Thrombosis Update 2 (2021) 100030

Contents lists available at ScienceDirect

Thrombosis Update

journal homepage: www.journals.elsevier.com/thrombosis-update

Novel biomarkers to detect occult cancer in patients with unprovoked venous thromboembolism: Rationale and design of the PLATO-VTE study

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ARTICLE INFO

Keywords:
Early detection of cancer neoplasms
- tumor biomarkers
- venous thromboembolism
- venous thrombosis

ABSTRACT

Occult cancer is detected in about 5% of patients with unprovoked venous thromboembolism (VTE) in the 12 months following VTE diagnosis. Current guidance suggests conducting a 'limited' cancer screening in these patients, consisting of medical history taking, physical examination, routine blood tests, chest X-ray, and age- and gender-specific testing, over full-body imaging. However, almost half of underlying cancers remain undetected with this approach. Blood-based liquid biopsies may provide an attractive addition or alternative to current cancer screening strategies, with a potentially higher detection rate while avoiding radiation or invasive testing.

The PLATO-VTE study is an ongoing, investigator-initiated, multinational, prospective, observational cohort study comparing the sensitivity of novel biomarkers for detecting cancer with that of limited cancer screening in the setting of unprovoked VTE. Patients older than 40 years with a first episode of unprovoked VTE are eligible, while those with major and minor transient provoking risk factors for VTE are excluded. Patients undergo standard-of-care 'limited' cancer screening and are followed for 12 months for the occurrence of cancer. A blood sample for biomarker analysis is drawn within 10 days; a second sample is taken at 3 months to assess test result consistency over time. Three biomarkers are assessed: platelet mRNA, circulating tumor DNA, and plasma

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https://doi.org/10.1016/j.tru.2020.100030
Received 1 November 2020; Received in revised form 16 December 2020; Accepted 16 December 2020

proteomics analysis. The sensitivity and predictive value of the biomarkers at baseline will be compared with those of limited screening.

The results from the PLATO-VTE study may lead to reconsider current approaches for cancer screening in patients with unprovoked VTE.

Abbreviations

AJCC	American Joint Committee on Cancer
ROC	receiver operating characteristic
CA-125	cancer antigen 125
CEA	carcinoembryonic antigen
CI	confidence interval
CT	computed tomography
ctDNA	circulating tumor DNA
DNA	deoxyribonucleic acid
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PET	positron emission tomography
PSA	prostate specific antigen
VTE	venous thromboembolism

1. Introduction

Patients presenting with unprovoked venous thromboembolism (VTE) are at substantial risk of occult cancer, a phenomenon first described in the 19th century by *Dr. Bouillaud* [1]. A systematic review and meta-analysis, including individual data of more than 2000 patients, estimated that the 12-month risk of occult cancer in patients with unprovoked VTE is about 5% [2]. The most frequently diagnosed underlying cancer types were colorectal (17%), lung (15%), and pancreatic cancer (11%) [2]. The risk of a new cancer diagnosis is known to be highest in the first 6–12 months following unprovoked VTE diagnosis; thereafter it becomes almost comparable with that of the general population [2,3].

Current guidance statements recommend a 'limited' cancer screening strategy in patients with unprovoked VTE by means of history taking, a thorough physical examination, routine blood tests, a chest X-ray, and age- and gender specific screening [4,5]. The aim is to detect cancer at an early, potentially curable stage, thereby reducing cancer-related morbidity and mortality. An important limitation of this strategy is that it misses almost half of the occult cancers [2].

Various studies have evaluated whether a more comprehensive diagnostic approach including additional imaging tests, such as computed tomography (CT)-scanning or positron emission tomography (PET)/CT-scanning, would be able to detect more underlying cancers than a 'limited' screening strategy. However, results of individual studies have not shown a clear benefit of an extended screening approach.

Recently, evidence synthesis of studies evaluating extended and limited screening strategies showed that more cancers were detected at initial screening in the extended screening group compared to limited screening (odds ratio, 2.0; 95% CI, 1.2 to 3.4) [2]. Additionally, a larger proportion of the detected cancers in the extended screening group were of an early stage (47% vs 30%), although this difference was not statistically significant (P = 0.30) [2]. However, extensive screening did not significantly reduce cancer-related mortality in any of these studies [6–10]. Other concerns about these imaging-based screening strategies include the regularly encountered false-positive findings necessitating potentially harmful invasive procedures, and radiation exposure. Given these disadvantages and lack of evidence of survival benefit, current international guidance does not recommend extended screening methods [4,5].

Liquid biopsies comprise a group of diagnostic methods based on the isolation and analysis of tumor-derived material obtained from blood or other bodily fluids. These assays could provide an attractive alternative to or an improvement of the current screening methods for occult cancer [11,12], as they are minimally invasive, not associated with radiation exposure, affordable, while potentially increasing the number of cancers detected. Additionally, some of these methods are able to discriminate between different primary tumor locations, identify cancer-specific mutations, and estimate cancer stage and prognosis [11,12]. In addition, cancer screening by imaging or endoscopy may be avoidable in patients with negative liquid biopsy results. Taken together, liquid biopsies could potentially improve or even replace current cancer screening strategies in patients with unprovoked VTE or, alternatively or additionally, allow for a better stratification of patients and a targeted selection of screening strategies.

Recently, platelet mRNA sequencing has been introduced as such a novel promising biomarker with a high sensitivity and specificity in detecting cancer [13]. In addition, the test was able to identify the tumor location with an overall accuracy of 70%. The PLATO-VTE study is an ongoing prospective cohort study which aims to evaluate the diagnostic accuracy of platelet mRNA sequencing, as well as circulating tumor DNA (ctDNA) and plasma quantitative proteomics analysis, in detecting occult cancer in patients with unprovoked VTE. The present manuscript describes the design and rationale of the PLATO-VTE study and discusses the strengths and limitations of the evaluated liquid biopsy techniques.

2. Study overview

The PLATO-VTE study is an investigator-initiated, multinational, prospective, observational cohort study (ClinicalTrials.gov; NCT02739867). The study protocol has been reviewed and approved by the ethical review boards of all participating centers and written informed consent is obtained from all participating patients.

Patients will be recruited and followed for 12 months for the occurrence of cancer with the initial protocol (see Table 1 for study design overview). After the baseline visit, patients undergo standard-of-care limited cancer screening, including at least a thorough medical history taking and physical examination, basic laboratory testing, and a chest X-ray if this had not been performed in the 6 months prior to VTE diagnosis, and if CT pulmonary angiography had not been performed for the index VTE event [4,5]. Age- and gender-specific testing (e.g. prostate specific antigen [PSA] or mammography) are left to the discretion of the treating physician, as is targeted testing following abnormalities of limited screening.

Blood for biomarker analysis is drawn at baseline within 10 days of the index VTE event. A second blood draw is performed at the 3-month visit to evaluate test result consistency over time. Follow-up visits are scheduled at 3 months (in person), and at 6 and 12 months (in person or telephone).

Study physicians and patients are not informed of the test results, since the diagnostic value of the biomarkers in clinical practice is still uncertain. Blood collection tubes are processed and stored in the freezers immediately after withdrawal (see Appendix A for laboratory protocol). All biomarker analyses will be performed in batches after study completion.

3. Patients

Patients aged 40 years or older, presenting with a first episode of

Table 1
Overview of the PLATO-VTE study.

Study visit	Assessments or procedures
Baseline *(clinic visit)*	Inclusion criteria
	First unprovoked symptomatic (distal or proximal) DVT or PE
	40 years or older
	Exclusion criteria
	Cancer diagnosis or cancer treatment within the past 5 years
	Transient risk factors for VTE in the previous 3 months: trauma or fracture of the leg, surgical procedures, general anesthesia, or immobilization greater than 3 days
	Previous unprovoked venous thromboembolism
	Known hereditary or acquired thrombophilia
	Current pregnancy or puerperium up to 3 months postpartum
	Current estrogen therapy
	Greater than 10 days after VTE diagnosis
	Inability for blood withdrawal or written informed consent
Day 1–10 *(clinic visit)*	First blood withdrawal for cancer biomarkers[a]
Day 1–30 *(clinic visit)*	Standard-of-care limited cancer screening
	Medical history and physical examination
	Laboratory testing[b]
	Chest X-ray
	Age- and gender-specific testing depending on local practice[c]
Month 3 *(clinic visit)*	Second blood withdrawal for cancer biomarkers[a]
	Assessment cancer diagnosis, recurrent venous thromboembolism, major bleeding, and mortality
Month 6 *(clinic or telephone visit)*	Assessment cancer diagnosis, recurrent venous thromboembolism, major bleeding, and mortality
Month 12 *(clinic or telephone visit)*	Assessment cancer diagnosis, recurrent venous thromboembolism, major bleeding, and mortality

Abbreviations: DVT: deep vein thrombosis, PE: pulmonary embolism.
[a] Cancer biomarkers include platelet mRNA sequencing, circulating tumor DNA, and proteomics analysis.
[b] Laboratory tests include at least haemoglobin, white blood cell count, platelet count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, lactate dehydrogenase (LD).
[c] Age- and gender-specific testing may include mammography, Papanicolaou (Pap) smear, prostate specific antigen testing, and fecal occult blood test.

unprovoked symptomatic (distal or proximal) deep vein thrombosis of the lower extremity or pulmonary embolism, are eligible to participate. Suspicion of cancer at VTE diagnosis is allowed if cancer is unconfirmed. Exclusion criteria include provoking risk factors for VTE and VTE diagnosed more than 10 days before possible inclusion (see Table 1 for full list of exclusion criteria).

4. Outcomes

The primary outcome is a diagnosis of solid or haematological cancer, confirmed by histology or cytology, or unequivocally diagnosed by imaging (e.g. PET/CT) or tumor markers (e.g. PSA) in exceptional cases when it is impossible to obtain tumor material. Non-melanoma skin cancer and myeloproliferative neoplasms are excluded from the primary outcome and will be reported separately. Secondary outcomes include early stage solid cancer (stage I or II according to the American Joint Committee on Cancer [AJCC] criteria), any solid cancer, any haematological cancer, cancer-associated mortality, and all-cause mortality. Recurrent VTE and bleeding events are also documented. All clinical outcome events will be centrally adjudicated by an independent expert adjudication committee. Solid cancers will be staged according to the AJCC criteria. Bleeding events will be classified as 'major' or 'clinically relevant non-major' as per the International Society on Thrombosis and Haemostasis criteria [14–16]. An overview with detailed definitions of the outcomes of interest is provided in Appendix B.

5. Sample size considerations

The study hypothesis is that platelet mRNA sequencing is more sensitive than limited screening in detecting cancer. In this first evaluation of platelet mRNA profiling in a clinical setting, the implicit assumption was made that platelet mRNA profiling would be a replacement test for limited screening, not an add-on test. Assuming a 12-month cancer detection rate of 5% [2], a sensitivity of limited screening of 50% [2], a conservatively considered sensitivity of platelet mRNA sequencing of 86% (which is 10% lower than the previously reported sensitivity [13]), a loss to follow-up of 5%, and 5% invalid baseline samples, it was estimated that 462 patients would need to be included to have 80% power for demonstrating superiority of the sensitivity of platelet mRNA sequencing, based on McNemar's test for paired proportions, at a two-sided alpha of 0.05.

6. Statistical analysis

The primary analysis will be a per-protocol analysis restricted to patients with a valid baseline blood sample and patients who were not lost to follow-up in the 12-month study period. The most recent evaluations of platelet RNA sequencing will be used to predefine the test cut-off to be applied in the present study. Based on these unpublished data, the test cut-off corresponding to a sensitivity of 86% will be selected and used for the analysis. Additionally, a second cut-off will be evaluated at which the specificity was 99% based on these unpublished data. The cumulative incidence of cancer will be presented as a proportion. The timing of the incidence will be displayed as a cumulative incidence curve.

The sensitivity of platelet mRNA sequencing at baseline and limited screening will be compared using McNemar's test for dependent testing of categorical data, with corresponding 95% CI for sensitivity using Wilson's score method.

In addition, the predictive values of the baseline platelet mRNA sequencing measurement will be used to assess the area under the receiver operating characteristic (ROC) curve for the diagnosis of cancer. Based on the standard error obtained by DeLong's method, it will be assessed whether this area under the ROC curve is greater than 0.5. A two-graph ROC-curve (also, cumulative distribution analysis) for the diagnosis of cancer will be plotted to depict the sensitivity and specificity of platelet RNA profiling against all test cut-off values, using the predictive values from the baseline platelet RNA profile.

In a subgroup analysis, results will be stratified by age categories. Several sensitivity analyses will be performed. First, all patients will be analyzed, assuming that patients without a valid baseline sample had a 'normal' platelet RNA profile and those lost to follow-up were not diagnosed with cancer. In a second sensitivity analysis missing test results (i.e., scores ranging from 0 to 1.0) and follow-up data will be replaced using multiple imputation methods assuming a 'missing at random' pattern. A third analysis will be performed excluding patients in whom cancer was clinically suspected at VTE diagnosis. Additionally, the analysis will be restricted to the first 6 months of follow-up as well as to the six cancer types which were used to develop the pan-cancer mRNA sequencing tool.

In secondary analyses, the clinical utility and corresponding net benefit of platelet mRNA sequencing for occult cancer screening at the prespecified test cut-offs will be explored by weighing the true positive rate (TPR; i.e. correct positive cancer classification by the test) against the false positive rate (FPR; i.e. initial test positive but no cancer diagnosis during follow-up). The net benefit will be analyzed using the approach provided by Pepe and colleagues, which includes disease (cancer) prevalence and weighs the absolute numbers of true and false positive results [17]. The minimally acceptable TPR/FPR ratio in the present study is set at approximately 3.0, based on standard-of-care 'limited' cancer screening, which is associated with a sensitivity of 50% and a positive predictive value of 15%, at a cancer prevalence of 5% [2]. If the cancer detection rate (i.e., sensitivity) of platelet mRNA

sequencing is found to be 75% or higher, an even lower positive predictive value of 10% will be considered acceptable, translating to a TPR/FPR ratio of approximately 2.0. A TPR/FPR ratio of platelet mRNA sequencing similar to or greater than the prespecified TPR/FPR ratio (either 2.0 or 3.0) will meet the criteria for clinical utility.

In the subgroups of patients with discordant test results (i.e., negative initial limited cancer screening work-up but a positive platelet mRNA sequencing result, or positive work-up with negative mRNA result), the proportion of occult cancers at the end of 12-month follow-up will be assessed, as well as cancer type and stage, and timing of cancer diagnosis. The cancers missed by both strategies will be reported separately.

Similar primary and secondary analyses will be performed for circulating DNA and proteomics biomarker analyses in the present study. Several substudies on recurrent VTE and bleeding in unprovoked VTE were prespecified in the protocol.

7. Two-step approach for sample sequencing

The platelet mRNA analyses will be conducted in a two-step approach (Appendix C). The first step will have a case-cohort design. All patients diagnosed with cancer will be selected, as well as three randomly selected patients without cancer within the PLATO-VTE study cohort. The samples will be sequenced and analyzed in a blinded fashion. If the sensitivity of platelet mRNA sequencing is approximately 70% or higher at a minimal specificity of 50%, the remaining samples of the patients without cancer will be sequenced as well to report the full results of the study. In case the sensitivity is lower than 70% and/or the specificity is lower than 50%, only the results of the first analysis step will be reported, after which the specificity and positive predictive value will be estimated by inflating the number of patients without cancer to the actual size within the cohort. In the latter case, not all proposed analyses can be performed. A similar approach will be applied to the other biomarkers of interest.

8. Platelet mRNA sequencing

The potential of tumor-educated platelets for blood-based detection of cancer has been supported by several studies [13,18–22]. Platelets play a vital role in hemostasis and are the second most abundant cell type in blood with concentrations usually ranging between 150 and 400 billion per liter. Originally, platelets are cytoplasmic fragments derived from megakaryocytes residing in the bone marrow or lungs [23]. Even though these cell fragments lack a nucleus, they do contain several types of RNA, including pre-mRNAs, microRNAs, long-noncoding RNAs, and circular RNA, as well as mitochondrial DNA, a spliceosome, and a protein translational machinery. Platelets usually circulate in the blood for approximately 7–10 days, after which they are degraded by the spleen [24].

The role of platelets in cancer patients has been widely acknowledged. A relationship between platelet numbers in blood and the presence of cancer was first revealed by a study including 14,000 individuals in the 1960's, which demonstrated an association between thrombocytosis and cancers from different organs [25]. These early findings suggested a possible interaction between tumor cells and platelets. In 2010, Zaslavsky et al. showed that the number of megakaryocytes in the bone marrow increases in response to cancer [26]. Kerr et al. showed that interaction between the tumor and potential metastasis sites in the bone is facilitated by platelets via granules containing tumor-derived proteins which are released upon platelet activation [27]. In the past decade, it became apparent that platelets are also involved in tumor growth and metastasis. Platelets can interact with tumor-resident cancer cells, as well as with cancer cells that circulate in the bloodstream. More recently, advanced technology further elucidated the complex interactions between cancer cells, thrombosis, megakaryocytes, and platelets, leading to the concept of 'tumor-educated platelets' [25,28–30].

Around 10 years ago, Calverley et al. and Nilsson et al. demonstrated that platelet RNA profiles of cancer patients are different from those of healthy individuals [18,31]. It is thought that the interaction between platelets and tumor cells alters the RNA expression of platelets, leading to their so-called education. This can be the result from sequestration of tumor-associated biomolecules, induction of queue-specific splice events by tumor cells, and direct ingestion of extracellular vesicles containing (spliced) circulating mRNA by platelets [31–35]. Therefore, platelets carry a dynamic and rich mRNA repertoire, which makes them a highly interesting liquid biopsy biosource. RNA-sequencing of tumor-educated platelets enables for characterization of the RNA profiles and may be used to distinguish cancer patients from healthy individuals [13,36,37]. Recently, a standardized protocol for platelet mRNA sequencing (thromboSeq), as well as 'tumor-educated platelet' classifier development software, have been made available to further evaluate the diagnostic accuracy of platelets [37]. In short, platelet pellets are obtained from whole blood, and RNA is subsequently isolated from the platelets. RNA-sequencing is then performed using an Illumina platform, allowing for assessment of the RNA profile and classification with a self-learning and swarm intelligence–based algorithm. All steps are quality-controlled by Bioanalyzer analysis.

In 2015, Best et al. performed extensive RNA sequencing to compare profiles of differentially spliced RNA in platelets from 228 cancer patients (with six types of localized and metastasized solid cancers) and 55 healthy individuals [13]. The discriminative performance of the test was found to be high, with an overall accuracy of 96%, sensitivity of 97%, and specificity of 94%, suggesting a role as a 'pan-cancer' detection tool. In addition, in a multiclass test across the six different tumor types (non--small-cell lung carcinoma, colorectal cancer, glioblastoma, pancreatic cancer, hepatobiliary cancer, and breast cancer), the location of the primary tumor was correctly identified with an overall accuracy around 70%, suggesting tumor type-specific splicing within platelets. A large follow-up study, including 402 patients with early- and late-stage non--small-cell lung carcinoma and 377 individuals without cancer, confirmed the diagnostic potential of thromboSeq, which was shown to be independent of patient's age, smoking habits, inflammatory conditions, and whole-blood storage time [38]. The PLATO-VTE study will be the first to prospectively evaluate platelet RNA-sequencing as a pan-cancer screening tool in a population at increased risk of occult cancer.

9. Circulating tumor DNA

Circulating tumor DNA assays revolve around tumor-derived cell-free DNA fragments circulating in the blood. These tests target cancer-associated mutations, copy number variations, or epigenetic changes affecting gene expression of promotor or enhancer regions, which already occur in early carcinogenesis. Multiple mechanisms are thought to contribute to the presence of ctDNA in blood, including passive release by apoptotic or necrotic tumor cells, as well as active secretion by tumor cells [39]. Generally, in healthy individuals, the plasma concentration of cell-free DNA ranges between 3 and 9 ng/mL. In cancer patients, the fraction of ctDNA relative to the total amount of cell-free DNA is low and highly dependent on tumor stage. For example, in patients with early-stage cancers the ctDNA fraction is usually less than 0.1%, whereas in those with advanced-stage disease cell-free DNA concentration can increase more than 10-fold [40].

Two main approaches are currently used for ctDNA analysis: an untargeted and a targeted sequencing strategy. The targeted approach focuses on specific mutations in cell-free DNA, such as *EGFR*, *KRAS*, *TP53*, *BRAF*, and *PIK3CA*. Most targeted strategies can detect known somatic variants even at very low levels and are based on real-time or digital polymerase chain reaction (PCR), including digital polymerase chain reaction (dPCR), bead, emulsion, amplification, and magnetics (BEAMing), and amplification-refractory mutation system PCR (ARMS-PCR) [41]. The untargeted genome-wide techniques are usually based on next generation sequencing, which are able to detect variants in a large number of genes [39].

As there is no standardized protocol for ctDNA analysis, the diagnostic accuracy for cancer may vary between assays due to test characteristic differences, such as lower detection limits or different gene regions targets [42]. Tumor-related factors may also play a role, since tumors not always harbor the mutations targeted by ctDNA assays, and ctDNA levels can remain undetected in those with a small tumor size [39]. Many studies have evaluated the diagnostic accuracy of ctDNA analysis to detect cancer in patients with both early- and late-stage disease, but their findings were heterogeneous. An overview of studies which evaluated the sensitivity of ctDNA for at least early-stage (I/II) or localized cancer is provided in Appendix D. Studies with a sample size of less than 50 patients are excluded from the overview. In the 32 included studies, the most frequently assessed cancer types were breast (n = 7; 22%), lung (n = 7; 22%), colorectal (n = 4; 13%), pancreatic (n = 4; 13%). Three studies (9.4%) included various cancer types. The laboratory methods used and genetic targets varied widely among the studies. Sensitivity for early-stage (I/II) or localized cancer varied widely, ranging from 0% to 97%, and as the sensitivity was lower than 50% in 16 of 28 studies (57%), it seems fair to conclude that the diagnostic accuracy of many ctDNA assays may be suboptimal for early cancer detection. However, as improvements for early cancer detection are the main focus in the field of ctDNA, it is expected that novel or improved assays with better accuracy will be available within the next coming years, which will be candidates for validation within the PLATO-VTE cohort.

10. Proteomics

Until recently, antibody assays (ELISA) measuring individual protein molecules have been common practice for the quantification of plasma proteins. Several single-protein biomarkers have been used in cancer patient management, but none of them can be used as a pan-cancer detection tool. For example, PSA is a frequently used biomarker to detect early stage prostate cancer, while other cancer-type-specific biomarkers, such as carcinoembryonic antigen (CEA) and cancer antigen 125 (CA-125), are mostly used to monitor disease activity only, as their sensitivity for the detection of early-stage disease is deemed too low [43,44].

Mass spectrometry-based targeted proteomics with internal standard has emerged as the method of choice for large-scale protein analysis, as it is able to precisely quantify protein contents of bio-fluids and tissue samples [45]. It enables the detection of subtle changes in the proteomic composition of the sample, which may possibly occur in the presence of cancer. Multiple/parallel reaction monitoring is the most sensitive targeted proteomics approach, which allows quantifying hundreds of peptides simultaneously using less than 10 μL plasma [46,47]. The absolute concentrations of endogenous proteotypic peptides, which are peptides that can be used to identify target proteins, are measured by adding internal standards. These are chemically synthesized peptides that have the same sequence as the endogenous peptides, but labeled with stable isotopes, usually carbon and/or nitrogen (i.e., ^{13}C and ^{15}N). Both peptides with and without labeled isotopes behave similarly throughout the measurement process. However, the labeled peptide has a mass shift that can be resolved in the mass spectrometer, allowing to calculate the exact quantity of the endogenous peptides, based on the relative abundance and added concentration of the labeled peptide [48].

A recent study by Mohammed et al. evaluated the concentrations of 31 coagulation- and fibrinolysis-related proteins in plasma from 25 VTE patients, 25 VTE patients who were also diagnosed with cancer (more than 10 types of solid or hematological cancer), and 25 healthy controls [47]. The ability to deliver high precision and specificity for quantifying multiple coagulation proteins in a rapid and simultaneous manner was demonstrated using only 5 μl plasma from each sample. Mass spectrometry results correlated well with the results of traditional ELISA and activity assays and went beyond by measuring multiple additional proteins. Remarkably, unsupervised hierarchical clustering grouped 17 of the 25 patients with VTE and cancer together, suggesting that protein profiles may possibly be used to distinguish cancer from non-cancer patients in the setting of VTE. Since the initial study, the panel has been extended to 270 validated plasma protein assays (Appendix E) [49,50], and will be used within the PLATO-VTE cohort to establish patient group specific protein signatures.

11. Rationale for patient eligibility criteria

The inclusion of patients 40 years or older was based on the assumption that the risk of yet undetected cancer in younger patients is negligible. In four previous cancer screening trials totalling 1,644 patients with unprovoked VTE without age restrictions, none of the patients with an age below 40 were diagnosed with cancer during follow-up [6,9,51,52]. A subsequent individual patient meta-analysis of contemporary studies assessing cancer screening in unprovoked VTE confirmed these figures with a 12-month risk of cancer in those younger than 40 years of 0.5% (95% CI, 0.03–8.2%) [2]. Patients with both distal and proximal deep vein thrombosis of the lower extremity are eligible, as the risk of cancer appears to be independent of the location and extent of the index VTE [53–55].

As the present study focuses on patients with an unprovoked VTE event, major and minor transient provoking risk factors for VTE, such as immobilization and estrogen therapy, are exclusion criteria. Similarly, patients with previous unprovoked VTE and known thrombophilia are excluded because the intrinsic thrombotic tendency is more likely to be the cause of VTE rather than cancer in these patients [56].

Patients who were diagnosed with cancer or received cancer-related treatment in the past 5 years are excluded given the likelihood of VTE being related to recurrent cancer. Patients with cancer treated before this 5-year period are considered to have achieved long-term remission, hence eligible for inclusion.

12. Rationale for a follow-up duration of 12 months

A follow-up duration of 12 months was chosen, since the risk of a new cancer diagnosis is highest in the first year after a VTE event, and rapidly declines thereafter. For example, a large Danish population-based study compared the risk of cancer in 77,247 VTE patients with the expected cancer risk in the general population and reported a relative risk (standardized incidence ratio) of 2.75 (95% CI, 2.6 to 2.9) with deep vein thrombosis and 3.27 (95% CI, 3.03 to 3.52) with pulmonary embolism in the first 12 months of follow-up [3]. Beyond 12 months, these numbers were 1.11 (95% CI, 1.07–1.16) and 1.15 (95% CI, 1.09–1.22), respectively [3]. An individual patient data meta-analysis of 2,316 patients with unprovoked VTE found similar results; the point prevalence of cancer was 3.5% (95%, 2.8 to 4.5) and 1.6% (95% CI, 1.0 to 2.6) between the initial screening and 12 month-follow-up for limited or extended cancer screening, respectively [2]. Thereafter, the point prevalence decreased to 1.0% (95% CI, 0.56 to 1.9) [2]. Although it is expected that the vast majority of cancers will be diagnosed in the first 12-month period, a subsequent 24-month visit will be planned to assess whether any cancer was diagnosed between 12 and 24 months. The 12-month analysis of the main manuscript will provide a conservative estimate of the diagnostic accuracy of the biomarkers assessed in case any cancer is missed. Results of the analysis at 24 months will be presented in a subsequent report.

13. Expected results and future steps

The PLATO-VTE study could be the first step towards a change in cancer screening practice in patients with unprovoked VTE by using a biomarker-based approach. If platelet mRNA sequencing, ctDNA, or proteomics analysis show to have a higher sensitivity for cancer detection than standard-of-care limited screening, one or more of these tests could be evaluated in a subsequent interventional clinical trial, aiming to improve or replace current screening strategies.

The liquid biopsies evaluated in PLATO-VTE target several different

biological analytes, including DNA, RNA, and proteins. It is conceivable that highest sensitivity for cancer is achieved when using a combination of biomarker tests, rather than only a single test. For example, a recent study by Cohen et al. introduced a multiparameter liquid biopsy test called CancerSEEK as a pan-cancer screening tool, by combining ctDNA mutation analyses and 8 protein biomarkers, such as CEA and CA-125 [57]. The diagnostic accuracy of this combination test appeared to be promising; at a specificity of 99%, the median sensitivity in patients with eight different types of solid cancer was reported to be 70%, ranging from 33% in breast cancers to 93% in ovarian cancers. These findings suggest that combining several biomarkers may be preferable over the use of single biomarker tests. Therefore, subsequent analyses within the PLATO-VTE study will combine the results of several biomarkers, which may lead to the development of a novel combined assay.

In the general population, the specificity of cancer screening tests is preferably 99% to minimize false positive test results. However, a high specificity inevitably comes at the cost of a lower sensitivity. Patients with unprovoked VTE differ from the general population as they carry a high risk of an underlying cancer. Therefore, the diagnostic accuracy trade-off is different in these patients. In the setting of unprovoked VTE, a high sensitivity is essential to minimize the proportion of false negative test results, which reflects the proportion of missed cancers. However, higher sensitivity of these tests increases the proportion of false positive test results in those without cancer (i.e., lower specificity). In other words, a lower test cut-off will lead to a higher cancer detection rate, but more patients without cancer will also have a positive test result and be referred for additional, targeted testing. The minimally acceptable trade-off between the true and false positive rate (i.e., net benefit) is a matter of debate. In the present study, two minimally acceptable net benefit cut-offs were prespecified, depending on the sensitivity of the biomarkers.

Biomarker testing is likely to be more cost-effective than previously assessed extended cancer screening strategies which involved expensive diagnostic imaging tests. The biomarkers assessed in the PLATO-VTE study are likely to be associated with costs ranging between $100 and $500 per assay. The question remains whether biomarkers for cancer will truly be able to detect tumors at an earlier stage, and whether this early detection actually reduces cancer-related morbidity and mortality. Moreover, the effects of overdiagnosis, for example of subclinical prostate cancer, should also be considered and weighed against the possible benefits of the screening strategies.

What could be the clinical implications of a biomarker-based screening strategy? Depending on the optimal sensitivity found in the present study, one or more biomarker tests could be performed immediately after unprovoked VTE is diagnosed, possibly leading to detection of cancers at an earlier stage. Importantly, biomarker testing is not intended as a diagnostic tool for cancer or for the assessment of cancer stage, but rather as a screening tool that guides further investigations. If sensitivity is high, a negative test result would rule out the cancer without the need for subsequent diagnostic testing, and a positive test result would indicate that further testing is needed (e.g. with imaging, endoscopies, or biopsies). We will consider a clinically valid sensitivity of platelet RNA sequencing of approximately 75% to proceed with a subsequent randomized trial comparing the two screening methods in an add-on design. The intervention arm will include both limited screening and platelet mRNA profiling, and predefined further diagnostic tests in case of a positive biomarker result. Possibly other biomarkers for cancer (e.g. circulating tumor DNA, proteomics) will be considered as well for a combined testing approach. In addition, as platelet mRNA sequencing might also be able to indicate cancer location, a more targeted approach for further testing may be developed. For example, in patients with high suspicion of colorectal cancer based on the biomarker test result, a colonoscopy could be performed as a first targeted screening test. In those with an inconclusive test result with regard to tumor location, a broader targeted testing approach (e.g. PET/CT) would apply.

If the sensitivity of the liquid biopsies assessed in PLATO-VTE is not superior to that of limited cancer screening, future improvements of these biomarkers or other biomarkers may still be useful to the VTE population. The quest for a high-sensitivity cancer screening biomarker in the VTE population should therefore not be aborted. Numerous liquid biopsies have been developed over the past decade. In the absence of head-to-head comparisons, we were unable to assess which biomarker could have the best diagnostic accuracy for cancer detection when selecting biomarkers for evaluation in the PLATO-VTE study. It is plausible that other biomarkers could also be highly accurate in our target population. In addition, as technology is improving at a rapid pace and the need of early cancer detection is high on the global research agenda, more sensitive biomarkers will likely be available soon.

The PLATO-VTE study could be the first step towards a non-invasive, biomarker-based cancer screening approach in patients with unprovoked VTE. If the sensitivity of platelet mRNA sequencing, ctDNA, and/or proteomics analysis is found to be higher than that of limited screening, potentially by combining these biomolecules, validation in an interventional clinical trial will be needed before the biomarker-based screening strategy can be applied in clinical practice.

Declaration of interest

N. Kraaijpoel, F.I. Mulder, A. van Lieshout, M. ten Wolde, Bart J.M. van Vlijmen, Yassene Mohammed, Hans-Martin Otten, Mike J.L. Peters, and Patrick M.M. Bossuyt report no conflicts of interest.

Marc Carrier has received research funding from BMS, Pfizer and Leo Pharma. He has also received Honoria from Bayer, BMS, Pfizer, Servier and Leo Pharma.

Tom Würdinger is an inventor on relevant patent applications, received funding from Illumina, Inc and is shareholder of GRAIL, Inc.

Myron G. Best is an inventor on relevant patent applications, Luis Jara-Palomares has received research funding from LEO Pharma and MSD. He has also received honoraria from Bayer Hispania, Actelion, Pfizer, Rovi, LEO Pharma, Menarini and MSD.

Pieter-Willem Kamphuisen received research grants from Daiichi Sankyo and Roche Diagnostics and the Tergooi academy.

Marcello Di Nisio declares personal consulting fees from Daiichi Sankyo, Bayer, BMS-Pfizer, Leo Pharma outside the submitted work.

Walter Ageno has received research funding from Bayer and honoraria from Bayer, Boehringer Ingelheim, BMS-Pfizer, Daiichi Sankyo, Aspen, Sanofi, Janssen, and Portola.

Jan Beyer-Westendorf has received research funding from Bayer, Daiichi Sankyo and Pfizer. He has also received Honoria from Bayer, Daiichi Sankyo, Pfizer and Portola.

Thomas Vannasche has served as a speaker and/or advisor for Boehringer Ingelheim, Daiichi Sankyo, BMS/Pfizer, Bayer, Sanofi, and Leo Pharma.

Frederikus A Klok reports research grants from Bayer, Bristol-Myers Squibb, Boehringer-Ingelheim, MSD, Daiichi-Sankyo, Actelion, the Dutch thrombosis association and the Dutch Heart foundation.

Benilde Cosmi reports speakers' fees from Daiichi Sankyo and Sanofi.

Harry Büller reports personal fees from Daiichi Sankyo, Bayer Healthcare, BMS/Pfizer, Boehringer Ingelheim, Portola, Medscape, Eli Lilly, Sanofi Aventis, and Ionis.

Nick van Es has received advisory board honoraria from Daiichi-Sankyo, Bayer, and LEO Pharma which were transferred to his institute.

Funding

Funding was granted by the Dutch Thrombosis Foundation, the Netherlands (nr: 2017–02) and by CanVECTOR, Canada (pilot trial grant, nr: 2017–01). The funders had no role the design of the study, nor in the conduct of the study and decision to submit the article for publication.

Authors' contributions

Study conception and design: NK, FIM, TW, MGB, BvV, YM, PMMB,

HRB, NvEDrafting of the manuscript: NK, FIM, TW, MGB, BvV, YM, NvE.

Critical revision of the manuscript for important intellectual content: all authors.

Final approval of the manuscript

All authors.

Ethics approval and consent to participate

Ethics approval was firs by the ethics committee of the coordinating center Amsterdam UMC, location AMC, Amsterdam, the Netherlands, on June 9th, 2016 (study nr 2016_110), followed by the ethics committees of all other participating centers. All patients provide written informed consent.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interest.

Acknowledgements

None.

Appendix A-E. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tru.2020.100030.

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