant factor found in EVs, only minute quantities of TF are normally present in human plasma. Therefore, we also investigated the effect of TF on the modified PPL assay. It was previously shown by Connor and coworkers that the XACT assay was insensitive to increasing concentrations (0–0.1%) of TF added to whole blood. Accordingly, we found that monocyte-derived EVs expressing
TF after LPS stimulation (pathophysiological conditions) did not affect the clotting time in our assay. However, we observed a dose-dependent decrease in the clotting times with increasing supra-physiological concentrations of relipidated TF. Taken together, our findings suggest that the modified PPL assay is not influenced by physiological concentrations of the clotting factors or TF+EVs in the test samples.

An assay designed for large-scale applications should be reproducible over time, and it should be possible to compare the results between different laboratories. To solve the latter challenge we proposed the introduction of the UPTT reagent, which is an inexpensive standardized preparation of rabbit brain cephalin, allowing for clotting times to be converted into a standardized unit of phospholipids. The XACT assay solves this problem by the inclusion of a synthetic PPL calibrator, while the Stago assay leaves it up to the users to create a reference range and standards for the clotting time.

There are two main considerations with the modified PPL assay. First, the results will be influenced by the presence of lupus anticoagulants as well as high concentrations of coagulation factors which may lead to falsely prolonged or shortened clotting times. This is common for all plasma-based assays, and should be accounted for when interpreting the results. Second, pre-analytical conditions and inter-individual variations might influence plasma concentrations of coagulation factors in PPL depleted plasma. However, our modified assay seems to be unaffected by variations within the pathophysiological range.
5.2.3 PPL clotting time and the risk of future incident VTE

In paper III, we investigated the association between plasma PPL\textsubscript{CT} and the risk of future VTE in a nested case-control study derived from a population-based cohort study. We found that prolonged PPL\textsubscript{CT} displayed a modest protective effect on VTE risk both when PPL\textsubscript{CT} was used as a continuous and as a categorized variable in the logistic regression models. In addition, we show that subjects with extremely prolonged PPL\textsubscript{CT} (above the 95\textsuperscript{th} percentile) had lowered risk of VTE (OR 0.35, CI 95\% 0.13-0.81) compared to those with PPL\textsubscript{CT} in the lowest quartile. We observed similar results in subgroup analysis for PE and DVT. However, our results appeared to be influenced by regression dilution bias, as the ORs for VTE by plasma PPL\textsubscript{CT} decreased substantially with shortened time between blood collection and the VTE events.

Our study is, to the best of our knowledge, the first to investigate the association between plasma PPL\textsubscript{CT} and the risk of future VTE in the general population. In a paper by Riva and coworkers, they report their findings from a hospital based cross-sectional study exploring PPL\textsubscript{CT} in relation to VTE. They measured the PPL\textsubscript{CT} of 100 patients referred to the Emergency Department under suspicion of VTE using the commercial STA Procoag PPL assay from Diagnostica Stago. They reported that PPL\textsubscript{CT} did not discriminate between patients with (n=31) and without VTE in their study\textsuperscript{183}. However, the lack of discriminatory diagnostic power by the PPL assay may have been diluted by other conditions associated with shortened PPL\textsubscript{CT} among the acute medical patients without VTE admitted to the hospital. Since our study population as well as study design differ, we believe that their findings does not exclude the potential association between plasma PPL\textsubscript{CT} and the risk of future VTE.

Ayers and colleagues published a cross-sectional study investigating if EV count, assessed by flow cytometry, correlated with their functional capacity\textsuperscript{185}. They measured the plasma PPL\textsubscript{CT} in 53 healthy individuals and 47 patients with obstructive sleep apnea, using the STA Procoag PPL assay, as well as thrombin generation by the calibrated automated thrombogram (CAT). They found that plasma PPL\textsubscript{CT} showed strong inverse correlations to parameters of thrombin generation, such as ETP (Spearman $r$=-0.77) and peak thrombin concentration (Spearman $r$=-0.72), using the addition of minimal amounts of phospholipids and TF (1 pM) to trigger thrombin generation\textsuperscript{185}. Accordingly, we demonstrated a clear dose-response
relationship between plasma PPL<sub>CT</sub> and parameters of the CAT assay. The PPL<sub>CT</sub> correlated strongly with both lag time (r= 0.99, p= <0.0001) and ETP (r= -0.98, p= <0.0001).

An association between plasma PPL<sub>CT</sub> and the risk of future VTE is supported by circumstantial evidence. First, the PPL<sub>CT</sub> is inversely associated with annexin V-positive EVs and high plasma levels of EVs are associated with VTE risk in most but not all studies. Second, in a cross-sectional study including plasma samples from 100 healthy individuals and patients with obstructive sleep apnea, plasma PPL<sub>CT</sub> showed strong and inverse correlations to parameters of thrombin generation, such as ETP and peak thrombin concentration, using the CAT assay. Accordingly, we demonstrated a clear dose-response relationship between plasma PPL<sub>CT</sub> and parameters of the CAT assay. In addition, several studies have shown that parameters of the CAT assay, particularly lag-time and ETP, are associated with incident and recurrent VTE. Third, carriers of rare (e.g. deficiencies of antithrombin, protein C and S) and common (e.g. factor V Leiden and the prothrombin mutation G20210A) prothrombotic genotypes had significantly shorter plasma PPL<sub>CT</sub> than non-carriers, providing indirect evidence for lower risk of VTE with prolonged plasma PPL<sub>CT</sub>.

The plasma levels of modifiable biomarkers such as PPL<sub>CT</sub>, are expected to change over time. Fluctuations in the exposure variable during follow-up will lead to the phenomenon called regression dilution bias, which usually results in an underestimation of the true association between exposure and outcome. We therefore chose to estimate the ORs for VTE among subjects with the highest (highest quartile) versus lowest (lowest quartile) plasma PPL<sub>CT</sub> as a function of time between blood sampling and the VTE events. We observed that the inverse association between high plasma PPL<sub>CT</sub> and VTE was stronger with shortened time between the blood sampling and the VTE events. In subgroup analysis the ORs for DVT and PE as a function of time between blood sampling and events showed similar patterns as the ORs for overall VTE. The explicit impact of regression dilution in our study may indicate that we report an underestimation of the true association between PPL<sub>CT</sub> and the risk of future VTE. Regression dilution has in a similar manner previously been reported to impact other modifiable biomarkers such as the terminal complement complex (TCC) and mannose-binding lectin, both markers of complement activation.
Strengths of our study include recruitment of VTE patients from a population-based cohort with age- and sex-matched controls from the same source population where blood samples were collected prior to the VTE event. This allows assumptions on the direction of the observed association between plasma PPL_{CT} and VTE. Additionally, the modified FXa-dependent PPL clotting assay is highly sensitive and displayed a low CV of ≤ 4%. A limitation of our study is that plasma samples used were collected in 1994/95 and stored at -80°C until analysis more than 20 years later. The long storage time, as well as freezing and thawing, might possibly affect the plasma PPL_{CT}. However, it is unlikely that it would impact our end results, as the potential effects would be similar for both cases and controls. Moreover, the PPL levels were only measured in baseline samples, while potential changes during follow-up were not accounted for. This might lead to an underestimation of the true association between plasma levels of PPL_{CT} and VTE risk due to regression dilution bias. In our study, some plasma samples were excluded due to either missing samples or poor plasma quality.
5.2.4 PPL activity and rosuvastatin treatment after a VTE event

In paper IV we investigated the effect of rosuvastatin treatment on plasma PPL activity, measured by a FXa-dependent PPL clotting assay in patients with a previous history of VTE. We observed that statin treatment caused a 22% reduction in PPL activity for all VTE patients and 37% reduction in PPL activity for PE patients compared to no treatment. The observed effect of rosuvastatin on PPL activity was not explained by changes in serum levels of total cholesterol or a parallel change in plasma levels of total- and platelet-derived microvesicles by statin treatment, measured by flow cytometry. The results from our study support the beneficial effect of statin treatment on coagulation factors and thrombin generation potential in plasma. As the presence of negatively charged phospholipids augment the activity of the extrinsic tenase complex TF-FVIIa by several orders of magnitude, the combined effect of reduced PPL activity and modest decline in several coagulation factors may reduce coagulation activation and contribute to the explanation of why rosuvastatin treatment lower the risk of VTE.

Clinical studies have previously shown that statin treatment, with simvastatin, atorvastatin, or cerivastatin caused a beneficial effect on the coagulation system by a moderate lowering of specific coagulation factors and thrombin generation. In previous publications from the START trial, rosuvastatin treatment showed favorable effects on the hemostatic system by reducing plasma levels of coagulation factors FVII, FVIII, and FXI by 4-6%, D-dimer by 3% and lowering the ex vivo thrombin generation potential by 10%. Additionally, rosuvastatin treatment was found to increase the fibrinolytic potential assessed by shortening of the mean plasma clot lysis time and a decrease in both plasmin inhibitor levels and thrombin-activatable fibrinolysis inhibitor (TAFI) activity. The treatment effects of rosuvastatin on thrombin generation and plasma D-dimer levels were mainly driven by an increase among non-statin users. In contrast, we found a more profound beneficial effect of rosuvastatin treatment that was mainly driven by a significant decline in the PPL activity among rosuvastatin users accompanied by a minor increase in the PPL activity among the non-users. The increase in hemostatic factors among non-statin users in our and previous studies from the START trial may be interpreted as a consequence of the rebound hypercoagulability often seen after discontinuation of anticoagulant treatment.
Previous studies have demonstrated that plasma PPL activity is mainly due to the presence of EVs \(^{184,185}\), and most \(^{186,191-193,267}\) but not all \(^{268}\) case-control studies have reported increased EV-related plasma PPL activity in VTE patients compared to controls. Therefore, our findings of a profound decrease in PPL activity by statin treatment may contribute to the explanation behind the reduction of incident and recurrent VTE by statin treatment \(^{260,269-276}\).

Microvesicles (MVs) are larger EVs (100-1000 nm in diameter), which bud directly from the plasma membrane of activated cells, and express surface markers of their cell of origin \(^{179,277}\). The largest proportion of MVs in circulating blood is derived from platelets \(^{278,279}\) and the subsequent procoagulant activity in plasma is mediated by platelet-derived MVs (PDMVs) \(^{267,278}\). A strong inverse correlation has also been reported between PPL clotting time and lactadherin positive EVs measured in PPP from healthy control subjects and patients with obstructive sleep apnoea (OSA), though the strength of the correlations was mainly driven by the OSA patients \(^{185}\). We therefore hypothesized that the reduction we observed in plasma PPL activity following rosuvastatin treatment was caused by a parallel decline in plasma MV levels, and particularly platelet-derived MVs. In order to test our hypothesis, we isolated EVs from platelet free plasma and measured the total count (lactadherin-positive) and platelet-derived MVs (lactadherin- and CD41-positive) by flow cytometry. Although we found that statin treatment lowered the PPL activity in the treatment group, we did not observe a reduction in total EV count, or platelet-derived EVs for comparisons between – or within study groups.

In our study, rosuvastatin treatment did not affect plasma MV levels in patients with a previous history of VTE. Contradicting our findings, previous observational studies have shown that patients with arterial cardiovascular diseases or risk factors (hyperlipidemia in particular) had higher plasma MV levels than control individuals, and that statin treatment lowered plasma MV levels in most, but not all studies \(^{280-285}\). Several factors may contribute to explain our findings. First, the effect of statin treatment on plasma MV levels may be limited to individuals with arterial cardiovascular diseases and risk factors, and not transferrable to VTE patients. Second, one might speculate that statin treatment could differentially influence EV formation from various intravascular cells and the subsequent process of externalization of PS to the outer leaflet of the cell membrane during EV formation \(^{286}\). Accordingly, in a placebo-controlled randomized double-blinded crossover study, the treatment of 19 patients with peripheral arterial occlusive disease for 8 weeks with 80 mg atorvastatin daily showed a
reduction in plasma MV levels expressing CD62P- and CD61-positive MVs without affecting plasma levels of lactadherin-positive EVs \(^{283}\). Third, a well-recognized limitation of flow cytometry as a method is the detection limit of the instrument. Even a sensitive flow cytometer will still only detect vesicles above approximately 200 nm in diameter, and thereby exclude the majority of EVs. Vesicles larger than 200 nm in diameter have been reported to only account for a minority of the EV population (< 5%) \(^{287}\). This may imply that a possible decrease in plasma EVs after statin treatment could have been masked by the unchanged level of the EVs >200 nm in diameter.

Some aspects of our randomized controlled trial need special considerations. Neither the patients nor the physicians were blinded to treatment. However, it is unlikely that knowledge of the treatment would affect the laboratory outcomes. Furthermore, the technicians conducting the laboratory analyses were blinded to sample treatment. In addition, despite randomization, the distribution of age and sex was uneven between the study arms. We decided \textit{a priori} to adjust analysis for age and sex as potential confounders, and adjustments did not influence the observed treatment effect. Even though results from subgroup analysis revealed the most pronounced decrease in plasma PPL activity in individuals with a history of PE, they should be interpreted with caution as the study was not originally powered to analyze differences in subgroups \(^{196}\). Lastly, as participants were recruited from an outpatient setting, it limits the risk of confounding diseases at randomization, and for analyses, participants were compared with themselves.
6. Conclusions

I. Isolation of EVs from plasma by high-speed centrifugation yielded similar concentrations and size distributions of EVs for the four anticoagulants tested (citrate, EDTA, CTAD and heparin). We found no statistical difference in concentration or size of EVs (measured by NTA) when plasma was prepared as PPP or PFP before freezing. Plasma VLDL particles interfered with EV measurements assessed by NTA, particularly under postprandial conditions due to an increase in the median particle diameter of VLDLs exceeding the lower detection limit of NTA.

II. The use of sequential centrifugation, including final ultracentrifugation, to deplete plasma of procoagulant phospholipids performed equal to enzymatic depletion of phospholipids from plasma in a FXa-based clotting assay to determine PPL clotting times. In addition, we introduced a standardized PPL reagent (UPTT) which allows for clotting times to be converted into a standardized unit of phospholipids. These modifications allowed us to establish a sensitive and reproducible in-house assay.

III. Results from our nested case-control study indicate an inverse association between plasma PPL\textsubscript{CT} (measured by a modified FXa-dependent PPL clotting assay) and the risk of future VTE. Subjects with PPL\textsubscript{CT} above the 95th percentile had particularly low risk of future VTE and the results were strongly influenced by regression dilution bias.

IV. Rosuvastatin treatment caused a substantial decrease in plasma PPL activity, suggesting that PPL-dependent attenuation of coagulation activation may contribute to a reduced risk of VTE recurrence by statin treatment.
7. References


Paper I
Impact of preanalytical conditions on plasma concentration and size distribution of extracellular vesicles using Nanoparticle Tracking Analysis

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Optimal pre-analytical handling is essential for valid measurements of plasma concentration and size distribution of extracellular vesicles (EVs). We investigated the impact of plasma preparation, various anticoagulants (Citrate, EDTA, CTAD, Heparin), and fasting status on concentration and size distribution of EVs measured by Nanoparticle Tracking Analysis (NTA). Blood was drawn from 10 healthy volunteers to investigate the impact of plasma preparation and anticoagulants, and from 40 individuals from a population-based study to investigate the impact of postprandial lipidemia. Plasma concentration of EVs was measured by NTA after isolation by high-speed centrifugation, and size distribution of EVs was determined using NTA and scanning electron microscopy (SEM). Plasma concentrations and size distributions of EVs were essentially similar for the various anticoagulants. Transmission electron microscopy (TEM) confirmed the presence of EVs. TEM and SEM-analyses showed that the EVs retained spherical morphology after high-speed centrifugation. Plasma EVs were not changed in postprandial lipidemia, but the mean sizes of VLDL particles were increased and interfered with EV measurements (explained 66% of the variation in EVs-concentration in the postprandial phase). Optimization of procedures for separating VLDL particles and EVs is therefore needed before NTA-assessment of EVs can be used as biomarkers of disease.

Extracellular vesicles (EVs), including exosomes (30–100 nm in diameter) and microvesicles (100–1000 nm in diameter), are bilayer membrane vesicles released from various cells into their surroundings1. The EVs are characterized by their size, mechanism of cellular release, and their content of proteins, RNA, and DNA molecules derived from the parental cell2. The functional properties of EVs are dependent on the cellular origin and pathological conditions3,4. Elevated plasma levels of EVs, and microvesicles in particular, have been associated with several disease states such as atherosclerosis5,6, diabetes7, cancer8,9, arterial cardiovascular diseases10–12 and venous thromboembolism13,14. Although the majority of EVs in plasma have diameters below 200 nm15,16, most studies relating plasma levels of EVs with diseases have been carried out using flow cytometry for EV measurements with lower detection limits above 200 nm. Therefore, it would be attractive to use Nanoparticle Tracking Analysis (NTA) to determine plasma concentration of EVs in the size range of 50–1000 nm15,17 in order to assess associations between the predominantly smaller EVs and disease states.

Measurement of plasma EVs by NTA could possibly provide novel information on the role of EVs as potential biomarkers of risk, diagnosis, and prognosis of various diseases. However, the clinical application is currently hampered by methodological concerns related to NTA assessment of EVs. In human plasma, more than...
98% of the particles detected by NTA are very low density lipoprotein (VLDL) particles\textsuperscript{15,18}, and the measured particle concentrations are associated with the triglyceride concentration both under fasting and postprandial conditions\textsuperscript{19}. Isolation of EVs from plasma is therefore an essential pre-analytical step before NTA measurement. However, isolation of EVs by differential centrifugation may affect EV morphology, promote aggregation of EVs, and to some extent pellet lipoproteins\textsuperscript{20–22}. In clinical and epidemiological studies, plasma is most often prepared by single centrifugation at 1500–3000 × g for 10–20 min, yielding platelet poor plasma (PPP), before storage in biobanks. High-speed centrifugation to achieve platelet free plasma (PFP) from PPP prior to freezing has been shown to lower the concentration of platelet-derived EVs compared to plasma subjected only to lower speed centrifugation (PPP) before freezing\textsuperscript{23}. However, it is not known whether high-speed centrifugation after thawing of PPP would affect concentration and size distribution of plasma derived EVs compared to PFP prepared by double centrifugation (including one low and one high-speed centrifugation step) before freezing. Furthermore, previous studies comparing the impact of various anticoagulants on plasma concentrations of EVs, assessed by flow cytometry, have shown higher levels of EVs in heparin than in sodium citrate, acid-citrate dextrose (ACD) or sodium citrate theophylline adenosine dipyridamole (CTAD)\textsuperscript{24,25}. Pre-analytical conditions such as centrifugation steps, choice of anticoagulant, and fasting status may impact the plasma concentration and size distribution of EVs determined by NTA. We therefore aimed to investigate the impact of plasma preparation, assessed by freezing plasma before (PPP) or after (PFP) a second high-speed centrifugation, various anticoagulants in commercial blood collection tubes (Citrated, EDTA, CTAD, and Heparin), and fasting status on plasma concentration and size distribution of EVs using NTA and SEM.

**Material and Methods**

**Study participants.** Ten healthy volunteers (5 men and 5 women, aged 28–55 years) were recruited from the research staff, and they donated blood used to investigate the method of blood collection, centrifugation steps for plasma preparation and EV isolation, and choice of anticoagulant on the concentration and size distribution of plasma EVs. The study was approved by the regional ethical committee (REK Nord), and was conducted in accordance with relevant guidelines and regulations. Informed written consent was obtained from all participants.

Forty healthy subjects, 20 to 80 years of age, were recruited from a general population-based study (the Tromso study) in order to investigate whether fasting and postprandial lipoproteins would affect the concentration and size distribution of plasma EVs. They underwent a screening visit including a complete medical history, physical examination, a self-administered questionnaire which also included dietary habits, physical exercise, and alcohol consumption, and blood samples were taken with special emphasis on exclusion criteria. Exclusion criteria were any of the following conditions: regular use of lipid-lowering drugs (statins, resins or nicotinic acid derivates), estrogen supplementation or oral anticoagulants, cancer or other serious life-threatening medical conditions, present or previous cardiovascular diseases, recurrent venous thrombosis, diabetes mellitus, hypothyroidism, renal, hepatic, or psychiatric disease, and current abuse of alcohol or drugs. Informed written consent was obtained from all participants, and the Regional Committee for Research Ethics approved the study. The study was performed at the Clinical Research Unit at the University Hospital of North-Norway.

**Blood collection.** Blood was drawn from an antecubital vein using a 21 Gauge needle in the morning (08:30 am). Tourniquet was only used to find a vein and was opened after needle insertion. Blood was drawn into regular commercially available blood collection tubes (BD Vacutainers) (BD Bioscience, New Jersey, US) with the following anticoagulants: Sodium citrate (2.7 ml, REF363048), Sodium heparin (6.0 ml, REF367876), Ethylenediaminetetraacetic acid (K2E-EDTA) (6.0 ml, REF367864) and buffered Sodium Citrate Theophylline Adenosine Dipyridamole (CTAD) (4.5 ml, REF367599). The first few millilitres of blood were drawn into a dummy tube that was discarded afterwards. The blood collection tubes were gently inverted several times in order to mix anticoagulants with blood. The blood collection tubes were not transported, as blood collection and plasma preparation was performed in the same laboratory. The blood collection tubes were held in upright racks until centrifuged at room temperature. Blood cell counts were performed at baseline using Micros60 (ABX Diagnostics, Montpellier, France).

**Plasma and EV Preparation.** Platelet poor plasma (PPP) was prepared from anticoagulated whole blood by centrifugation at 3000 × g for 10 minutes at room temperature within 30 minutes after blood collection. PPP was then either subjected to a second high-speed centrifugation at 13,500 × g for 2 minutes to achieve platelet free plasma (PFP) or aliquoted and stored at −70 °C for at least 48 hours. After thawing, PPP was then centrifuged at 13,500 × g for 2 minutes to get rid of platelets and cell debris. PPP was then diluted with Hanks/Hepes buffer (130 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl\textsubscript{2}, 0.8 mM MgSO\textsubscript{4}, 0.44 mM Na\textsubscript{2}HPO\textsubscript{4}, 20 mM HEPES, pH 7.4) (1:10 dilution) (to be used for electron microscopy) or sterile filtered Dulbecco’s phosphate buffered saline (DPBS) without CaCl\textsubscript{2} and MgCl\textsubscript{2} (Sigma-Aldrich, St. Luis, MO, USA) (1:20 dilution) (to be used for EV concentration and size distribution)\textsuperscript{26}. EVs were pelleted from PFP by centrifugation at 20,000 g for 30 minutes at room temperature (Beckman OptimaTM LE-80K Ultracentrifuge, swinging bucket rotor SW 40 TI). The supernatant was discarded and the EV pellet re-suspended in DPBS, snap frozen\textsuperscript{27} and stored at −70 °C until further analysis. For electron microscopy the EV pellet was re-suspended in double filtered Hanks/Hepes buffer and fixed in 4% formaldehyde until further analysis.

**Nanoparticle Tracking Analysis (NTA).** EV concentration and size distribution were determined using NanoSight NS300 (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 488 nm blue laser and a CMOS camera. EV samples were thawed in 37 °C water immediately prior to analysis and diluted (50–100 ×) in DPBS. Samples were manually introduced to the instrument using a syringe. Samples were captured at ambient
temperature with automatic temperature monitoring. Three separate dilutions of the samples were used for analysis. Each dilution was captured 3 times 60 seconds (camera level 16), and the sample was refreshed between captures. The gasket was cleaned between each sample. The nine resulting videos were analysed with NTA software version 3.0 (detection threshold 3). Mean values for concentration and size distribution were calculated.

**Transmission Electron Microscopy (TEM).** TEM was performed on isolated EVs. The EV pellets were re-suspended in 50 µl of Hanks/Hepes buffer and fixed in 4% formaldehyde in 200 ml Hepes overnight.

**TEM of Ultrathin sections.** To improve localization of EVs for TEM, the EVs were adsorbed onto an epoxy resin substrate containing colloidal gold particles. The substrate was prepared by adsorbing 15 nm gold particles (Department cell biology, University of Utrecht, the Netherlands) on formvar/carbon coated copper specimen grids and then embedded the gold-coated grids in a thin layer of epoxy resin between two layers of Aclar film and polymerized at 60 °C for 48 hours. The EV suspension was placed on the gold-loaded, epoxy-embedded specimen grids and in 1% glutaraldehyde, postfixed in 1% OsO4 and stained with 1% aqueous uranyl acetate. The EVs on the epoxy-embedded grids were dehydrated in a graded series of ethanol, infiltrated in an Epon Equivalent (AGAR 100, DDSA, MNA and DMP-30, Agar Scientific, UK) and polymerized at 60 °C for 48 hrs. Ultrathin sections of the embedded EVs were prepared using Ultracut S ultramicrotome (Lieca Microsystems, Vienna, Austria) and a Diatome diamond knife (Diatome, Biel, Switzerland). Images using a JEOL JEM 1010 transmission electron microscope (Tokyo, Japan) were acquired with a Morada camera system (Olympus Soft Imaging System, Münster, Germany).

**Immune electron microscopy.** EVs were fixed with 1% buffered glutaraldehyde and adsorbed onto carbon-formvar coated specimen grids before immunolabelling. In short, unspecific labelling was blocked on 0.1% cold water fish skin gelatin (CWFG) (Sigma G-7765) and 1.5% bovine serum albumin. Samples were incubated with anti-annexin V (Anx5) antibody (abcam, Cat# ab14196), diluted in Anx5 binding buffer (BD Pharmingen, Cat#556454) and protein A-gold (University of Utrecht, The Netherlands). All immunoreagents were diluted in CWFG and the grids washed in PBS between each step. The grids were finally fixed in 1% glutaraldehyde, washed in distilled water and dried in 1.8% Methylcellulose containing 0.3% uranyl acetate.

**Scanning Electron Microscopy (SEM).** To obtain an overview of the morphology and measure particle size of EVs, isolated EVs were prepared for SEM analysis. To study the surface size, shape, and features of EVs, they were negatively stained on formvar/carbon coated copper grids. Grids floated on sample drops for 30 minutes were treated with 1% glutaraldehyde, washed in PBS and distilled water and contrasted/dried with the addition of 1.8% methyl cellulose and 0.3% uranyl acetate according to Tokuyasu28,29. EV size measurements were performed in the iTEM program (Olympus Soft Imaging Solutions, Münster, Germany) by measuring shortest diameter of at least 200 EV on SEM pictures. The start and end of every diameter was set manually and the diameter was calculated by the program. Grids were mounted on specimen holder and coated with gold/palladium before examination in the SEM. The images were obtained using a Zeizz Merlin VP compact scanning microscope.

**Fat tolerance test.** A fat-tolerance test was conducted using a test meal prepared from standard porridge cream containing 70% of calories from fat of which 66% saturated fat, 32% monounsaturated fat and 2% polyunsaturated fat. The test meals were served with two teaspoons of sugar, cinnamon, and two glasses (150 ml each) of sugar-free juice. The test meals were freshly prepared each morning. A weight-adjusted meal (1 gram fat per kg body weight) was served at 8:00 a.m. and consumed over a 15-minute period. The participants were allowed to drink 350 ml calorie-free beverages and eat an apple during the following 8 hrs. Blood was drawn from an antecubital vein in the morning at 7:45 a.m., after a 12-hour overnight fast and a 48-hour refrain of exhaustive physical exercise and alcohol consumption, and then 2, 4, 6, and 8 hours after the meal, using a 19-gauge needle in a vacutainer system with minimal stasis for serum and plasma preparations. Blood for plasma preparation was collected into 4.5 ml vacutainers (Becton Dickinson, Meylan Cedex, France) containing 0.129 M sodium citrate (1 vol anticoagulant and 9 vol whole blood) or EDTA (K3–EDTA 40 gr), centrifuged at 2000 g for 15 minutes at 22 °C, and transferred into cryovials (Greiner Laboratory, GmbH). Plasma was re-suspended in 50 µl of Hanks/Hepes buffer and fixed in 4% formaldehyde overnight at −70 °C until further analysis.

Serum lipids were analyzed on an ABX Pentra 400 (Horiba ABX Diagnostics, Montpellier, France) with reagents from Horiba ABX Diagnostics (Montpellier, France). Proton nuclear magnetic resonance (NMR) spectroscopy was used to determine mean particle sizes of the main lipoprotein classes (very-low-density lipoprotein (VLDL), low-density-lipoprotein (LDL), and high-density lipoprotein (HDL)) along with concentrations of 10 lipoprotein subclasses (chylomicron/large VLDL, medium VLDL, small VLDL, intermediate-density lipoprotein (IDL), large LDL, small LDL) also reported as medium small LDL and very small LDL, large HDL, medium HDL, and small HDL) in fasting and 4-hours postprandial citrated plasma at Liposoft Imaging Solutions, Railegh, NC, USA. Plasma concentrations of EVs in fasting and 4-hours postprandial citrated plasmas were determined using NTA as previously described.

**Statistics.** Median values and interquartile ranges for continuous data (EV concentrations) were presented as data was not normally distributed. To test for differences in EV concentrations between anticoagulants, we used Friedman’s test for non-parametric and dependent continuous data. Bar graphs were used to display (i) EV concentrations according to size categories of EVs (<100 nm, 100–199 nm, 200–299 nm, 300–1000 nm), and (ii) mean sizes of EVs measured by NTA and SEM in the different anticoagulants. The correlation between
triglycerides and EV concentrations, as well as VLDL and EV concentrations, was calculated using Pearson correlation coefficient. All analyses were performed using IBM SPSS Statistics version 22 (Armonk, NY, USA).

Results

The baseline characteristics of the ten healthy volunteers (aged 28–55 years) recruited from the research staff and the forty subjects (aged 28–76 years) recruited from the general population health study are shown in Table 1.

Table 1. Characteristics of the volunteers recruited from the research staff (n = 10) and the participants of the cohort study (n = 40). Values are means ± standard deviations or numbers with percentage in brackets.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Volunteers (n = 10)</th>
<th>Cohort (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women, n (%)</td>
<td>5 (50)</td>
<td>20 (50)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>41 ± 9</td>
<td>56 ± 14</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.0 ± 4.0</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dL)</td>
<td>13.9 ± 1.2</td>
<td>14.4 ± 1.3</td>
</tr>
<tr>
<td>Leukocytes (10⁹/L)</td>
<td>5.1 ± 0.9</td>
<td>6.3 ± 1.7</td>
</tr>
<tr>
<td>Platelet count (10⁹/L)</td>
<td>229 ± 47</td>
<td>249 ± 62</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.69 ± 1.35</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.32 ± 0.45</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.33 ± 0.82</td>
<td></td>
</tr>
</tbody>
</table>

The impact of freezing PPP compared to PFP on concentration and size distribution of plasma-derived EVs is shown in Fig. 1, panel A–D. There were no statistical differences in total concentration and size distribution of EVs between plasmas prepared as PPP and PFP before freezing in any of the anticoagulants used (Fig. 1).

The impact of the four different anticoagulants on concentration and size distribution of plasma EVs are displayed in Table 2. Neither the total plasma concentration, nor concentrations of EVs within defined size categories (<100 nm, 100–199 nm, 200–299 nm and 300–1000 nm) differed significantly between the anticoagulants. However, although not statistically significant, blood anticoagulated with sodium citrate showed the lowest plasma concentration of EVs across all size categories, whereas heparinized plasma yielded an almost 2-fold higher concentration of large EVs (300–1000 nm) compared to the other anticoagulants (Table 2). The majority of EVs were in the smaller size categories (<100 nm: 76.4% to 78.2%, 100–199 nm: 18.0% to 20.2%, 200–299 nm: 2.7% to 3.5%, 300–1000 nm: 0.7% to 1.3%) and the size distribution did not differ between anticoagulants (Table 2).

The mean size of the EVs was marginally lower (Fig. 2, upper panel) and the size distribution was shifted towards smaller EVs (<199 nm) when EVs were sized by SEM compared to NTA (Fig. 2, lower panel). Similar to NTA, the majority of EVs determined by SEM were below 200 nm in diameter and large vesicles were rarely seen.

TEM was applied to confirm the presence of EVs after isolation by high-speed centrifugation from plasma samples. TEM of negatively stained (Fig. 3A) and ultrathin sections (Fig. 3B) revealed that the majority of isolated vesicles were EVs, characterized by the bilayer phospholipid membrane. Even though concern has been raised about EV aggregation and morphological changes as a result of high-speed centrifugation, we could not identify EV aggregates, nor large variations in the EV morphologies, as the main proportion of vesicles identified were spheroidal (Fig. 3C) and presented uneven non-smooth surfaces (Fig. 3D). Lipoprotein vesicles, characterized by the lack of a bilayer surface membrane, were rarely seen.

In order to determine if the concentration of triglycerides would change, and lipoproteins would sediment with EVs after a high speed centrifugation (20,000 x g for 30 minutes), we measured the levels of plasma lipoproteins before and after centrifugation, in both diluted and undiluted plasma. We found that the plasma concentration of triglycerides was equal before and after high-speed centrifugation for both undiluted and diluted (1:5 in DPBS) plasma (Fig. 4, panel A). Despite the minimal sedimentation of VLDL particles by high-speed centrifugation due to unchanged plasma concentration of triglycerides, we found moderate correlations between EVs and plasma concentrations of triglycerides (Fig. 4, panel B) and VLDL particles (Fig. 4, panel C) under fasting conditions. Concentrations of serum triglycerides and VLDL particles explained (r²) 13% and 19%, respectively, of the plasma variation of EVs.

Ingestion of a standardized high-fat meal (1 g/kg body weight) was accompanied by a significant increase in serum triglycerides, which peaked at 4 hours, and returned to baseline levels within 8 hours after the meal (Fig. 5A). The postprandial lipemia was not accompanied by significant changes in the concentration of EVs in plasma determined by NTA (Fig. 5A). Similarly, the plasma concentration of VLDL particles did not change from the fasting to the postprandial state (Fig. 5B), but the median diameter of VLDL particles increased from 42 ± 6 nm in fasting plasma to 55 ± 9 nm in postprandial plasma (p < 0.0001) (Fig. 5C). However, serum triglycerides and the concentration of VLDL particles in plasma collected 4 hours after ingestion of the meal showed a strong correlation with the plasma concentration of EVs and explained 59–66% of the variation in plasma EVs (r² = 0.59 and 0.66, respectively) (Fig. 5D and E).
Figure 1. Panels of bar graphs showing concentrations across size categories of plasma EVs frozen before (platelet poor plasma, PPP) and after (platelet free plasma, PFP) high-speed centrifugation in order to get rid of cell debris and platelets. After thawing, plasmas frozen as PPP were subjected to high-speed centrifugation before isolation of plasma EVs. The panels represent blood anticoagulated with citrate (panel A), CTAD (panel B), EDTA (panel C), and heparin (panel D). EV concentrations were measured by nanoparticle tracking analysis (NTA). Values are means with standard error of the means (SEM) (n = 10 in each group). EDTA: Ethylenediaminetetraacetic acid, CTAD: sodium citrate theophylline adenosine dipyridamole.

<table>
<thead>
<tr>
<th>EV size category</th>
<th>Citrate</th>
<th>EDTA</th>
<th>CTAD</th>
<th>Heparin</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100 nm</td>
<td>1133 (1066–2034)</td>
<td>1514 (1074–2039)</td>
<td>1420 (928–1926)</td>
<td>1314 (928–2207)</td>
<td>0.1</td>
</tr>
<tr>
<td>100–199 nm</td>
<td>262 (200–450)</td>
<td>402 (270–443)</td>
<td>319 (265–474)</td>
<td>313 (287–372)</td>
<td>0.2</td>
</tr>
<tr>
<td>200–299 nm</td>
<td>41 (19–71)</td>
<td>53 (38–65)</td>
<td>63 (40–105)</td>
<td>54 (35–76)</td>
<td>0.7</td>
</tr>
<tr>
<td>300–1000 nm</td>
<td>13 (2–20)</td>
<td>14 (6–19)</td>
<td>13 (5–36)</td>
<td>22 (9–52)</td>
<td>0.5</td>
</tr>
<tr>
<td>Total Concentration</td>
<td>1588 (1331–2403)</td>
<td>2017 (1510–2528)</td>
<td>1980 (1320–2514)</td>
<td>1658 (1342–2700)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 2. Plasma concentration (10^7 per mL) of extracellular vesicles (EVs) according to type of anticoagulant and size categories of EV. Values are expressed as medians with interquartile ranges (n = 10). EDTA: Ethylenediaminetetraacetic acid, CTAD: sodium citrate theophylline adenosine dipyridamole.
Discussion

We aimed to investigate the impact of various preanalytical conditions such as centrifugation steps for plasma preparation and EV isolation, choice of anticoagulant, and fasting status on plasma concentration and size distribution of EVs determined by NTA. The size distribution of plasma EVs was similar when determined by NTA and SEM, and the majority of EVs were round-shaped. The plasma concentration and size distribution of EVs were essentially similar in plasmas isolated from blood with various anticoagulants, even though the plasma concentration of large EVs was almost 2-fold higher in heparinized plasma compared to the other anticoagulants.

A bilayer membrane of EVs was demonstrated in the majority of particles in the EV pellet by two separate TEM methods. The plasma concentration and size distribution of EVs, isolated from plasma by high-speed centrifugation and determined by NTA, were similar in blood collected before and 4 hours after ingestion of a high-fat meal. Even though triglyceride levels remained unchanged after high-speed centrifugation, serum concentrations of triglycerides and plasma concentrations of VLDL particles correlated with the concentration of EVs, particularly in the postprandial state.

Isolation of EVs from plasma by high-speed centrifugation may elicit shape change, aggregates and/or damage of EVs. In our study, the mean diameter of EVs isolated from plasma by high-speed centrifugation was 80–90 nm. Our results were very similar to those reported by Dragovic et al. who measured vesicles directly in plasma after labelling with a specific cell tracker dye. We used two independent methods (NTA and SEM) to determine the size distribution of the EV fraction, and the results were similar for the two methods. NTA assessment of the size distribution revealed that only 3.5–5% of the EVs had a diameter above 200 nm, and that only 0.7–1.3% of the EVs had a diameter above 300 nm. The low proportion of EVs with a diameter above 2–300 nm, which is the lower detection limit of conventional flow cytometers, may explain the huge differences in observed plasma concentrations of EVs measured by flow cytometry and NTA.

Previous studies comparing the impact of various anticoagulants on plasma concentrations of EVs assessed by flow cytometry, have shown higher levels of EVs in heparin than in sodium citrate, ACD or CTAD as anticoagulants. In line with these studies, we found that the plasma level of large EVs (>300 nm), which is detectable by flow cytometry, was 2-fold higher in plasma anticoagulated by heparin compared to plasma containing the other anticoagulants. In our study, blood anticoagulated with EDTA, a stronger chelator than citrate, displayed highest
plasma concentrations of EVs, supporting the notion that EDTA promotes formation of artefactual microvesicles due to platelet activation. Citrated plasma displayed the lowest median concentrations of EVs across all size categories. Citrate plays a role in membrane phospholipid remodelling and may thereby partially inhibit vesiculation. Our findings of a lower EV concentration in samples with citrate indicates that in vitro vesiculation may occur to a larger extent with the other anticoagulants, and support the recommended use of citrate as anticoagulant for studies on EVs.

Previous studies using Nanoparticle Tracking Analysis of PFP have reported a particle concentration of approximately $1.5 \times 10^{12}$/mL in plasma. However, NTA does not allow for distinction between EVs and other particles within the same size range in plasma. Triglyceride-rich lipoproteins (chylomicrons and very-low-density lipoproteins) are in molar excess, but of similar size to cell-derived EVs, and can be detected in light scatter. Previous studies showed that the apparent plasma concentration of EVs measured by NTA was highly correlated to the plasma concentration of triglycerides, and that the concentration of EVs declined by more than 98% when only vesicles labelled with a cell tracker dye were counted. To avoid interference of triglyceride-rich lipoproteins on the NTA measurements of EVs, we isolated EVs from plasma by high-speed centrifugation and re-suspended the pellet in a particle-free buffer. In our study, the median concentrations of EVs isolated from plasma varied between $1.6-2.0 \times 10^{10}$/mL with an interquartile range from 1.3 to $2.7 \times 10^{10}$/mL, which is in line with the results based on specific labelling of EVs by a cell tracker dye.

Our findings provide several lines of evidence for at least partial separation of EVs from triglyceride-rich lipoproteins by high-speed centrifugation of plasma. First, the concentration of triglycerides in the plasma supernatant remained unchanged after high-speed centrifugation, suggesting that VLDL-particles in fasting blood samples were not pelleted and mainly remained floating. Second, even though serum triglycerides and the concentration of VLDL particles in plasma from fasting individuals displayed a moderate correlation with the plasma

Figure 3. Visualization of Extracellular Vesicles (EVs) by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). (Panel A) EVs with phospholipid bilayer membrane after ultra-thin sectioning of the Epon blocks. (Panel B) Representative transmission electron micrograph of purified, negatively stained annexin positive and negative bilayer membrane extracellular vesicles. (Panel C) Representative transmission electron micrograph of plasma purified, negatively stained EVs. (Panel D) Representative micrograph of an EV by SEM, isolated from EDTA peripheral blood of a healthy donor at the working distance of 2.6 mm and an accelerating voltage of 2.00 kV (Original magnification 61.12 KX). EVs were spherical and presented uneven non-smooth surfaces.
concentration of EVs, it only explained 13–19% of the variation in plasma EVs, suggesting that the particle count, measured by NTA, was not dominated by VLDL particles. Third, we developed a novel procedure to section the EVs and could thereby clearly visualize the bilayer phospholipid membrane which distinguishes EVs from lipoproteins. Fourth, the results of our TEM analysis confirmed that the majority of isolated vesicles in our samples were in fact EVs (characterized by the bilayer phospholipid membrane) and not lipoproteins or protein complexes.

Figure 4. Bar graphs showing concentration of triglycerides before and after high-speed centrifugation (20,000 × g for 30 minutes) in undiluted (PFP) and diluted (dPFP, diluted 1:5 in DPBS) samples (panel A, bar graphs are means with 1 standard deviation), and dot-plots showing correlations between plasma concentration of EVs and serum triglycerides (panel B) and plasma VLDL particles (panel C) under fasting conditions. Proton nuclear magnetic resonance (NMR) spectroscopy was used to determine mean particle sizes of the main lipoprotein classes, and EV concentration was measured by NTA.
Ingestion of a standardized high-fat meal was accompanied by a significant increase in serum triglycerides which peaked at 4 hours and returned to baseline levels 8 hours after the meal without affecting the plasma levels of EVs determined by NTA. However, serum triglycerides and the concentration of VLDL particles in plasma collected 4 hours after ingestion of the meal showed a strong correlation with the plasma concentration of EVs and explained 59–66% of the variation in plasma EVs. These findings suggest that the particle count, measured by NTA, was highly influenced by VLDL particles under postprandial conditions. The reason(s) for the apparent differential impact of VLDL particles on the vesicle count measured by NTA under fasting and postprandial conditions is not known. Even though the plasma concentration of VLDL particles did not increase in postprandial plasma, the mean particle size increased significantly from 42 ± 6 nm in fasting plasma to 55 ± 9 nm in postprandial plasma. With a detection limit of a particle diameter of around 50 nm for EVs by NTA, even a marginal sedimentation of VLDL particles during high-speed centrifugation may affect the particle counts, particularly under postprandial conditions. Thus, our measurements of EVs isolated from plasma by high-speed centrifugation, should be interpreted with caution due to a potential partial interference by VLDL particles, particularly

**Figure 5.** Bar graphs showing concentrations of serum triglycerides and plasma EVs before (fasting) and every second hour after ingestion of a standardized high fat meal (panel A, bar graphs are means with 1 standard deviation, ***p < 0.0001 from fasting levels). Dot plots showing median and 25 to 75% percentiles of plasma concentration of VLDL particles (panel B) and mean sizes of VLDL particles (panel C); and dot plots showing correlations between plasma concentration of EVs (measured by NTA) and serum triglycerides (panel D) and plasma VLDL particles (panel E) 4 hours after ingestion of a standardized high fat meal.
under postprandial conditions. Recently, Mork et al. aimed to prevent VLDL interference during NTA measurements of EVs by antibody-mediated removal of ApoB-exposing lipoproteins from plasma using magnetic beads. However, antibody-mediated depletion of ApoB-containing lipoproteins (including VLDLs) was accompanied by removal of EVs most probably due to the formation of LDL-EV complexes, and by a shift in particle size due to the formation of aggregates of antibodies and lipoproteins. Future studies should optimize centrifugation procedures, antibody-mediated removal or combined procedures in order to separate EVs and VLDL particles, so that NTA can accurately and reliably be applied to measure plasma concentrations of EVs.

In conclusion, isolation of EVs from plasma by high-speed centrifugation yielded similar concentrations and size distributions of EVs for the four anticoagulants tested (citrate, EDTA, CTAD and heparin). We found no statistical difference in concentration nor size of EVs (measured by NTA) when plasma was prepared as PPP or PFP before freezing. Plasma VLDL particles interfered with EV measurements assessed by NTA, particularly under postprandial conditions due to an increase in the median particle diameter of VLDLs exceeding the lower detection limit of NTA. Future studies are warranted to optimize the separation of VLDL particles and EVs in plasma in order to promote the utility of EVs determined by NTA as potential biomarkers of risk, diagnosis and prognosis of diseases.

References


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**Author Contributions**

S.J. contributed with data collection and drafted the manuscript. C.R. and N.L. collected data, performed NTA analyses and revised the manuscript. R.O. and P.W. provided expertise and facilities in electron scanning microscopy and revised the manuscript. T.S. collected data and performed statistical analyses, and revised the manuscript, S.K.B. collected data and revised the manuscript, J.B.H. designed the study, collected and interpreted data, and revised the manuscript.

**Additional Information**

**Competing Interests:** The authors declare no competing interests.

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Paper II
A modified clot-based assay to measure negatively charged procoagulant phospholipids

Cathrine Ramberg1, S. Jamaly1, N. Latysheva1, L. Wilsgård1, T. Sovershaev1, O. Snir1 & J.-B. Hansen1,2

Growing evidence supports a role for extracellular vesicles (EVs) in haemostasis and thrombosis due to exposure of negatively charged procoagulant phospholipids (PPL). Current commercial PPL-dependent clotting assays use chemically phospholipid depleted plasma to measure PPL activity. The purpose of our study was to modify the PPL assay by substituting the chemically phospholipid depleted plasma with PPL depleted plasma obtained by ultracentrifugation. This in order to get readily access to a sensitive and reliable assay to measure PPL activity in human plasma and cell supernatants. The performance of the assay was tested, including the influence of individual coagulation factors and postprandial lipoproteins and compared to a commercial PPL assay (STA-Procoag-PPL). The two PPL assays displayed similar sensitivity to exogenously added standardized phospholipids. The PPL activity measured by the modified assay strongly correlates with the results from the commercial assay. The intraday- and between-days coefficients of variation ranged from 2–4% depending on the PPL activity in the sample. The modified PPL assay was insensitive to postprandial lipoprotein levels in plasma, as well as to tissue factor (TF) positive EVs from stimulated whole blood. Our findings showed that the modified assay performed equal to the comparator, and was insensitive to postprandial lipoproteins and TF+ EVs.

Procoagulant phospholipid (PPL) activity has regained interest in recent years, mainly due to the increased understanding of the role of extracellular vesicles (EVs) in thrombosis and haemostasis. As early as 1946, Chargaff and West observed that the clotting time of plasma was prolonged by applying high-speed centrifugation to remove "thromboplastic substances". Accordingly, Connor and colleagues demonstrated that the amount of annexin A5 positive EVs, measured by flow cytometry, showed a significant and inverse correlation with clotting time. These findings suggest that EVs play a significant role in coagulation, apparently due to exposure of phospholipids, and phosphatidylserine (PS) in particular, on their surface. The increase in surface expression of negatively charged PPL will facilitate the assembly of coagulation factors upon cell activation or apoptosis. This is crucial for several stages of the coagulation pathway, namely the formation of the intrinsic and extrinsic tenase complexes, as well as the conversion of prothrombin to thrombin by coagulation factor Xa (FXa). The activity of the extrinsic tenase complex, the tissue factor (TF)—factor VIIa (FVIIa) complex, is increased by several orders of magnitude in the presence of negatively charged membrane phospholipids.

Several assays have been developed to measure the PPL activity in human plasma. While some are based on the ability of annexin A5 to bind PS in the presence of Ca2+, others are clot-based, utilizing the ability of PPL to accelerate the conversion of prothrombin to thrombin. Annexin A5-based assays are widely used, often in a flow cytometry setting, a method that is time consuming, requires expensive equipment and experienced personnel. In addition, annexin A5 is commonly used in chromogenic FXa assays, where the activity measured is based on the EVs exposing PS that are bound to the microplate. These EVs are then able to accelerate the cleavage of the chromogenic substrate by FXa.

Compared to FXa chromogenic assays, which measure procoagulant activity of EVs in a purified system, clotting assays involve a more complex reaction and a physiological end-point as they measure the PPL activity of plasma, and not only captured PS-positive EVs. To the best of our knowledge, there are currently two clot-based assays commercially available, the STA-Procoag-PPL assay from Diagnostica Stago (Asnières sur Seine Cedex, France) and the XACT assay from Haematex (Hornsby, NSW, Australia). Both assays use chemical phospholipase treatment to deplete phospholipids from plasma, but differ with regard to the phospholipase used, plasma origin,
and the use of a phospholipid calibrator. The XACT assay uses a snake phospholipase and porcine plasma, while an unspecified phospholipase and human plasma is used for the Stago assay.

In this study, we aimed to develop a modified PPL-dependent clotting assay, capable of measuring the PPL activity in human plasma and cell supernatants of in vitro experiments, by removing PPL from plasma by sequential centrifugation, including final ultracentrifugation. The performance of the modified assay was then validated against the commercially available Stago STA-Procoag-PPL assay.

Results
Impact of stepwise EV depletion on PPL clotting times. The removal of PPL from plasma is fundamental for the clot-based assays. As EVs are the main source of PPL, we first tested whether sequential centrifugation reliably depleted EVs from plasma. To achieve this, we compared the PPL clotting times (PPLCT) of plasma samples (n = 6) subjected to sequential centrifugation procedures (Fig. 1). Plasma prepared by centrifugation at 2500×g for 15 min caused clotting times of 51.8 ± 4.7 s (mean ± 1 SD). A second centrifugation step of 2500×g for 15 min resulted in a prolongation of the clotting times to 92.5 ± 6.3 s (mean ± 1 SD). Pelleting larger EVs (e.g. microvesicles) from platelet free plasma (PFP) by an additional spin of 20,000×g for 30 min at room temperature (RT) further prolonged the clotting times to 127.8 ± 16.9 s (mean ± 1 SD). The final spin at 100,000×g for 60 min to remove the smallest and lightest EVs (e.g. exosomes) further prolonged the clotting times to 159.3 ± 7.1 s (mean ± 1 SD).

Procoagulant phospholipid depleted plasma (PPL depleted plasma) prepared from pooled PFP (2500×g for 15 min twice) by ultracentrifugation (100,000×g for 60 min) resulted in a mean clotting time of 163.2 ± 7.5 s (mean ± 1 SD) (Supplementary Figure S1).

PPL depletion by ultracentrifugation does not affect standard coagulation assays. To test whether the preparation of PPL depleted plasma affected standard coagulation assays, we measured activated partial thromboplastin time (aPTT) and prothrombin time (PT). The aPTT was 30.2 (normal range 25–37 s) and PT 21.3 s, corresponding to a PT-INR of 1.00 (normal range < 1.1).

Comparison of the modified PPL assay and the STA-Procoag-PPL assay. In order to demonstrate that PPL depleted plasma prepared by ultracentrifugation was comparable to enzymatic depletion of PPL, we tested the sensitivity of the STA-Procoag-PPL assay and the modified PPL assay using serial dilutions of a standardized phospholipid reagent (UPTT reagent). The addition of UPTT shortened the clotting times in a concentration dependent manner (Fig. 2). The two assays performed similarly throughout the tested concentration range. Dilution curves of bovine FXa (bFXa) tested in PPL depleted plasma and pooled PFP supported the use of 0.1 U/ml bFXa for the modified PPL assay (Supplementary Figure S2). Despite a ten-fold lower concentration of bFXa used in the STA-Procoag-PPL assay, it consistently displayed shortened clotting times compared to the modified assay.

To compare the performance of the modified PPL and STA-Procoag-PPL assays, plasma samples from ten healthy blood donors were tested on both assays (Fig. 3). A strong correlation (r = 0.76, p < 0.01) was found

![Figure 1. The effect of sequential centrifugation on plasma PPL clotting times. Citrated blood collected from 6 healthy volunteers was subjected to consecutive centrifugations: 2500×g for 15 min to obtain platelet poor plasma (PPP), 2500×g for 15 min to obtain platelet free plasma (PFP), 20,000×g for 30 min and 100,000×g for 1 h to deplete for EVs. A sample of plasma after each centrifugation was analyzed with the modified PPL assay. The PPL activity is presented in seconds (s) of clotting time. Dot plot with mean ± 1 SD (n = 6 for each condition).](https://www.nature.com/scientificreports/)
between the assays, confirming their comparable performance. PPL\textsubscript{CT} measured by the modified assay were consistently longer than for the STA-Procoag-PPL assay.

**Assay reproducibility.** To assess the coefficients of variation (CV) of the modified PPL assay, we used the two plasma standards provided by the STA-Procoag-PPL assay, one resulting in PPL\textsubscript{CT} of 16 s, and the other PPL\textsubscript{CT} of 54 s, as well as a pooled PFP sample with PPL\textsubscript{CT} of 85 s. The day-to-day CV for the Stago standards (n = 30) was 3.9% and 3.2%, respectively, whereas the within-a-day CV (n = 20) was 3.7% and 2.8%, respectively. Pooled PFP had a day-to-day CV of 4.1% and a within-a-day CV of 3.3%.

The reproducibility of three independent preparations of PPL depleted plasma from the same six donors was tested, and demonstrated highly comparable PPL\textsubscript{CT} measurements without the addition of UPTT and with the addition of two different concentrations of UPTT (PPL depleted plasma without UPTT: 163.2 ± 7.5 s, 1:3200 U/ml of UPTT: 102.3 ± 5.7 s, 1:100 U/ml of UPTT: 30.6 ± 0.9 s) (Supplementary Figure S1).

**Impact of different PFP preparation protocols on PPL\textsubscript{CT.}** We wanted to investigate to what extent two different centrifugation protocols (Protocol A: 2500×g for 15 min twice; protocol B: 3000×g for 10 min followed by 13,500×g for 2 min) affected PPL\textsubscript{CT} (Fig. 4). Plasmas obtained by protocol A from 10 healthy individuals displayed prolonged PPL\textsubscript{CT} compared to plasmas obtained by protocol B (101.2 ± 10.9 s versus 76.5 ± 9.1 s, p < 0.0001).

**Postprandial lipemia does not affect PPL\textsubscript{CT} in the modified PPL assay.** To test the sensitivity of the modified PPL assay to postprandial lipemia, we compared the PPL\textsubscript{CT} of PFP prepared from blood collected before (0 h) and 4 h after a standardized high fat meal (1 g fat/kg body weight) (Fig. 5). This caused a prompt
increase in total serum triglycerides that peaked after 4 h and almost reached baseline concentrations 8 h after the meal\(^1\). However, postprandial lipemia was not accompanied by a significant change in the \(\text{PPL}_{\text{CT}}\) of fasting and postprandial plasma samples (51.1 ± 12.4 s and 48.9 ± 9.3 s, respectively). This indicates that the modified PPL assay was insensitive to postprandial lipemia measured in PPP samples subjected to a second high speed centrifugations after thawing, that generate mean CT values around 50 s.

**The impact of FVIIa, FVa and surface PS on the modified PPL assay.** Since the presence of coagulation factors in human PPL depleted plasma may provide a source of preanalytical variation of the modified
PPL assay, we tested the assay with the addition of various concentrations of coagulation factors V/Va and VIIa (Fig. 6). The addition of FVa resulted in a dose dependent shortening of PPLCT (Fig. 6A). The addition of increasing levels of FVIIa had minor influence on PPLCT, although the initial addition of 5 nM FVIIa slightly shortened the PPLCT (Fig. 6B).

To further prove that coagulation in the modified assay is mainly driven by PPL, we used lactadherin to neutralize the negatively charged phospholipids. Pretreatment with lactadherin reduced the PPLCT dose-dependently of EVs derived both from A23187-stimulated PRP and EVs from unstimulated PRP (Fig. 6C). Pretreatment of

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**Figure 6.** The effect of coagulation factors on the performance of the modified PPL assay. Exogenous coagulation factors were added to the reaction buffer in concentrations from 0.02 to 3.0 nM for FVa (panel A) and from 5 to 80 nM for FVIIa (panel B). Panel (C) shows the effect of lactadherin pretreatment on the PPL activity of PRP-derived EVs generated by stimulation of PRP by calcium ionophore A23187 for 15 min. Values are means ± 1 SD (n = 4).
both PPL depleted plasma and PFP with 107 nM lactadherin drastically reduced their PPL_CT with clotting times exceeding the maximum clotting time of the coagulometer.

**Blood-borne TF has no effect on PPL_CT in the modified PPL assay.** Addition of recombinant relipidated TF to PPL depleted plasma resulted in a dose-dependent shortening of clotting time (Fig. 7A). Since the artificial nature of TF and supra-pathological levels of TF may have caused the observed effect, we decided to test a more physiological source of TF. Whole blood was stimulated with a combination of LPS and PMA to induce the release of TF EVs from monocytes. Then, isolated EVs were run on the PPL assay with or without inhibitory TF antibody (clone HTF-1). As seen in Fig. 7B, stimulation of whole blood resulted in significant shortening of the clotting time. However, addition of anti-TF antibody (26 µg/ml final concentration) did not alter clotting times, suggesting that TF did not affect coagulation times in the modified PPL assay.

**Discussion**

The procoagulant potential of plasma phospholipids is modified under pathological conditions. Currently, two commercial assays, namely XACT and STA-Procoag PPL are widely used to monitor PPL activity plasma. We thoroughly validated a modified and easy to use PPL assay for the measurement of procoagulant phospholipids in test specimens (plasma samples or isolated EVs) and compared its performance with the STA-Procoag-PPL assay. Our primary modification included preparation of PPL-depleted plasma by ultracentrifugation to remove
EVs, assumed to be the main source of negatively charged phospholipids in plasma. This modification allowed for the establishment of an accessible and in-house assay with a comparable performance to that of the commercial assay. The addition of a standardized phospholipid reagent (UPTT) allows for clotting times to be converted into a standardized unit of phospholipids.

The commercially available PPL assays are well established in research and provide a reliable tool for assessing PPL activity in human plasma. Such assays are dependent on chemically phospholipid-depleted plasma provided by the assay as a reagent. Their performance has been assessed under different pre-analytical and experimental settings such as quality control of cell storage\(^\text{13}\), plasma EVs\(^\text{14,15}\), and monitoring of plasma EV levels in various disease states\(^\text{16–19}\). Assuming that the vast majority of procoagulant phospholipids in plasma are EV-bound, we examined whether sequential centrifugation, including final ultracentrifugation, might substitute phospholipase treatment for production of a suitable assay plasma. Indeed, subjecting plasma to sequential centrifugation resulted in prolonged clotting times (i.e. reduced PPL activity), thus supporting that the major part of PPL activity in plasma is mediated by EVs, as originally suggested by Chargaff and West\(^\text{3}\).

Since ultracentrifugation may leave residual amounts of PPL in the assay plasma, it was crucial to compare the properties of our PPL-depleted plasma with the phospholipase-depleted plasma. Therefore, the performance of the modified PPL assay and the STA-Procoag-PPL assay were compared using a standardized UPTT reagent. The results revealed a high degree of coherence between assays. Further, measurement of endogenous plasma PPL in PFP obtained from 10 volunteers demonstrated strong correlations between assays assessed by PPL\(_{\text{CT}}\). These findings support that the modified assay provide comparable performance to the STA-Procoag PPL assay with regard to sensitivity and measurement of levels of PPL in plasma.

It is important to note the effects of pre-analytical parameters on the performance of the modified PPL assay. Recently, Lacroix and colleagues used the STA-Procoag PPL assay to investigate the effect of different centrifugation settings such as quality control of cell storage\(^\text{13}\), plasma EVs\(^\text{14,15}\), and monitoring of plasma EV levels in various disease states\(^\text{16–19}\). They found that PFP prepared according to the International Society of Thrombosis and Haemostasis (ISTH) recommended protocol (2500xg for 15 min twice) yielded similar PPL\(_{\text{CT}}\) to another common centrifugation protocol (1500xg for 15 min followed by 13,500xg for 2 min) in fresh PFP. However, PFP obtained by the second, less intensive centrifugation protocol, displayed a substantial shortening of the PPL\(_{\text{CT}}\) after a freeze–thaw cycle (−154%). A minor effect on PPL activity (−5%) was observed when analyzing frozen-thawed PFP using the ISTH recommended centrifugation protocol\(^\text{14}\). Accordingly, we found that a routine protocol for PFP preparation (3000xg for 10 min followed by 13,500xg for 2 min) yielded a 24% shorter PPL\(_{\text{CT}}\) than PFP prepared as recommended by ISTH. Hence, the plasma centrifugation protocol should be taken into consideration when comparing plasma PPL\(_{\text{CT}}\) between studies. As plasma depleted of PPL is an essential reagent in the modified assay, the importance of not introducing assay variations is critical. We demonstrated that three independent batches of PPL-depleted plasma prepared from the same donors performed similarly, supporting the use of ultracentrifugation as a method for PPL depletion.

An additional pre-analytical challenge for population-based studies is the availability of fasting blood samples as the PPL activity in plasma may be affected by plasma levels of triglyceride-rich lipoproteins. We therefore tested the PPL activity in plasma isolated before (0 h) and 4 h (4 h) after a high fat meal on the modified PPL assay. PPL\(_{\text{CT}}\) was unchanged in fasting and postprandial plasma, suggesting that PPL\(_{\text{CT}}\) is independent of postprandial lipemia and that there is no need to use fasting blood samples to obtain reliable PPL activity in plasma samples. Similar studies on the effect of lipemia should be repeated using more stringent pre-analytical conditions of plasma collection to see whether lipemia has a slight albeit detectable effect on the procoagulant properties of EVs. However, our findings are supported by Silveira et al. who reported no effect of postprandial lipemia on the overall PPL measures, using the STA-Procoag PPL assay\(^\text{20}\). Using the same assay, Mark and colleagues showed that even a non-standardized meal and a shorter time interval between the ingestion of the meal and blood draw (75 min) resulted in no change in PPL between fasting and postprandial samples\(^\text{31}\).

Procoagulant phospholipids, and PS in particular, affect the activity of both the intrinsic and extrinsic tenase and prothrombinase complexes, as well as the activation of FXI by thrombin\(^\text{1}\). The inter-individual variability may affect the degree of activation of several coagulation factors in the test samples. We therefore tested a wide range of FVIIa and FVa concentrations, where high concentrations of coagulation factors proved to shorten the clotting time in the modified PPL assay. However, these effects occurred only at supra-physiological concentrations significantly higher than those observed in vivo. Another aspect is the presence of TF. While only minute quantities are normally present in human plasma, TF is thought to be a major procoagulant factor found in EVs\(^\text{22}\). Previously, Connor and coworkers showed that increasing concentrations (0–0.1%) of TF added to whole blood were insensitive to the XACT assay\(^\text{1}\). Accordingly, we found that monocyte-derived EVs expressing TF after LPS stimulation (pathophysiological conditions) didn't affect the clotting time in our assay. However, we observed a dose-dependent decrease in the clotting times with increasing supra-physiological concentrations of relipidated TF. Taken together, our findings suggest that the modified PPL assay is not influenced by physiological concentrations of the clotting factors in the test samples.

Over the last decade the interest in assays measuring the negatively charged phospholipid fraction of plasma has increased along with the growing interest and knowledge about EVs. Elevated levels of EVs, most frequently measured by flow cytometry, were found in venous thromboembolism\(^\text{23–25}\), arterial cardiovascular diseases\(^\text{25,26}\), cancer\(^\text{27,28}\), atherosclerosis\(^\text{29,30}\), and diabetes\(^\text{31}\). In vascular disorders, the procoagulant properties of EVs are of particular interest. It has been shown that PS is the main phospholipid contributing to the procoagulant function\(^\text{32}\). In the presence of PS, the negatively charged phospholipid fraction of plasma has increased along with the growing interest and knowledge about EVs. Elevated levels of EVs, most frequently measured by flow cytometry, were found in venous thromboembolism\(^\text{23–25}\), arterial cardiovascular diseases\(^\text{25,26}\), cancer\(^\text{27,28}\), atherosclerosis\(^\text{29,30}\), and diabetes\(^\text{31}\). In vascular disorders, the procoagulant properties of EVs are of particular interest. It has been shown that PS is the main phospholipid contributing to the procoagulant function\(^\text{32}\). The abundance of PS on the EV surface is often used to characterize EVs, by exploiting the ability of annexin A5 or lactadherin to bind PS on the outer leaflet of the membrane. Lactadherin is a small glycoprotein that binds PS in a calcium-independent manner and with higher affinity than annexin A5\(^\text{33}\). It has been demonstrated that lactadherin is an effective anticoagulant blocking the activity of PS, and inhibits the procoagulant activity of blood cells, endothelial cells and extracellular vesicles by 80%\(^\text{34}\). Here we showed that PPL\(_{\text{CT}}\) was prolonged with increasing concentrations of lactadherin to the extent that it was no longer measurable in the assay, implying that...
the measured PPL activity is largely dependent on PS in the test samples. Similarly, using the XACT assay Aung et al. showed that pre-treatment of packed red blood cell supernatants by lactadherin prolonged clotting times13.

Any assay designed for large-scale applications should be reproducible over time, and it should be possible to compare the results between different laboratories. To solve the latter challenge we propose the introduction of the UPTT reagent—an inexpensive standardized preparation of rabbit brain cephalin, which allows for clotting times to be converted into a standardized unit of phospholipids. The XACT assay solves this problem by inclusion of a synthetic PPL calibrator, while the Stago assay leaves it up to the users to create a reference range and standards for the clotting time. Further, our modified PPL assay displayed minor variation in the assay performance. CVs obtained with either the standards from the Stago STA-Procoag-PPL or in-house pooled PFP range from 2.8 to 4.1%, well within recommended acceptable limits for within-day and between-day variability. Similar results were shown by van Dreden and colleagues in the XACT assay, with intra-assay CVs of 3.3% and 3.1% for normal pooled plasma and patient plasma, respectively, and inter-assay CVs of 3.9% and 4.2%10.

There are two main considerations with the modified PPL assay. First, the results will be influenced by the presence of lupus anticoagulants as well as high concentrations of coagulation factors which may lead to falsely prolonged or shortened clotting times. This is common for all plasma-based assays, and should be accounted for when interpreting the results. The creators of the XACT assay tried to overcome this issue by using plasma of porcine origin. However, while it significantly decreased the assay sensitivity to some of the lupus anticoagulants, it failed to completely eliminate the problem35. Second, pre-analytical conditions and inter-individual variations might impact plasma concentrations of coagulation factors in PPL depleted plasma. However, our modified assay seems to be unaffected by variations within the pathophysiological range.

In conclusion, the use of sequential centrifugation, including final ultracentrifugation, to deplete plasma of procoagulant phospholipids performed equal to enzymatic depletion of phospholipids from plasma in a FXa-based clotting assay to determine PPL clotting times. In addition, we introduced a standardized PPL reagent (UPTT) which allows for clotting times to be converted into a standardized unit of phospholipids. These modifications allowed us to establish an accessible and convenient in-house assay.

Materials and methods

Study subjects and sample preparations. For the calibration of the modified PPL assay, blood was drawn from healthy volunteers (n = 25) aged 25–79 years old, by venipuncture of an antecubital vein using a 21-gauge needle and minimal stasis. Blood was collected into 3 mL tubes containing 3.2% sodium citrate (0.109 M, 1:9 v/v) (Vacutte, Greiner Bio-One, Kremsmünster, Austria), 3 mL K2EDTA Vacutte tubes (Greiner Bio-One, Kremsmünster, Austria) for cell count or Fragmin (Sigma-Aldrich, St. Louis, Missouri, USA) for the Ca-ionophore experiments (preparation of EVs from PRP). The first 3 mL of blood were discarded. Blood was mixed with anticoagulant by gentle inversions of the tube. The samples were kept at room temperature (20–24 °C) and processed within 15 min of collection. Blood was centrifuged twice at 2500×g for 15 min to obtain platelet free plasma (PFP). Both individual PFP samples and pooled PFP (n = 11) were prepared. PFP samples were aliquoted and stored at − 80 °C until use.

Procoagulant phospholipid depleted plasma was prepared by sequentially centrifuging citrated blood (n = 18) using the following protocol: 2500×g for 15 min twice followed by 100,000×g for 60 min at 16 °C (Beckman Optima LE-80 K Ultracentrifuge, rotor SW40TI, Beckman Coulter, Indianapolis, Indiana, USA). Supernatants were pooled, aliquoted and stored at − 80 °C until further use.

Activated partial thromboplastin time (aPTT) and prothrombin time (PT-INR) were determined for the pooled PFP depleted plasma used in the modified assay. Both aPTT and PT-INR were measured on ACL TOP 750 CTS (Instrumentation Laboratory, Bedford, MA, USA), using the kits SynthASil (Instrumentation Laboratory, Bedford, MA, USA), and STA-SPA+ (Diagnostica Stago, Asnières sur Seine Cedex, France), respectively.

The reproducibility of three independent preparations of PFP depleted plasma from the same six donors were tested. Citrated PFP from six volunteers were centrifuged at 100,000×g for 60 min at 16 °C, pooled, aliquoted and frozen at − 80 °C. PPLCT was measured using the modified PPL assay. Clotting tests were perform in PFP depleted plasma alone, or with UPTT added to mimic normal CT (1:3200 U/ml UPTT) and short CT (1:100 U/ml UPTT).

In order to investigate the impact of postprandial lipemia, forty study participants donated blood for plasma and serum analysis in a previously described study11. Briefly, blood was drawn from an antecubital vein using a 19-gauge needle in a Vacutainer system with minimal stasis in the morning after 12 h fasting and then 2, 4, 6, and 8 h after a standardized high fat meal (1 g fat/kg body weight). Blood for plasma preparation was collected into 4.5-mL Vacutainers (Becton Dickinson, Meylan Cedex, France) containing 0.129 M sodium citrate (1:9 v/v). Serum was prepared by letting blood clot for 1 h in a glass tube at room temperature. Plasma and serum were centrifuged at 2000×g for 15 min at 22 °C, transferred into cryovials (Greiner Labortecnich, Nütringen, Germany) and stored at − 80 °C until further analysis. Blood samples collected before (fasting) and 4 h after the meal were selected for the present study. For analysis, samples were thawed, centrifuged 13,500×g for 2 min and measured on the modified PPL assay. Informed written consent was obtained from all participants, and the regional committee for medical and health research ethics (REC North) approved the study. The study was conducted in accordance with relevant guidelines and regulations.

Assay reagents. The UPTT reagent was purchased from BioData Corporation (Horsham, Pennsylvania, USA). Bovine FXa (bFXa), FVIIa, bovine FV/Va (bFV/Va) were purchased from Enzyme Research Laboratory (South Bend, Indiana, USA). Human recombinant tissue factor was purchased from Sekisui Diagnostics, LLC (Stamford, Connecticut, USA) and lactadherin from Haematologic Technologies Inc. (Essex Junction, Vermont, USA). The STA-Procoag-PPL assay was purchased from Diagnostica Stago Inc. (Asnières sur Seine Cedex, France). All other chemicals were from Sigma-Aldrich (St. Louis, Missouri, USA).
Test procedure for the modified PPL assay. Clotting tests were carried out in duplicate by using a Start Max instrument from Diagnostica Stago (Asnières sur Seine Cedex, France). Twenty five µl of test plasma or EV suspension was mixed with 25 µl of PPL depleted plasma in a Start-cuvette containing a steel ball, and pre-warmed for 2 min to 37 °C. The reaction was initiated by the addition of 100 µl of pre-warmed assay buffer with a cabled pipette that automatically starts the timer upon pipetting, and clotting time was measured. The assay buffer contains bFXa (0.1 U/ml) in 15 mM calcium chloride, 100 mM sodium chloride and 20 mM HEPES buffer (pH 7.0). The STA-Procoag-PPL assay (Asnières sur Seine Cedex, France) was performed according to the manufacturer's protocol.

Assay calibration. Citrated blood samples from six individuals were subjected to sequential centrifugation in order to remove an increasing amount of EVs. The centrifugation protocol was as follows: 2500×g for 15 min, 2500×g for 15 min twice, 20,000×g for 30 min and 100,000×g for 60 min. Clotting time was measured after each centrifugation step.

Concentration of bFXa for the modified assay was determined by serially diluting bFXa from 0.01 to 2 U/ml. Clotting time was measured using the modified PPL assay. Pooled PFP added to PPL depleted plasma or PPL depleted plasma alone were used as test plasma.

Standardized UPTT reagent containing 0.1% of rabbit brain cephalin in a buffered solution was used as a calibrator. The UPTT reagent was reconstituted in milliQ water according to the manufacturer's protocol, and set at 1 U/ml. To obtain a standard curve, serial dilutions of the UPTT reagent (1/50 to 1/12,800) in the assay buffer were added to phospholipid-depleted plasma. UPTT dilution curves were measured for both the modified PPL assay and STA-Procoag-PPL assay. The effect of PFP preparation was tested using two different centrifugation protocols. Citrated blood samples from ten volunteers were split in two, one fractions was centrifuged 2500×g for 15 min twice (75,000 minutes) and the second fraction 3000×g for 10 min, followed by 13,500×g for 2 min (57,000 minutes). Clotting time was measured using the modified PPL assay.

The effect of coagulation factor V, VII, and blocking surface PS on PPL activity. The effect of varying levels of coagulation factors Va and VIIa were tested on the modified PPL assay. The coagulation factors were added to the reaction buffer in concentrations from 0.02 to 3.0 nM for FVa, and from 5 to 80 nM for FVIIa, and tested in pooled PFP. The effect of lactadherin pretreatment of PRP-derived EVs on PPL activity was tested on the modified PPL assay. Plasma EVs were isolated from pooled citrated PFP, diluted with PBS (without Ca²⁺ and Mg²⁺) 1:3 v/v and centrifuged at 20,000×g for 30 min at 22 °C. The supernatant was discarded and the EV-pellet was resuspended in phospholipid-depleted plasma. PRP-derived EVs were prepared by incubation of PRP (anticoagulated with Fragmin) with 10 μM of calcium ionophore A23187 for 15 min at 37 °C, followed by the isolation procedure described above. The concentrated EV suspension was serially diluted with PPL depleted plasma and assayed within 1 h.

The effect of TF and TF + EVs on PPL activity. Recombinant relipidated TF was added in the reaction buffer to pooled PFP in concentrations from 0.01 to 1.38 pM and clotting time was measured on the modified PPL assay.

Whole blood was stimulated with a combination of LPS (5 ng/ml, Dako, strain 026:B6, Diło Lab., Detroit, MI, USA) and PMA (30 ng/ml, Sigma-Aldrich, Oslo, Norway) at 37 °C for 4 h with gentle agitation. EVs were isolated as described above. TF activity was blocked with an inhibitory antibody at a final concentration of 26 µg/ml (Purified Mouse Anti-Human CD142, Clone HTF-1, Catalog No.550252, BD Biosciences, Pharmingen, NJ). Clotting time was measured on the modified PPL assay.

Serum lipid analysis. Serum triglycerides were measured by the use of an enzymatic photometric method on the ABX Pentra 400 instrument (Horiba ABX Diagnostics, Montpellier, France).

Statistical analysis. Statistical analysis were performed in Graph Pad Prism 9.0.0 (GraphPad Software, Inc. La Jolla, CA, USA). ANOVA was used to test for differences in performance between the modified PPL assay and the STA-Procoag-PPL assay.


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Author contributions
C.R. planned and performed experiments, analyzed data, wrote and revised the manuscript. S.J. analyzed data and revised the manuscript. N.L. and L.W. planned and performed experiments, analyzed data, participated in writing and revising the manuscript. T.S. analyzed data, participated in writing and revising the manuscript. O.S. analyzed data, participated in writing and revising the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.
Additional information

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A modified clot-based assay to measure negatively charged procoagulant phospholipids

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Supplementary Figures

Supplementary Figure S1. The batch-to-batch reproducibility of EVDP. Citrated blood samples from six volunteers were centrifuged twice at 2500xg for 15 minutes, then 100,000xg for 1 hour to prepare EVDP. EVDP samples were pooled and stored at -80°C until analysis. Three separate batches were prepared and the clotting times (CT) were measured using the modified PPL assay for EVDP alone, and with the addition of UPPT concentrations of 1:3200 (normal range CT) and 1:100 (short range CT). Values are mean of duplicate measurements ± 1 SD.
Supplementary Figure S2. Bovine FXa dilution curve. Clotting times were measured from serial dilutions of bFXa added to EVDP alone, or in combination with pooled platelet free plasma (PFP). Clotting times for EVDP alone using 0.01 U/ml bFXa exceeded the range of the instrument (300 s) and artificial values of 301 seconds were plotted. Values are mean of three experiments ± 1 SD.
Paper III
Plasma Procoagulant Phospholipid Clotting Time is Inversely Associated with Future Risk of Incident Venous Thromboembolism

PPL clotting time is associated with risk of VTE

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ESSENTIALS

- It is not known whether plasma levels of procoagulant phospholipids are associated with VTE risk
- The association between PPL and risk of VTE was investigated in a nested case-control study
- PPL clotting time above the 95th percentile was associated with lower risk of future VTE
- The association was stronger when analyses were restricted to samples taken shortly before the VTE
ABSTRACT:

**Background:** Negatively charged procoagulant phospholipids, phosphatidylserine (PS) in particular, are vital to coagulation and expressed on the surface membrane of extracellular vesicles. No previous study has investigated the association between plasma procoagulant phospholipid clotting time (PPL_CT) and future risk of venous thromboembolism (VTE).

**Objectives:** To investigate the association between plasma PPL_CT and the risk of incident VTE in a nested case-control study.

**Methods:** We conducted a nested case-control study in 296 VTE patients and 674 age- and sex-matched controls derived from a general population cohort (The Tromsø Study 1994-2007). PPL_CT was measured in platelet free plasma using a modified factor Xa-dependent clotting assay. Logistic regression was used to estimate odds ratio (OR) with 95% confidence intervals (CI) for VTE with PPL_CT modelled as a continuous variable, across quartiles and in dichotomized analyses.

**Results:** There was a weak inverse association between plasma PPL_CT and risk of VTE per one standard deviation increase of PPL_CT (OR 0.93, 95% CI 0.80-1.07) and when comparing those with PPL_CT in the highest quartile (OR 0.89, 95% CI 0.60-1.30) with those in the lowest quartile. Subjects with PPL_CT > 95th percentile had substantially lowered OR for VTE (OR 0.35, 95% CI 0.13-0.81). The inverse association was stronger when the analyses were restricted to samples taken shortly before the event. The risk estimates by categories of plasma PPL_CT were similar for deep vein thrombosis and pulmonary embolism.

**Conclusion:** Our findings suggest that high plasma PPL_CT is associated with reduced risk of VTE.
KEY WORDS

biological assay, extracellular vesicles, phosphatidylserines, phospholipids, venous thromboembolism
INTRODUCTION

Venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common disease with an annual incidence of 1-2 per 1000 individuals [1, 2]. VTE is associated with severe short- and long-term complications, such as recurrent events [3], post-thrombotic syndrome (PTS) [4], post-PE syndrome [5] and death [6]. The incidence of VTE has been stable [7] or slightly increased during the last two decades [8, 9], and VTE has become a major economic burden and challenge to health care systems [10, 11]. Therefore, there is a great need to identify novel biomarkers to improve risk-stratification and unravel disease mechanisms to tailor preventive and treatment strategies with the long-term goal to lower the incidence of the disease.

Phosphatidyl serine (PS) is a negatively charged phospholipid with procoagulant potential expressed at the surface of activated platelets and extracellular vesicles (EVs) [12]. The presence of PS on the membrane surface facilitates the assembly of coagulation factors VII (FVII), FIX, FX and prothrombin (FII) [13], and accelerates the activity of the TF:FVIIa complex by several orders of magnitude [14]. Furthermore, a strong relationship between plasma levels of PS-positive EVs and procoagulant phospholipid (PPL) activity has been reported [15]. Hence, plasma PPL clotting time (PPL_CT) may be considered as a measure for the potential to facilitate coagulation activation when blood is exposed to triggers of the coagulation system.

As most [16-19], but not all [20] observational studies have reported elevated plasma levels of EVs in VTE, we hypothesized that prolonged PPL_CT was associated with lowered risk of VTE. We therefore aimed to investigate the association between plasma PPL_CT, measured by
a modified factor Xa-dependent clotting assay, and the risk of incident VTE in a nested case-control study derived from the general population.

MATERIAL AND METHODS

Study population

The Tromsø Study is a large prospective single-center population-based cohort study with repeated health surveys of inhabitants of Tromsø, Norway [21]. The study participants were recruited from the fourth survey (1994-95) of the Tromsø Study. All inhabitants aged 25 years and older living in the municipality of Tromsø were invited to participate, and 77% of those invited participated (n=27,158). The participants were followed from the date of inclusion until an adjudicated incident VTE event, migration, death, or end of follow-up (September 1, 2007). All first lifetime events of VTE occurring among the participants in this period were identified using the hospital discharge diagnosis registry, the autopsy registry, and the radiology procedure registry from 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 adjudicated and recorded each VTE by extensively reviewing medical records. The identification and adjudication process of VTEs has previously been described in detail [22]. In short, the adjudication criteria for VTE were presence of signs and symptoms of DVT or PE combined with objective confirmation by radiological procedures, which resulted in initiation of treatment (unless contraindications were specified). A VTE occurring in the presence of one or more provoking factors was classified as provoked. Provoking factors were: surgery or trauma (within eight weeks before the event), acute medical condition (acute myocardial infarction, acute ischemic stroke, acute infections), immobilization (bed rest > 3 days or confinement to wheelchair within the last 8 weeks, or long distance travel ≥4 hours
within the last 14 days), or other factors specifically described as provoking by a physician in the medical record (e.g. intravascular catheter).

There were 462 individuals who experienced a VTE event during the follow-up period (1994-2007). For each case, two age- and sex-matched controls, who were alive at the index date of the corresponding VTE-case, were randomly sampled from the source cohort (n=924). In total, 349 (140 cases and 209 controls) lacked plasma samples and 67 (26 VTE cases and 41 controls) had plasma samples of insufficient quality (e.g. hemolysis). Hence, our study population consisted of 296 subjects with incident VTE and 674 age- and sex-matched controls. The regional committee for medical and health research ethics approved the study, and all participants provided informed written consent.

**Baseline measurements**

At inclusion in Tromsø 4 (1994/95), baseline information was collected by physical examinations, blood samples and self-administered questionnaires. All participants had their height (to the nearest cm) and weight (to the nearest 0.5 kg) measured, wearing light clothing and no shoes. Body mass index (BMI) was calculated using weight in kilograms divided by height in meters squared (kg/m²). Information on previous cardiovascular disease and cancer was collected from the self-administered questionnaires.

**Handling of blood samples**

Non-fasting blood samples were collected by venipuncture of an antecubital vein, with minimal stasis, into blood collection tubes containing ethylenediaminetetraacetic acid (K₃-
EDTA 40 µL, 0.37 mol/L per tube) (Becton Dickinson, Meylan Cedex, France). Cell counts were performed using a Coulter Counter (Coulter Electronics, Luton, UK). Platelet poor plasma (PPP) was prepared by centrifugation at 3000 x g for 10 minutes at room temperature. Plasma aliquots were transferred to 1 mL cryovials (Greiner Laboratechnik, Nürtingen, Germany) and stored at -80°C until analysis.

**Measurement of procoagulant phospholipid clotting time (PPL-CT) in plasma**

PPL clotting time was measured in platelet free (PFP) EDTA plasma using a modified factor Xa-dependent clotting assay. In short, PPP samples were thawed and centrifuged at 13,500 x g for 2 minutes to generate PFP. Phospholipid-depleted plasma (PPLDP) used as a reagent in the assay was prepared from pooled citrated PFP (n = 18) centrifuged at 100,000 x g for 60 minutes at 16°C (Beckman Optima LE-80K Ultracentrifuge, rotor SW40TI, Beckman Coulter, Indianapolis, Indiana, USA). PPLDP was aliquoted and stored at -80°C until use. Twenty-five μl of sample PFP was mixed with 25 μl of PPLDP, and incubated for 2 minutes at 37°C, before the reaction was initiated by the addition of 100 μl pre-warmed factor Xa reagent containing bovine Factor Xa (0.1 U/ml) in 15 mM calcium chloride, 100 mM sodium chloride and 20 mM HEPES buffer (pH 7.0). Clotting tests were carried out in duplicate on a StarT4 instrument from Diagnostica Stago (Asnières sur Seine Cedex, France) and measured in seconds of clotting time. The variation between runs were adjusted for by an internal standard. The technician carrying out the measurements was blinded to the identity and case-control status of the samples. The PPL assay displayed low intra- and inter-series coefficients of variations (CV) ranging from 2.8% to 4.1%.
Measurements of thrombin generation in plasma

Thrombin generation was assessed using a calibrated automated thrombinoscope, and was performed as described by Hemker et al [23] and manufacturer’s instructions (Thrombinoscope BV, Maastricht, the Netherlands). Thrombin generation was measured in a Fluoroscan Ascent Fluorometer (Thermolabsystems OY, Vantaa, Finland) equipped with a dispenser. Fluorescence intensity was detected at wavelengths of 355 nm (excitation filter) and 460 nm (emission filter). Briefly, 40 µl of plasma was mixed with 40 µl Hepes buffer (20 mM Hepes and 140 mM NaCl) and pipetted into the wells of round bottom 96-well microtiter plates (Immulon, Lab Consult, Lillestrøm, Norway). Ten µl of TF solution (final concentration of 3 pM) (Innovin, Bade Behring) and 10 µl of a standardized phospholipid in solution (diluted 1:20) (UPTT, BioData Corporation, Horsham, Pennsylvania, USA.) was added as triggers. Both TF and UPTT were diluted to the stated concentrations in Hepes buffer. The plasma samples measured were a combination of pooled citrated PFP and PPLDP added in ratios of 100:0, 80:20, 60:40, 40:60, 20:80, 10:90, and 0:100, respectively. For each experiment, a fresh mixture of 2.5 mM fluorogenic substrate (Z-Gly-Gly-Arg-AMC from Bachem, Bubendorf, Switzerland), 0.1 M CaCl₂, 20 mM Hepes (Sigma Aldrich, St Louis, USA) and 60 mg/ml BSA (A-7030, Sigma Aldrich) with pH 7.35 was prepared. Each dilution of PFP/PPLDP was assigned its own calibrator (Thrombinoscope BV, Maastricht, The Netherlands). The computer software calculated lag time (LG) (min), the time to peak (TTP) (min), the peak of thrombin generation (PEK) (nM) and the area under the thrombin generation curve (nM*min) and endogenous thrombin potential (ETP). Plasma samples were run in duplicate and each experiment was repeated three times.
Statistical analysis

Statistical analyses were performed using R (Version 4.0.4. for Windows; R Foundation). Unconditional logistic regression models were used to estimate odds ratios (ORs) for VTE with 95% confidence intervals (CIs) with plasma PPL\textsubscript{CT} used as a continuous variable, discretized to quartiles and dichotomized according to PPL\textsubscript{CT} ≤ 25th percentile versus PPL\textsubscript{CT} > 95th percentile. The analyses were adjusted for age, sex and BMI. The PPL\textsubscript{CT} quartile cut offs were determined using the control group.

As the follow-up time in the source cohort was long (more than 12 years for many persons), the results based on baseline PPL\textsubscript{CT} measurements could be influenced by regression dilution bias [24]. To investigate this, we performed analyses where we restricted the maximum time from blood sampling in Tromsø 4 to the VTE events, while keeping all controls in the analyses. The logistic regression analyses on time restrictions were set to require at least 10 VTE events, and ORs were generated at every 0.1 year increase and plotted as a function of time from blood sampling to VTE.

RESULTS

The distribution of characteristics of the study population at baseline across quartiles of plasma PPL\textsubscript{CT} is presented in Table 1. The mean age and BMI were similar, while the percentage of men (42.9% in Q1 to 47.8% in Q4) and the proportion of individuals with self-reported CVD (13.5% in Q1 to 18.8% in Q4) increased across quartiles. In contrast, the proportion of individuals with self-reported cancer decreased (7.1% in Q1 to 2.9% in Q4) across quartiles of PPL\textsubscript{CT}. 
Characteristics of patients at VTE diagnosis are shown in Table 2. The mean age at the time of VTE was 68 years, 47% were men, and 59% of the events were deep vein thrombosis. The majority of the VTE events were provoked (60.1%), and the leading provoking factors were active cancer (27.7%), surgery or trauma (21.3%), and immobilization (18.2%).

The ORs of VTE across categories (quartiles and >95th percentile) and per one standard deviation (i.e. 14.5 s) increase in plasma PPL_{CT} are shown in Table 3. There was a weak inverse association between plasma PPL_{CT} and risk of VTE per one standard deviation increase of PPL_{CT} (OR 0.93, 95% CI 0.80-1.07), and in subjects with PPL_{CT} in the highest quartile (OR 0.89, 95% CI 0.60-1.30) compared to those in the lowest quartile, in analyses adjusted for age, sex, and BMI. However, subjects with particularly prolonged PPL_{CT} (>95th percentile) had lower OR for VTE (OR 0.35, 95% CI 0.13-0.81) than those with PPL_{CT} ≤25th percentile in analyses adjusted for age, sex, and BMI. Similar results were found for DVT and PE (Table 3), but the OR for PE (OR 0.14, 95% CI 0.01-0.69) was lower than for DVT (OR 0.50, 95% CI 0.17-1.25) in analyses comparing individuals with PPL_{CT} >95th percentile versus ≤25th percentile. The ORs for unprovoked and provoked VTE, DVT and PE, were similar to those found in the overall analyses (data not shown).

To consider the possibility of underestimating ORs due to regression dilution bias, we estimated ORs for VTE among subjects with the highest (highest quartile) versus lowest (lowest quartile) plasma PPL_{CT} as a function of time between blood sampling and the VTE events (Figure 1). The inverse association between high plasma PPL_{CT} and VTE was stronger with shortened time between the blood sampling and the VTE events. The ORs for DVT and
PE as a function of time between blood sampling and events showed similar patterns as the ORs for overall VTE (data not shown).

To study the effect of plasma procoagulant phospholipids on thrombin generation, mixtures of pooled PFP and PPLDP were analyzed using the CAT assay. As shown in Figure 2A, thrombin generation declined in a dose-dependent manner with declining percentage of PFP added (i.e. declining levels of PPL). A clear dose-response relationship was observed between plasma PPL levels and parameters of the CAT assay (i.e. lag-time and ETP) (Figures 2B and C). The PPL\textsubscript{CT} correlated strongly with both lag time (r= 0.99, p= <0.0001, Figure 2D) and ETP (r= -0.98, p= <0.0001, Figure 2E).

**DISCUSSION**

We investigated the association between plasma PPL\textsubscript{CT} and future risk of VTE in a population based nested case-control study. Prolonged PPL\textsubscript{CT} displayed a modest protective effect on VTE risk both when PPL\textsubscript{CT} were used as a continuous and as a categorized variable in the logistic regression models. However, subjects with extremely prolonged PPL\textsubscript{CT} (above the 95\textsuperscript{th} percentile) had lowered risk of VTE (OR 0.35, CI 95% 0.13-0.81) compared to those with PPL\textsubscript{CT} in the lowest quartile. Similar results were observed in subgroup analysis for PE and DVT. The results appeared to be influenced by regression dilution bias, as the ORs for VTE by plasma PPL\textsubscript{CT} decreased substantially with shortened time between blood collection and the VTE events. Our findings support the hypothesis of an inverse association between plasma PPL\textsubscript{CT} and VTE risk.
Our study is, to the best of our knowledge, the first to investigate the association between plasma PPL_CT and future risk of VTE in the general population. In a recent cross-sectional study including 100 patients referred to the Emergency Department under suspicion of VTE, plasma PPL_CT, assessed by the STA Procoag PPL assay (Diagnostica Stago), did not discriminate between patients with (n=31) and without VTE [25]. The lack of discriminatory diagnostic power by the PPL assay may have been diluted by other conditions associated with shortened PPL_CT among patients without VTE. However, this does not exclude the potential association between plasma PPL_CT and future risk of VTE. Further, plasma levels of modifiable biomarkers, such as PPL_CT, are expected to change over time. Fluctuations in the exposure variable during follow-up will lead to the phenomenon called regression dilution bias [24], which usually results in an underestimation of the true association between exposure and outcome. Accordingly, we found that the risk estimates for VTE declined substantially with shortened time between measurement of plasma PPL_CT and the VTE events.

Circumstantial evidence support an association between plasma PPL_CT and the risk of future VTE. First, the PPL_CT is inversely associated with annexin V-positive EVs [15, 26] and high plasma levels of EVs are associated with VTE risk in most [16-19], but not all studies [20, 27]. Second, in a cross-sectional study including plasma samples from 100 healthy individuals and patients with obstructive sleep apnea, plasma PPL_CT showed strong and inverse correlations to parameters of thrombin generation, such as ETP and peak thrombin concentration, using the CAT assay with the addition of minimal amounts of phospholipids and tissue factor (1 pM) to trigger thrombin generation [26]. Accordingly, we demonstrated a clear dose-response relationship between plasma PPL_CT and parameters of the CAT assay. In addition, several studies have shown that parameters of the CAT assay, particularly lag-time
and ETP, are associated with incident [28-32] and recurrent [33-35] VTE. Third, carriers of rare (e.g. deficiencies of antithrombin, protein C and S) [36] and common (e.g. factor V Leiden and the prothrombin mutation G20210A) [37, 38] prothrombotic genotypes had significantly shorter plasma PPL<sub>CT</sub> than non-carriers, providing indirect evidence for lower risk of VTE with prolonged plasma PPL<sub>CT</sub>.

Strengths of our study include recruitment of VTE patients from a population-based cohort with age- and sex-matched controls from the same source population where blood samples were collected prior to the VTE event. This allows assumptions on the direction of the observed association between plasma PPL<sub>CT</sub> and VTE. Further, the modified FXa-dependent PPL clotting assay is highly sensitive and displayed a low CV of ≤4%. A limitation with our study is that plasma samples used were collected in 1994/95 and stored at -80°C until analysis >20 years later. The long storage time, as well as freezing and thawing, might possibly affect the plasma PPL<sub>CT</sub>. However, it is unlikely that it would impact our end results, as the potential effects would be similar for both cases and controls. Moreover, the PPL levels were only measured in baseline samples, while potential changes during follow-up were not accounted for. This might lead to an underestimation of the true association between plasma levels of PPL<sub>CT</sub> and VTE risk due to regression dilution bias [24]. In our study, some plasma samples were excluded due to either missing samples or poor plasma quality. The plasma samples were missing completely at random, hence there was no selection bias.

In conclusion, results from our nested case-control study indicate an inverse association between plasma PPL<sub>CT</sub> (measured by a modified FXa-dependent PPL clotting assay) and the risk of future VTE. Subjects with PPL<sub>CT</sub> above the 95<sup>th</sup> percentile had particularly low risk of future VTE and the results were strongly influenced by regression dilution bias. Further
studies are needed to validate our findings and unravel the mechanisms behind this observation.

**Addendum**

C. Ramberg planned experiments, analyzed data, wrote and revised the manuscript. L. Wilsgård and N. Latysheva planned and performed experiments and revised the manuscript. S.K. Brækkan analyzed data and revised the manuscript. K. Hindberg performed statistical analysis and revised the manuscript. O. Snir planned experiments, analyzed data and revised the manuscript. T. Sovershaev revised the manuscript. J-B. Hansen conceived and designed the study, analyzed data, participated in writing, and revision of the manuscript.

**Conflict of interest**

The authors state that they have no conflict of interests.

**Acknowledgements**

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References


### Table 1: Baseline characteristics of the study population (n=970) across quartiles of PPL clotting time.

<table>
<thead>
<tr>
<th>Clotting time (seconds)</th>
<th>Q1 (26.5-51.7)</th>
<th>Q2 (51.7-60.0)</th>
<th>Q3 (60.0-71.5)</th>
<th>Q4 (71.5-148.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>252</td>
<td>243</td>
<td>230</td>
<td>245</td>
</tr>
<tr>
<td>Age, years</td>
<td>59 ± 14</td>
<td>61 ± 13</td>
<td>61 ± 14</td>
<td>61 ± 14</td>
</tr>
<tr>
<td>Sex, % men (n)</td>
<td>42.9 (108)</td>
<td>47.7 (116)</td>
<td>46.5 (107)</td>
<td>47.8 (117)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.1 ± 4.5</td>
<td>26.6 ± 4.0</td>
<td>26.6 ± 4.4</td>
<td>26.5 ± 4.1</td>
</tr>
<tr>
<td>CVD*, % (n)</td>
<td>13.5 (34)</td>
<td>16.0 (39)</td>
<td>15.2 (35)</td>
<td>18.8 (46)</td>
</tr>
<tr>
<td>Cancer*, % (n)</td>
<td>7.1 (18)</td>
<td>6.2 (15)</td>
<td>5.2 (12)</td>
<td>2.9 (7)</td>
</tr>
<tr>
<td>WBC, 10⁹/L</td>
<td>7.2 ± 1.9</td>
<td>7.1 ± 3.3</td>
<td>6.8 ± 1.7</td>
<td>6.8 ± 1.9</td>
</tr>
<tr>
<td>Platelet Count, 10⁹/L</td>
<td>259 ± 57</td>
<td>247 ± 53</td>
<td>240 ± 47</td>
<td>230 ± 52</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>41.5 ± 3.3</td>
<td>41.7 ± 3.6</td>
<td>41.4 ± 3.0</td>
<td>41.5 ± 3.4</td>
</tr>
</tbody>
</table>


*Self-reported history of disease.

Values are mean ± 1 standard deviation or percentage with absolute numbers in parenthesis.
**Table 2:** Characteristics of the VTE patients (n=296).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>%</th>
<th>Absolute Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at VTE, years</td>
<td>68 ± 14</td>
<td></td>
</tr>
<tr>
<td>Sex, % men</td>
<td>47.0 (139)</td>
<td></td>
</tr>
<tr>
<td>Deep vein thrombosis</td>
<td>58.8 (174)</td>
<td></td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>41.2 (122)</td>
<td></td>
</tr>
<tr>
<td>Unprovoked VTE</td>
<td>39.9 (118)</td>
<td></td>
</tr>
<tr>
<td>Provoked VTE</td>
<td>60.1 (178)</td>
<td></td>
</tr>
<tr>
<td>Active cancer</td>
<td>27.7 (82)</td>
<td></td>
</tr>
<tr>
<td>Surgery/ Trauma</td>
<td>21.3 (63)</td>
<td></td>
</tr>
<tr>
<td>Immobilization</td>
<td>18.2 (54)</td>
<td></td>
</tr>
<tr>
<td>Acute medical condition</td>
<td>15.9 (47)</td>
<td></td>
</tr>
<tr>
<td>Other factors</td>
<td>4.1 (12)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± 1 standard deviation or percentage with absolute numbers in parenthesis.
Table 3. Odds ratios (OR) with 95% confidence intervals (CI) for venous thromboembolism (VTE), deep vein thrombosis and pulmonary embolism, per standard deviation (SD) increase and across increasing quartiles of PPL clotting time (seconds).

<table>
<thead>
<tr>
<th>PPL clotting time (sec)</th>
<th>Cases</th>
<th>Controls</th>
<th>Model 1 (95% CI)</th>
<th>Model 2 OR (95% CI)</th>
<th>Model 3 OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venous thromboembolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per SD</td>
<td>296</td>
<td>674</td>
<td>0.94 (0.81-1.08)</td>
<td>0.94 (0.81-1.08)</td>
<td>0.93 (0.80-1.07)</td>
</tr>
<tr>
<td>Q1 (26.5-54.3)</td>
<td>83</td>
<td>169</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Q2 (54.3-63.5)</td>
<td>74</td>
<td>169</td>
<td>0.89 (0.61-1.30)</td>
<td>0.89 (0.61-1.30)</td>
<td>0.87 (0.59-1.27)</td>
</tr>
<tr>
<td>Q3 (63.5-74.5)</td>
<td>63</td>
<td>167</td>
<td>0.77 (0.52-1.13)</td>
<td>0.77 (0.52-1.13)</td>
<td>0.75 (0.51-1.12)</td>
</tr>
<tr>
<td>Q4 (74.5-148.5)</td>
<td>76</td>
<td>169</td>
<td>0.92 (0.63-1.34)</td>
<td>0.92 (0.63-1.34)</td>
<td>0.89 (0.60-1.30)</td>
</tr>
<tr>
<td>≤ 25% (26.5-54.3)</td>
<td>83</td>
<td>169</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>&gt;95% (89.9-148.5)</td>
<td>6</td>
<td>34</td>
<td>0.36 (0.13-0.83)</td>
<td>0.36 (0.13-0.83)</td>
<td>0.35 (0.13-0.81)</td>
</tr>
<tr>
<td>Deep vein thrombosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per 1 SD increase</td>
<td>174</td>
<td>674</td>
<td>0.98 (0.83-1.16)</td>
<td>0.98 (0.83-1.17)</td>
<td>0.97 (0.82-1.15)</td>
</tr>
<tr>
<td>Q1 (26.5-54.3)</td>
<td>50</td>
<td>169</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
<td>1.00 (ref)</td>
</tr>
<tr>
<td>Q2 (54.3-63.5)</td>
<td>32</td>
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Model 1: Crude analysis

Model 2: adjusted for age and sex

Model 3: adjusted for age, sex and body mass index

1 standard deviation (SD) of PPL<sub>CT</sub> = 14.5 s
Figure 1. Estimated odds ratios (OR) for VTE for having PPL\textsubscript{CT} in quartile 4 compared to quartile 1. Each circle represents an analysis for a given maximum time between blood draw and VTE. At each time restriction, only VTE cases with a time below this maximum were included in the analysis, while all controls were included. The analysis are adjusted for age, sex and BMI.
Figure 2. Pooled platelet free plasma (PFP) was mixed in varying ratios (100%, 80%, 60%, 40%, 20%, 10% and 0% PFP) with phospholipid depleted plasma (PPLDP) and run on the CAT assay. Thrombin generation curves are shown in 2A. A dose dependent relationship was observed for both lagtime (2B) and ETP (2C) and % PFP added to the reaction. PPL clotting time (PPLCT) correlate strongly with both lagtime (2D) and ETP (2E).
Paper IV
Rosuvastatin treatment decreases plasma procoagulant phospholipid activity after a VTE: A randomized controlled trial

Rosuvastatin decreases PPL activity after VTE

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Number of figures: 5
References: 53
KEY POINTS

- Rosuvastatin treatment caused a 22% decrease in plasma PPL activity in participants with a previous history of VTE.
- The effect was not explained by changes in total cholesterol nor changes in plasma levels of total or platelet derived EVs.
ABSTRACT

Venous thromboembolism (VTE) is a frequent cardiovascular disease with severe complications, including recurrence and death. There is a great need for alternative prophylactic treatment options as anticoagulation is accompanied by increased bleeding risk. Statins are reported to reduce the risk of incident and recurrent VTE, but the mechanisms are elusive. Procoagulant phospholipids (PPL), and phosphatidylserine in particular, are crucial for efficient coagulation activation, but no study have investigate the effect of statin treatment on plasma PPL activity. We aimed to investigate the impact of rosuvastatin treatment on plasma PPL activity and levels of extracellular vesicles (EVs) in subjects with a history of VTE. Participants of the STA tín s Reduce Thrombophilia (START) (NCT01613794) trial were randomized to either 20 mg/day of rosuvastatin treatment or no treatment for 28-days. Plasma sample were collected at baseline and study end. The PPL activity was measured in samples from 245 participants using a FXa-dependent assay and plasma EV levels by a sensitive flow cytometer. Rosuvastatin treatment yielded an overall 22% (95% CI -38.2 to -5.8) reduction in PPL activity, and 37% (95% CI -62.9 to -11.2) reduction in PPL activity in participants with a history of pulmonary embolism. The effect of rosuvastatin on plasma PPL activity was not explained by changes in total cholesterol nor change in plasma levels of total- or platelet-derived EVs. Rosuvastatin treatment caused a substantial decrease in plasma PPL activity, suggesting that a PPL-dependent attenuation of coagulation activation may contribute to a reduced VTE risk following statin treatment.
INTRODUCTION

Venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), is a frequent cardiovascular disease with severe short- and long-term complications, including recurrence and death. At present, anticoagulation is the treatment of choice for primary and secondary prophylaxis of VTE. Although highly efficient, anticoagulation is accompanied by increased bleeding risk, where 1-4% annually experience major bleeding events, depending on type of anticoagulant, dose and duration of treatment.

As VTE recurs in up to 30-40% of patients within 10 years of the initial event, there is a need for alternative prophylactic treatment options in patients with high bleeding risk.

HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase inhibitors, known as statins, are a class of cholesterol-lowering drugs with cardioprotective effects independent of LDL cholesterol lowering, including protection against endothelial dysfunction, anti-inflammatory- and antithrombotic effects. Observational and randomized studies have reported a 14-54% reduction in the risk of a first VTE and a 27-50% reduction in recurrent events following statin treatment. However, there is only limited knowledge on the pleiotropic effects of statins that may explain their beneficial effects on the risk of VTE. The STAtins Reduce Thrombophilia (START) trial was established to address this knowledge gap. Previous results from the START trial have shown that rosuvastatin treatment modestly reduced the plasma level of several coagulation factors, in particular factor VIII, lowered the tissue factor (TF)-induced potential for thrombin generation in plasma by 10%, and increased the fibrinolytic potential in plasma. However, the beneficial effect of statins on VTE could not be explained by reduced platelet reactivity, measured by thromboxane-A2 mediated platelet aggregation.

Negatively charged phospholipids, and phosphatidylserine (PS) in particular, are vital to coagulation. PS is located on the surface of activated platelets as well as on extracellular vesicles (EVs), and they facilitate the assembly of coagulation factors VII (FVII), FIX, FX and prothrombin (FII) in blood. The presence of negatively charged phospholipids augment the activity of the extrinsic tenase complex, TF-FVIIa, by several orders of magnitude. The procoagulant phospholipid (PPL) activity of plasma samples can be measured using a factor Xa (FXa)-dependent clotting assay. Previously, an inverse correlation has been established...
between PPL, measured by FXa-dependant clotting assays and plasma levels of PS-positive (PS+) extracellular vesicles (EVs)\textsuperscript{28,29}. However, the effect of statin treatment on plasma PPL activity has previously not been investigated. In the present study, we aimed to (i) investigate the impact of rosvastatin treatment on plasma PPL activity in individuals with a history of VTE in a randomized controlled trial, and (ii) explore the effect of statin treatment on total- and platelet-derived EV counts using a sensitive flow cytometer.

**MATERIAL AND METHODS**

**Trial design**

STAtins Reduce Thrombophilia, or the START trial (NCT01613794), is a multicenter, randomized, controlled, open label clinical trial aimed to investigate the impact of rosvastatin treatment on the coagulation profile of individuals with a previous history of VTE. The study has been described in detail elsewhere\textsuperscript{22}. In brief, participants were recruited from three Dutch anticoagulation clinics (Leiden, Hoofddorp, and Rotterdam) which monitor anticoagulant treatment of VTE patients within a geographical area. Subjects with confirmed initial or recurrent symptomatic proximal DVT or PE allowed to stop oral anticoagulation treatment by their treating physician and aged 18 years or older, were invited to participate. Exclusion criteria were as following: individuals already using statins or other lipid lowering drugs, or if contraindications for 20 mg/day rosvastatin use were present, based on information provided by the instruction leaflet of the drug manufacturer. Participants were randomly assigned to either 20 mg/day of rosvastatin or no study medication for the 28-day study duration. Compliance to treatment was assessed by measurements of total cholesterol levels at baseline and at study end in all participants. The START trial was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands, and all study participants gave written informed consent prior to participation.

**Baseline measurements**

The study baseline was set as the last regular visit of the participant to the anticoagulation clinic. All participants were screened on acquired risk factors for VTE through a questionnaire, in addition to being tested on kidney and liver function. VTE patients stopped using vitamin K antagonist one month prior to baseline blood draw. Non-fasting blood samples were collected
in Vacutainer tubes containing 3.2% sodium citrate (Becton Dickinson, Meylan Cedex, France) at baseline and at study end (i.e. 28 days later). Samples were centrifuged at 2,500 x g for 15 min at 18°C and platelet poor plasma (PPP) was stored at -80°C until analysis.

Measurement of procoagulant phospholipid (PPL) clotting activity in plasma

A modified factor Xa-dependent PPL clotting assay (in-house) was used to measure plasma levels of PPL in citrated platelet free plasma (PFP) (n=247). Briefly, PPP samples were thawed and centrifuged 13,500 x g for 2 min to generate PFP. Phospholipid depleted plasma (PPL depleted plasma, PPLDP), was prepared from pooled citrated PFP (n = 18) centrifuged at 100,000 x g for 60 min at 16°C (Beckman Optima LE-80K Ultracentrifuge, rotor SW40TI, Beckman Coulter, Indianapolis, Indiana, USA). PPLDP was aliquoted and stored at -80°C until further use. Twenty-five μl of test plasma was mixed with 25μl PPLDP, incubated for 2 min at 37°C, before the reaction was initiated by the addition of 100μl of pre-warmed FXa reagent (0.1 U/ml bovine FXa in 15 mM calcium chloride, 100 mM sodium chloride and 20 mM HEPES buffer, pH 7.0). A commercially available standardized reagent containing 0.1% of rabbit brain cephalin in a buffered solution was used as calibrator (UPTT from BioData Corporation, Horsham, Pennsylvania, USA). Clotting tests were carried out in duplicate on a StarT4 instrument from Diagnostica Stago. PPL levels were measured in seconds of clotting time, and converted to mU/ml, by the use of the UPTT calibrator. The PPL assay displayed low inter and intra CVs of ≤ 4% and variation between runs was adjusted for by an internal standard.

Analysis of total- and platelet-derived microvesicles in plasma by flow cytometry

Plasma samples were selected from 40 participants from the rosuvastatin treatment group and 20 participants from the non-statin group. Participants with the largest decrease in plasma PPL activity were selected from the rosuvastatin treatment group as measurement of microvesicles by flow cytometry was expected to be less sensitive for changes than the plasma PPL activity. One sample from the no treatment group was later excluded due to technical failure. Plasma samples were thawed and centrifuged a second time for 2,500 x g for 15 min. Two hundred microliter of PFP was diluted 10x in pre-filtered (Amicon Ultra-15 filters, 10 kDa cutoff) Dulbecco's phosphate-buffered saline (DPBS) that is free of Ca²⁺/Mg²⁺ (Thermo Fisher Scientific). Samples were centrifuged at 20,000 x g for 30 min at 4°C to pellet EVs.
Supernatants were carefully aspirated and the EV pellets were divided and stained for PS using FITC-labeled bovine lactadherin (Haematologic Technologies, Vermont, USA) and CD41 APC-H7 clone HIP8 (Biolegend, San Diego, USA), or with FITC-labeled bovine lactadherin and matched isotype controls. All antibodies and isotype controls were filtered using 0.22 µm ultrafree-MC centrifugal filter (Merck, Millipore, Carrigtwohill, Ireland) before use. EV pellets were incubated with antibody or isotype control mixture for 20 min at 4°C in the dark. Samples were washed with 1 ml pre-filtered DPBS and centrifuged at 20,000 x g for 30 min at 4°C. Pellets were resuspended in 200 µl pre-filtered DPBS and samples were analyzed using CytoFLEX (Beckman Coulter, Indianapolis, USA) at the rate of 10 µl/min. Data analysis was performed using CytExpert 2.0 (Beckman Coulter, Indianapolis, USA). The EV gate was set using Rosetta calibration beads (Exometry, Amsterdam, The Netherlands). EVs were defined according to size and lactadherin-positive staining. The total number of EVs was calculated from the number of detected lactadherin-positive events in every sample, and further converted to EV number per microliter plasma (EV/µl) using the original volume of analyzed plasma (150 µl).

**Statistical analysis**

Statistical analysis were performed using R (Version 4.0.3 for Windows; R Foundation). Descriptive statistics were used to describe the baseline difference between the intervention and the control group. For the results tables, the treatment and non-treatment group, as well as subgroups, were compared using two-sample t-tests with equal variance assumed and standard multivariate linear regression models adjusting for age and sex. Pearson’s correlation coefficient was used to estimate correlation.
RESULTS

Study population

Between December 2012 and December 2016 255 participants were randomized to either the rosuvastatin treatment group (n=131) or the no treatment group (n=124). A study flowchart is shown in Figure 1 with reasons for exclusion. Two participants did not start rosuvastatin treatment, and another six randomized participants did not complete the study, three in each study arm due to various reasons. The PPL assay measurements could not be performed in two participants due to technical failure, one in each study arm. The baseline characteristics of the study population are shown in Table 1. Participants allocated to no treatment were slightly older (mean age 59 years) compared to the statin-users (mean age 57 years), and were more often male (69% vs 54%). Other characteristics of the participants associated with VTE risk were equally distributed among the groups.

Outcomes

Table 2 and Figure 2 show absolute and changes in plasma PPL activity levels within and between the study arms. Plasma PPL activity levels decreased significantly from baseline to study end for rosuvastatin users (mean change, -0.48 mU/ml; 95% CI -0.81 to -0.15), while a minor increase was observed for non-users (mean change, 0.17 mU/ml; 95% CI -0.18 to 0.53) for overall VTE. Similar trends were observed in subgroup analyses of participants with a history of provoked and unprovoked VTE, as well as for DVT and PE. However, a pronounced change in plasma PPL activity was observed for the PE patients in the rosuvastatin group (mean difference, -0.94 mU/ml; 95% CI -1.52 to -0.36), and particularly for provoked PE (mean difference, -1.14 mU/ml; 95% CI -2.13 to -0.16) (Table 2 and Figure 2). The absolute and relative changes in PPL activity between the two study arms are shown in Table 3. Rosuvastatin treatment yielded a 22% (95% CI -38.2 to -5.8) reduction in PPL activity among all VTEs, and 37% (95% CI -62.9 to -11.2) reduction in PPL activity in participants with a history of PE. The treatment effect of rosuvastatin on PPL activity for overall VTEs, DVTs and PEs are further illustrated in Figure 3. The treatment effect was also investigated in a linear model adjusted for age and sex, as these parameters were not balanced between groups at baseline. Adjustments for age and sex only marginally altered the mean differences between groups as well as the treatment effects (Table 3).
Total cholesterol levels were reduced from baseline to study end in the rosuvastatin treatment group by 35% (1.96 mmol/L) and by 3% (0.17 mmol/L) in the non-treatment group. In order to explore whether the reduction in PPL activity by statin treatment was explained by the statin-dependent decrease in serum cholesterol, we plotted the absolute changes in total cholesterol against changes in PPL activity (Figure 4). A weak and Pearson’s correlation coefficient of -0.10 (p-value 0.28) indicates that the reduction in PPL activity by statin treatment was independent of the cholesterol-lowering effect.

To assess whether the observed effect of statin treatment on PPL activity could potentially be explained by alterations in EV count, EVs were isolated from plasma by ultracentrifugation, labeled with lactadherin (binds to membranes expressing PS) and CD41, a platelet specific marker, and counted using a sensitive flow cytometer. Plasma levels of lactadherin positive and platelet-derived EV, in number of EVs per µL, for the two study groups are shown as box-plots in Figure 5. EV counts are presented for the baseline sample, and compared to the end of study sample. The box-plots show no change in total EV count for the no treatment group (Figure 5A), nor the statin treatment group (Figure 5B). Similar results were found for platelet-derived EVs (Figure 5C and D). The mean differences in lactadherin positive and CD41 positive EV counts are listed in Supplementary Table 1 and 2, respectively.
DISCUSSION

In the present study, we investigated the effect of rosuvastatin treatment on plasma PPL activity, measured by a FXa-dependent PPL clotting assay, in patients with a history of VTE. Statin treatment caused a 22% reduction in PPL activity for all VTE patients and 37% reduction in PPL activity for PE patients compared to no treatment. The observed effect of rosuvastatin on PPL activity was not explained by changes in serum levels of total cholesterol or a parallel changed in plasma levels of total- and platelet-derived microvesicles by statin treatment. The results from our study support the beneficial effect of statin treatment on coagulation factors and thrombin generation potential in plasma. As the presence of negatively charged phospholipids augment the activity of the extrinsic tenase complex, TF-FVIIa, by several orders of magnitude \(^{27}\), the combined effect of reduced PPL activity and modest decline in several coagulation factors may reduce coagulation activation and contribute to the explanation why rosuvastatin treatment lower the risk of VTE \(^{16}\).

Clinical studies have shown that statin treatment, either with simvastatin \(^{30}\), atorvastatin \(^{31,32}\), or cerivastatin \(^{33}\) caused a beneficial effect on the coagulation system by a moderate lowering of specific coagulation factors and thrombin generation. In the START trial, rosuvastatin treatment showed favorable effects on the hemostatic system by reducing plasma levels of coagulation factors FVII, FVIII, and FXI by 4-6% \(^{22}\), D-dimer by 3% \(^{22}\), lowered the ex vivo thrombin generation potential by 10% \(^{23}\), and increased the fibrinolytic potential assessed by shortening of the mean plasma clot lysis time and a decrease in both plasmin inhibitor levels and thrombin-activatable fibrinolysis inhibitor (TAFI) activity \(^{24}\). The treatment effects of rosuvastatin on thrombin generation and plasma D-dimer levels were mainly driven by an increase among non-statin users \(^{22,23}\). In contrast, we found a more profound beneficial effect of rosuvastatin treatment that was mainly driven by a significant decline in the PPL activity among rosuvastatin users accompanied by a minor increase in the PPL activity among the non-users. The increase in hemostatic factors among non-statin users in our and previous studies from the START trial may be interpreted as a consequence of the rebound hypercoagulability often seen after discontinuation of anticoagulant treatment \(^{34,35}\).

Previous studies have demonstrated that plasma PPL activity is mainly due to the presence of extracellular vesicles \(^{28,29}\), and most \(^{36-40}\), but not all \(^{41}\) case-control studies have reported
increased EV-related plasma PPL activity in VTE patients compared to controls. Therefore, our findings of a profound decrease in PPL activity by statin treatment may contribute to the reduction of incident and recurrent VTE by statin treatment 13-21. Microvesicles (MVs) are larger EVs (100-1000 nm in diameter), which bud directly from the plasma membrane of activated cells, and express surface markers of their cell of origin 42-43. The largest proportion of MVs in circulating blood is derived from platelets 44,45 and the subsequent procoagulant activity in plasma is mediated by platelet-derived MVs (PDMVs) 36,44. A strong inverse correlation has also been reported between PPL clotting time and lactadherin positive EVs measured in PPP from healthy control subjects and patients with obstructive sleep apnoea (OSA), though the strength of the correlations was mainly driven by the OSA patients 29. We therefore hypothesized that the reduction we observed in plasma PPL activity following rosuvastatin treatment was caused by a parallel decline in plasma MV levels, and particularly platelet-derived MVs. In order to test our hypothesis, we isolated EVs from platelet free plasma and measured the total count (lactadherin-positive) and platelet-derived MVs (lactadherin- and CD41-positive) by flow cytometry. Although we found statin treatment to lower the PPL activity in the treatment group, we did not observe a reduction in total EV count, nor platelet derived EVs, for comparisons between – or within study groups.

Our results show that rosuvastatin treatment did not affect plasma MV levels in patients with a history of VTE. Contradicting our findings, previous observational studies have shown that patients with arterial cardiovascular diseases or risk factors (hyperlipidemia in particular) had higher plasma MV levels than control individuals, and that statin treatment lowered plasma MV levels in most, but not all studies 46-51. Several factors may contribute to explain our findings. First, the effect of statin treatment on plasma MV levels may be limited to individuals with arterial cardiovascular diseases and risk factors, and not transferrable to VTE patients. Second, one might speculate that statin treatment could differentially influence EV formation from various intravascular cells and the subsequent process of externalization of PS to the outer leaflet of the cell membrane during EV formation 52. Accordingly, in a placebo-controlled randomized double-blinded crossover study, the treatment of 19 patients with peripheral arterial occlusive disease for 8 weeks with 80 mg atorvastatin daily showed a reduction in plasma MV levels expressing CD62P- and CD61-positive MVs without affecting plasma levels of lactadherin-positive EVs 49. Third, a well-recognized limitation of flow cytometry as a method is the detection limit of the instrument. Even a sensitive flow cytometer will still only detect
vesicles above approximately 200 nm in diameter, and thereby exclude the larger population of EVs. Larger vesicles (> 200 nm in diameter) have been reported to only account for a minority of the EV population (< 5%) \(^{53}\). This may imply that a possible decrease in plasma EVs after statin treatment could have been masked by the unchanged level of the larger EVs (>200 nm in diameter).

Some aspects of our randomized controlled trial need attention. Neither the patients nor the physicians were blinded to treatment. However, it is unlikely that knowledge of the treatment would affect the laboratory outcomes. Furthermore, the technicians conducting the laboratory analyses were blinded to sample treatment. In addition, despite randomization, the distribution of age and sex was uneven between the study arms. We decided \textit{a priori} to adjust analysis for age and sex as potential confounders, and adjustments did not influence the observed treatment effect. Even though results from subgroup analysis revealed the most pronounced decrease in plasma PPL activity in individuals with a history of PE, they should be interpreted with caution as the study was not originally powered to analyze differences in subgroups \(^{22}\). Last, as participants were recruited from an outpatient setting, it limits the risk of confounding diseases at randomization, and for analysis, participants were compared with themselves.

In conclusion, rosuvastatin treatment caused a substantial decrease in plasma PPL activity, suggesting that PPL-dependent attenuation of coagulation activation may contribute to a reduced VTE risk by statin treatment. Further studies are warranted to validate our findings and unravel underlying mechanisms.

\textbf{Acknowledgements}

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Authorship

C.R planned experiments, analyzed data, wrote and revised the manuscript. K. Hindberg performed the statistical analysis and revised the manuscript. O. Snir planned experiments, analyzed data and participated in writing and revising the manuscript. J.S. Biederman, S.C. Cannegieter, F.J. van der Meer, F.W.G. Leebeek, M.J.H.A. Kruip and W.M. Lijfering revised the manuscript. W.M. Lijfering was responsible for the START study concept. J.B. Hansen conceived and designed the study, analyzed data, participated in writing and revising the manuscript.

Conflict of interest disclosures

The authors state that they have no conflict of interests.

REFERENCES


### FIGURES AND TABLES

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<td>21 (17.5)</td>
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<tr>
<td>Diabetes</td>
<td>3 (2.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Overweight (25 ≤ BMI &lt; 30)</td>
<td>53 (42.4)</td>
<td>51 (42.5)</td>
</tr>
<tr>
<td>Obese (30 ≤ BMI)</td>
<td>29 (23.2)</td>
<td>35 (29.2)</td>
</tr>
</tbody>
</table>

Continuous variables denoted as mean (range) and categorical variables as number of (%).
Table 2. Mean difference in measures of PPL activity (mU/ml) from baseline to study end within the treatment and no treatment group and between groups for all VTE and subgroups provoked and unprovoked VTE, DVT and PE.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Treatment group (T) (n=125)</th>
<th>No treatment group (NT) (n=120)</th>
<th>t-test</th>
<th>Linear model*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Study end</td>
<td>Change over study</td>
<td>Baseline</td>
</tr>
<tr>
<td>VTE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>3.30±2.47</td>
<td>2.82±2.08</td>
<td>-0.48 (-0.81,-0.15)</td>
<td>2.64±1.81</td>
</tr>
<tr>
<td>Provoked</td>
<td>3.32±2.56</td>
<td>2.88±2.24</td>
<td>-0.44 (-0.93,0.06)</td>
<td>2.62±1.65</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>3.29±2.37</td>
<td>2.75±1.88</td>
<td>-0.54 (-0.97,-0.10)</td>
<td>2.66±1.95</td>
</tr>
<tr>
<td>DVT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>2.99±2.10</td>
<td>2.85±2.09</td>
<td>-0.13 (-0.51,0.24)</td>
<td>2.50±1.64</td>
</tr>
<tr>
<td>Provoked</td>
<td>2.74±1.97</td>
<td>2.85±2.24</td>
<td>0.11 (-0.31,0.52)</td>
<td>2.76±1.53</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>3.29±2.24</td>
<td>2.86±1.92</td>
<td>-0.43 (-1.10,0.24)</td>
<td>2.29±1.72</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>3.72±2.85</td>
<td>2.78±2.09</td>
<td>-0.94 (-1.52,-0.36)</td>
<td>2.81±1.98</td>
</tr>
<tr>
<td>Provoked</td>
<td>4.07±3.05</td>
<td>2.92±2.29</td>
<td>-1.14 (-2.13,-0.16)</td>
<td>2.49±1.78</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>3.28±2.58</td>
<td>2.60±1.85</td>
<td>-0.68 (-1.21,-0.15)</td>
<td>3.16±2.16</td>
</tr>
</tbody>
</table>

Values are means ± 1 standard deviation (SD) or the mean difference between groups with 95% confidence intervals in parenthesis.

* Adjusted for age and sex.

PPL: procoagulant phospholipids, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism
Table 3. Treatment effect of rosuvastatin on measures of PPL activity in percentage change and as a linear model for all VTE and subgroups provoked- and unprovoked VTE, DVT and PE.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Delta T - Delta NT</th>
<th>Percentage change (%)</th>
<th>Linear model adjusted effect*</th>
<th>Adjusted percentage effect* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-0.66 (-1.14,-0.17)</td>
<td>-22.0 (-38.2,-5.8)</td>
<td>-0.63 (-1.12,-0.14)</td>
<td>-21.0 (-37.5,-4.6)</td>
</tr>
<tr>
<td>Provoked</td>
<td>-0.59 (-1.35,0.17)</td>
<td>-19.7 (-45.0, 5.6)</td>
<td>-0.22 (-0.77,0.32)</td>
<td>-18.0 (-43.8, 7.7)</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>-0.73 (-1.33,-0.12)</td>
<td>-24.7 (-45.1,-4.2)</td>
<td>-0.72 (-1.33,-0.10)</td>
<td>-24.3 (-45.1,-3.5)</td>
</tr>
<tr>
<td>DVT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-0.23 (-0.77,0.32)</td>
<td>-8.2 (-28.0,11.6)</td>
<td>-0.22 (-0.77,0.32)</td>
<td>-8.1 (-27.9,11.8)</td>
</tr>
<tr>
<td>Provoked</td>
<td>0.11 (-0.58,0.80)</td>
<td>3.9 (-21.2,29.0)</td>
<td>0.13 (-0.57,0.83)</td>
<td>4.6 (-20.8,30.0)</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>-0.59 (-1.45,0.27)</td>
<td>-21.4 (-52.5, 9.7)</td>
<td>-0.60 (-1.47,0.27)</td>
<td>-21.7 (-53.3, 9.9)</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>-1.21 (-2.05,-0.37)</td>
<td>-37.0 (-62.9,-11.2)</td>
<td>-1.13 (-2.01,-0.26)</td>
<td>-34.8 (-61.7,-8.0)</td>
</tr>
<tr>
<td>Provoked</td>
<td>-1.45 (-2.86,-0.04)</td>
<td>-43.9 (-86.8,-1.1)</td>
<td>-1.34 (-2.85,0.18)</td>
<td>-40.7 (-86.6, 5.3)</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>-0.91 (-1.77,-0.05)</td>
<td>-28.4 (-55.2,-1.6)</td>
<td>-0.90 (-1.79,-0.00)</td>
<td>-27.9 (-55.7,-0.1)</td>
</tr>
</tbody>
</table>

Values are mean differences between groups with 95% confidence intervals in parenthesis or percentage change calculated using the mean difference and dividing it by the mean baseline levels of PPL for both groups.

*Adjusted for age and sex

PPL: procoagulant phospholipids, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism
Figure 1. Flow chart of the study participants with numbers at enrolment, randomization, follow-up and reasons for withdrawal.

* Hospitalization with acute asthma exacerbation
Figure 2. Forest plots of changes in plasma PPL activity (mU/ml) (after minus before) within the rosvastatin treatment and the no treatment group and between groups for all VTEs, DVTs and PEs. Values are means with 95% confidence intervals.

PPL: procoagulant phospholipids, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism
**Figure 3.** Forest plot of the treatment effects (change within the statin group minus the change in the no treatment group) as percentage change in PPL activity for all VTEs, DVTs, and PEs. Values are means with 95% confidence intervals.

PPL: procoagulant phospholipids, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism
Figure 4. Change in individual total cholesterol levels from baseline to study end plotted against change in individual PPL activity for the treatment group.
**Figure 5.** The effect of rosuvastatin treatment on extracellular vesicle count, measured by flow cytometry. The changes in lactadherin positive EV count (A and B) and platelet-derived EV count (C and D) are shown for the two study groups, i.e. no treatment group (A and C) and the rosuvastatin treatment group (B and D). The larger circles represent the mean, and a line is drawn to connect the means between baseline and study end within the groups.

PPL: procoagulant phospholipids, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism
**Supplementary Table 1.** Mean difference in lactadherin positive EV count (count/μl) from baseline to study end within the treatment and no treatment group and between groups for all VTE and subgroups provoked and unprovoked VTE, DVT and PE.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Treatment group (T) (n=40)</th>
<th>No treatment group (NT) (n=19)</th>
<th>t-test</th>
<th>Linear model*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Study end</td>
<td>Change over study</td>
<td>Baseline</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VTE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>833±580</td>
<td>776±463</td>
<td>-57 (-274,159)</td>
<td>532±410</td>
</tr>
<tr>
<td>Provoked</td>
<td>768±492</td>
<td>795±454</td>
<td>27 (-234,289)</td>
<td>511±351</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>899±662</td>
<td>757±483</td>
<td>-142 (-508,224)</td>
<td>569±526</td>
</tr>
<tr>
<td><strong>DVT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>812±758</td>
<td>717±472</td>
<td>-95 (-530,341)</td>
<td>331±235</td>
</tr>
<tr>
<td>Provoked</td>
<td>425±275</td>
<td>654±418</td>
<td>229 (-336,994)</td>
<td>374±279</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>988±850</td>
<td>746±511</td>
<td>-242 (-833,349)</td>
<td>246±96</td>
</tr>
<tr>
<td><strong>PE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>847±441</td>
<td>815±463</td>
<td>-32 (-278,213)</td>
<td>713±459</td>
</tr>
<tr>
<td>Provoked</td>
<td>882±501</td>
<td>842±470</td>
<td>-40 (-341,261)</td>
<td>648±385</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>789±338</td>
<td>770±476</td>
<td>-20 (-538,499)</td>
<td>812±603</td>
</tr>
</tbody>
</table>

Values are means ± 1 standard deviation or the mean difference between groups with 95% confidence intervals in parenthesis.

* Adjusted for age and sex

PPL: procoagulant phospholipids, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism
### Supplementary Table 2.
Mean difference in platelet-derived extracellular vesicle count (count/μl) from baseline to study end within the treatment and no treatment group and between groups for all VTE and subgroups provoked and unprovoked VTE, DVT and PE.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Treatment group (T) (n=40)</th>
<th>No treatment group (NT) (n=19)</th>
<th>t-test</th>
<th>Linear model*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Study end</td>
<td>Change over study</td>
<td>Baseline</td>
</tr>
<tr>
<td>VTE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>419±347</td>
<td>407±312</td>
<td>-12 (-150,126)</td>
<td>245±253</td>
</tr>
<tr>
<td>Provoked</td>
<td>386±315</td>
<td>408±306</td>
<td>22 (-146,190)</td>
<td>231±205</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>452±382</td>
<td>406±327</td>
<td>-46 (-281,189)</td>
<td>267±337</td>
</tr>
<tr>
<td>DVT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>411±424</td>
<td>381±296</td>
<td>-30 (-287,227)</td>
<td>121±86</td>
</tr>
<tr>
<td>Provoked</td>
<td>206±176</td>
<td>340±247</td>
<td>134 (-314,582)</td>
<td>150±91</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>504±476</td>
<td>399±325</td>
<td>-105 (-458,248)</td>
<td>62±27</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>424±295</td>
<td>424±328</td>
<td>0 (-171,171)</td>
<td>356±304</td>
</tr>
<tr>
<td>Provoked</td>
<td>446±332</td>
<td>431±328</td>
<td>-15 (-216,185)</td>
<td>312±262</td>
</tr>
<tr>
<td>Unprovoked</td>
<td>387±233</td>
<td>413±348</td>
<td>26 (-351,403)</td>
<td>421±391</td>
</tr>
</tbody>
</table>

Values are means ± 1 standard deviation or the mean difference between groups with 95% confidence intervals in parenthesis.

* Adjusted for age and sex

PPL: procoagulant phospholipids, VTE: venous thromboembolism, DVT: deep vein thrombosis, PE: pulmonary embolism