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ORIGINAL ARTICLE

Platelet RNA sequencing for cancer screening in patients with unprovoked venous thromboembolism: a prospective cohort study

Frits I. Mulder[1,2,3] | Noémie Kraaijpoel[1,3] | Marc Carrier[4] | Noori A. Guman[1,2,3] | Luis Jara-Palomares[5,6] | Marcello Di Nisio[7] | Walter Ageno[8] | Jan Beyer-Westendorf[9] | Frederikus A. Klok[10] | Thomas Vanassche[11] | Hans-Martin B. Otten[12] | Benilde Cosmi[13] | Marije ten Wolde[14] | Sjors G. J. G. In 't Veld[15,16,17] | Edward Post[15,16,17] | Jip Ramaker[15,16,17] | Kenn Zwaan[15,16,17] | Mike Peters[18] | Aurélien Delluc[4] | Pieter W. Kamphuisen[1,2] | Veronica Sanchez-Lopez[5,6] | Ettore Porreca[19] | Patrick M. M. Bossuyt[20] | Harry R. Büller[1,3] | Thomas Wurdinger[15,16,17] | Myron G. Best[15,16,17] | Nick van Es[1,3]

[1]Amsterdam UMC location University of Amsterdam, Department of Vascular Medicine, Amsterdam, the Netherlands

[2]Department of Internal Medicine, Tergooi Hospital, Hilversum, the Netherlands

[3]Amsterdam Cardiovascular Sciences, Pulmonary Hypertension & Thrombosis, Amsterdam, the Netherlands

[4]Department of Medicine, The Ottawa Hospital Research Institute, Ottawa, Ontario, Canada

[5]Medical Surgical Unit of Respiratory Diseases, Virgen del Rocio Hospital, Seville, Spain

[6]Centro de Investigación Biomédica en Red de Enfermedades Respiratorias, Instituto de Salud Carlos III, Madrid, Spain

[7]Department of Medicine and Ageing Sciences, Gabriele D'Annunzio University, Chieti, Italy

[8]Department of Medicine and Surgery, University of Insubria, Varese, Italy

[9]Thrombosis Research Unit, Department of Medicine I, Division Hematology, University Hospital "Carl Gustav Carus," Dresden, Germany

[10]Department of Medicine - Thrombosis and Hemostasis, Leiden University Medical Center, Leiden, the Netherlands

[11]Department of Cardiovascular Sciences, University Hospitals Leuven, Leuven, Belgium

[12]Department of Internal Medicine, Meander Medisch Centrum, Amersfoort, the Netherlands

[13]Department of Angiology and Blood Coagulation, S. Orsola-Malpighi University Hospital, IRCSS -University of Bologna, Bologna, Italy

[14]Department of Internal Medicine, Flevo Hospital, Almere, the Netherlands

[15]Amsterdam UMC Location Vrije Universiteit Amsterdam, Department of Neurosurgery, Amsterdam, the Netherlands

[16]Cancer Center Amsterdam and Liquid Biopsy Center, Amsterdam, the Netherlands

[17]Brain Tumor Center Amsterdam, Amsterdam, the Netherlands

[18]Department of Internal Medicine, University Medical Center Utrecht, Utrecht, the Netherlands

[19]Department of Innovative Technologies in Medicine and Dentistry, University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy

[20]Department of Epidemiology and Data Science, Amsterdam UMC, University of Amsterdam, Amsterdam, the Netherlands

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Frits I. Mulder, Noémie Kraaijpoel, Myron G. Best, and Nick van Es contributed equally to this study.

Correspondence
Frits I. Mulder, Department of Vascular Medicine, Amsterdam Cardiovascular Sciences, Amsterdam UMC/University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, the Netherlands.
Email: f.i.mulder@amsterdamumc.nl

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Abstract

Background: Platelet RNA sequencing has been shown to accurately detect cancer in previous studies.

Objectives: To compare the diagnostic accuracy of platelet RNA sequencing with standard-of-care limited cancer screening in patients with unprovoked venous thromboembolism (VTE).

Methods: Patients aged ≥40 years with unprovoked VTE were recruited at 13 centers and followed for 12 months for cancer. Participants underwent standard-of-care limited cancer screening, and platelet RNA sequencing analysis was performed centrally at study end for cases and selected controls. Sensitivity and specificity were calculated, using the predefined primary positivity threshold of 0.54 for platelet RNA sequencing aiming at 86% test sensitivity, and an additional predefined threshold of 0.89 aiming at 99% test specificity.

Results: A total of 476 participants were enrolled, of whom 25 (5.3%) were diagnosed with cancer during 12-month follow-up. For each cancer patient, 3 cancer-free patients were randomly selected for the analysis. The sensitivity of limited screening was 72% (95% CI, 52-86) at a specificity of 91% (95% CI, 82-95). The area under the receiver operator characteristic for platelet RNA sequencing was 0.54 (95% CI, 0.41-0.66). At the primary positivity threshold, all patients had a positive test, for a sensitivity estimated at 100% (95% CI, 87-99) and a specificity of 8% (95% CI, 3.7-16.4). At the secondary threshold, sensitivity was 68% (95% CI, 48-83; *p* value compared with limited screening 0.71) at a specificity of 36% (95% CI, 26-47).

Conclusion: Platelet RNA sequencing had poor diagnostic accuracy for detecting occult cancer in patients with unprovoked VTE with the current algorithm.

KEYWORDS
early detection of cancer, neoplasms, tumor biomarkers, venous thromboembolism, thrombosis, blood platelets

1 | INTRODUCTION

Liquid biopsies can be used for screening, diagnosis, or prognostication of cancer by detecting tumor-related material or tumor biomarkers in body fluids, such as blood plasma or urine. These novel methods are considered an attractive alternative to the current screening tools for cancer as they are minimally invasive, affordable, not associated with radiation exposure, and may differentiate between primary tumor locations [1,2].

Evidence shows that platelets play an important role in the response to cancer growth and metastasis [3–5], and that their interaction with cancer cells leads to substantial changes in platelet RNA expression patterns by specific splicing events, distant cell signaling interactions, and ingestion of tumor RNA [3,6–8]. The RNA expression profiles of these so-called tumor-educated platelets can be distinguished from those of healthy individuals, rendering platelets an interesting biomarker for cancer [3,9–13]. In a study [9] including 228 patients with 6 types of local or metastasized cancers and 55 healthy controls, platelet RNA sequencing was shown to accurately distinguish

Essentials

- Platelet RNA sequencing previously showed very promising results as a pan-cancer screening tool
- This technique was evaluated in a cohort of 476 patients with unprovoked venous thromboembolism
- In our cohort study, platelet RNA sequencing had a poor diagnostic accuracy
- Potential causes are preanalytical factors, inflammation, and heterogeneity in diagnosed cancers

both groups with very high diagnostic accuracy (sensitivity, 97%; specificity, 94%; area under the receiver operator curve [AUROC], 0.99). The primary cancer type was correctly identified in 71% of patients [9]. Similar findings were reported in a study with 402 patients with nonsmall-cell lung cancer and 377 matched cancer-free controls with various other conditions [10]. Although these findings indicate that platelet RNA sequencing may be a useful cancer screening tool, its diagnostic accuracy has not yet been evaluated in a prospective cohort study in patients at moderately elevated risk of cancer.

There is a strong association between cancer and venous thromboembolism (VTE) and patients with unprovoked VTE are at substantial risk of having occult cancer [14]. Studies have shown that the 12-month cancer risk is approximately 5%, which is 5-fold higher than the general population [15,16]. Therefore, international guidelines recommend a limited screening strategy to detect occult malignancies. This strategy relies on medical history, physical examination, routine laboratory measurements, chest x-ray, and age- and gender-specific additional tests according to national guidelines (eg, mammography, prostate-specific antigen, or fecal occult blood test) [17,18]. However, almost half of the underlying cancers are missed with this strategy and become clinically overt during follow-up [16]. Earlier detection of occult cancers with platelet RNA sequencing could lead to timely cancer treatment and consequently decrease cancer-related morbidity and mortality.

We here report a multicenter prospective cohort study evaluating the clinical performance of platelet RNA sequencing as a pan-cancer screening tool in patients with unprovoked VTE, and comparing it with the currently used limited screening strategy.

2 | METHODS

This investigator-initiated, multinational, prospective cohort study was registered before its initiation at ClinicalTrials.gov (NCT02739867), registered April 15, 2016. A more detailed description of the rationale and study design has been made available elsewhere [19]. The study protocol, which was approved by the ethical review boards of all participating institutions, is available as a Supplementary File. This report was prepared according to the Standards for Reporting Diagnostic accuracy studies (STARD) guidance for reporting diagnostic accuracy studies (Supplementary Table S1) [20].

2.1 | Study overview and design

Consecutive patients presenting with a first episode of unprovoked VTE, which was confirmed using imaging, were invited to participate in the study; consenting participants were followed for 12 months for cancer occurrence. Participants underwent a limited cancer screening strategy, as recommended by international guidelines, consisting of medical history, physical examination, basic blood work, chest x-ray, and additional age- and gender-specific tests, as per local protocol (eg, mammography, prostate-specific antigen, and fecal occult blood test) [17,18]. Following the baseline visit, clinic or telephone follow-up visits were scheduled at 3, 6, and 12 months, during which patients were asked about any cancer diagnosis. Whole-blood samples were collected by venepuncture from the antecubital vein at baseline (within 10 days after the VTE event) in ethylenediaminetetraacetic acid and cell free DNA (Streck) tubes. Samples were locally processed and stored at −80 °C according to the study laboratory protocol. As platelet RNA sequencing was performed centrally at study end, all study physicians, investigators, and participants were unaware of the test results during the study.

2.2 | Study group

Patients were eligible if they were aged ≥40 years and had a first episode of symptomatic, objectively-confirmed, unprovoked VTE, ie, lower-extremity deep vein thrombosis (DVT) and/or pulmonary embolism. VTE was considered unprovoked if it was not related to pregnancy or puerperium, recent immobilization for ≥3 days (<3 months), recent surgery (<3 months), recent hospitalization (<3 months), known genetic or acquired thrombophilia, or use of systemic estrogen therapy. Exclusion criteria were a known malignancy in the previous 5 years and enrolment >10 days after the VTE event. Patients with suspected cancer at presentation were only allowed to participate if the cancer had not yet been objectively confirmed by histology or cytology. All participants provided written informed consent prior to enrolment.

2.3 | Outcomes

The primary outcome was a solid or hematological cancer diagnosis over 12 months of follow-up, objectively confirmed by histology or cytology, excluding non–melanoma skin cancer and myeloproliferative neoplasms [19]. All cancer diagnoses were centrally adjudicated by 2 independent expert oncologists (H.W.M.v.L. and J.W.W.) unaware of the platelet RNA sequencing results. When histological or cytological material could not be collected, the adjudication committee only considered the cancer as being confirmed in case of a very strong clinical, radiological, and/or biochemical suspicion, taking all data into account. The committee also adjudicated cancer type and stage and whether cancers were detected as a result of abnormal limited screening tests or detected during regular follow-up [19].

2.4 | Platelet RNA sequencing

The analytical procedures of platelet RNA sequencing have been described previously in detail [9–11]. In short, following blood sample collection in ethylenediaminetetraacetic acid-coated tubes, samples were centrifuged for 20 minutes (120 × *g*, room temperature 16-22 °C), followed by a second centrifugation step of the platelet-rich plasma for 20 minutes (360 × *g*, room temperature).

After removal of the supernatant, the acquired platelet pellet was stored for 12 to 24 hours at 4 °C in 30 µL RNAlater (Ambion), and stored at −80 °C in each participating center. After shipment of all samples to the Amsterdam University Medical Center, RNA was isolated from the pellets with the MicroRNA isolation kit (mirVana, Ambion, Thermo Scientific, AM1560), reverse transcribed, amplified using a sequencing RNA kit (SMARTer Ultra Low RNA kit for Illumina Sequencing v3 and v4, Clontech), and barcoded for sequencing using an interrogation of samples kit (Truseq Nano DNA Sample Prep Kit, Illumina). Following equimolar pooling of the barcoded samples, sequencing libraries were subsequently sequenced on a sequencing platform (HiSeq 4000, Single End 100bp) (Illumina platform). These steps were quality controlled using an automated electrophoresis tool (Bioanalyzer, Agilent). Sample quality was assessed by the RNA integrity number score and visual inspection of 2 distinct ribosomal RNA peaks in the digital gel electrophoresis. The samples used for algorithm development were generated using SMARTer v3 chemistry. The samples collected and included in this study were sequenced using the SMARTer v4 chemistry (Clontech Laboratories) due to the reduced availability of the former v3 chemistry version by the manufacturer.

2.5 | Platelet RNA classification algorithm

Raw sequencing files were subjected to the standardized FASTQ-file processing and mapping pipeline [10,11], including Trimmomatic read quality assessment, STAR splice-aware read mapping, and HTseq read summarization. All subsequent analyses on RNA classification were performed in R-software (v3.3.2, R Foundation for Statistical Computing https://www.R-project.org). For the current study, a newly developed pan-cancer thromboSeq classification algorithm [21] was used, which was developed using the previously described particle swarm optimization-enhanced support vector machine algorithm [11]. In short, this pan-cancer thromboSeq classification algorithm was developed using 391 samples (asymptomatic controls, n = 121; patients with cancer, n = 270) in the training series and 385 samples (asymptomatic controls, n = 123; patients with cancer, n = 262) for algorithm optimization. Subsequently, the algorithm was validated in another 1575 samples (asymptomatic controls, n = 146; symptomatic controls, n = 333; patients with cancer, n = 1096, including 18 different tumor types, ie, breast cancer, cholangiocarcinoma, colorectal cancer, endometrial cancer, esophageal cancer, glioma, head and neck squamous cell carcinoma, hepatocellular carcinoma, lymphoma, melanoma, multiple myeloma, nonsmall-cell lung cancer, ovarian cancer, pancreatic ductal adenocarcinoma, prostate cancer, renal cell carcinoma, sarcoma, and urothelial carcinoma) reaching an overall detection accuracy of 64% at 99% specificity (AUROC of 0.91, 95% CI: 0.89-0.92), with a 493 RNA biomarker panel size. Sequencing files from patients were processed in parallel and checked for sample quality, which was considered sufficient when the detected number of RNAs was >1500 and intersample cross-correlation was >0.5 [10,11]. The support vector machine classification predictive strength was expressed as a cancer probability score ranging from 0 to 1. All laboratory procedures and processing of the RNA sequencing test results were performed by researchers unaware of the clinical study outcomes.

2.6 | Statistical analysis

For each patient diagnosed with cancer during the 12-month follow-up period, 3 patients without a cancer diagnosis were selected from the noncancer group. Controls were selected randomly from the study group without matching, to obtain overall, unselected estimates of specificity. Cancers were considered to be detected by limited screening if detected directly at initial limited screening or by targeted (additional) testing that was triggered by this screening. The limited screening. was considered false positive in case of targeted (additional) testing without a subsequent cancer diagnosis, and false negative in case of a negative limited screening result and a subsequent cancer diagnosis within 12 months.

Patients with cancer with an absent platelet sample were not included in the analysis. Patients with cancer of whom the sample had a poor quality were included in the main analysis. Participants without a cancer diagnosis and an invalid result were replaced with a randomly selected participants without a cancer diagnosis. Patients who died within 12 months without a cancer diagnosis were not included in the analysis.

The cumulative incidence of cancer was estimated using the Kaplan–Meier method. Sensitivity was defined as the cumulative proportion of patients with a cancer diagnosis at 12 months with a positive test result. Specificity was defined as the dynamic proportion of patients without a cancer diagnosis at 12 months who had a negative test result [22].

The diagnostic accuracy of the continuous platelet RNA sequencing test for cancer was evaluated by calculating the AUROC with 95% CIs obtained by DeLong method. Sensitivity and specificity estimates of platelet RNA sequencing test at all test positivity thresholds were calculated to generate a nonparametric receiver operating characteristic curve.

The accuracy of platelet RNA sequencing was additionally evaluated at 2 thresholds of the cancer probability score, which were predefined based on unpublished work using patients with cancer and asymptomatic controls: a positivity threshold of 0.54, expected to generate a 86% sensitivity, and a positivity threshold of 0.89, expected to generate a specificity of 99% [19]. Patients with a calculated cancer probability above the threshold were considered true positives if diagnosed with cancer during the 12-month follow-up period and false positives if not diagnosed with cancer. Using this positivity threshold, sensitivity and specificity estimates of platelet RNA sequencing were calculated with 95% CIs based on Wilson method.

The difference in sensitivity between platelet RNA sequencing and the limited screening strategy was tested using McNemar test statistic for paired proportions. Details of cancers missed by either limited screening or platelet RNA sequencing were analyzed. The potential clinical utility of platelet RNA sequencing was evaluated by

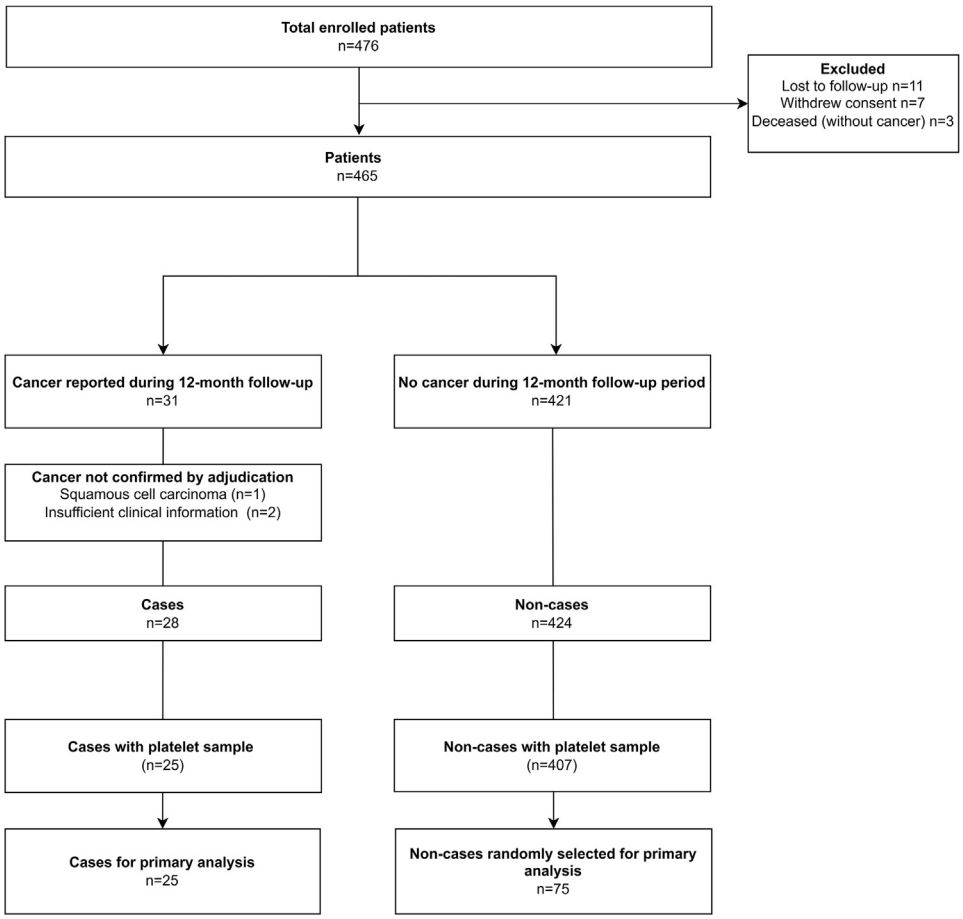

FIGURE 1 Flow chart.

weighing sensitivity (the proportion of cancer cases with a positive test result) against 1 − specificity (the proportion of noncancer cases with a positive test result) using a predefined minimally acceptable ratio of 3.0 [19,23].

The performance of platelet RNA sequencing was reported separately for patients aged <65 and ≥65 years. Sensitivity analyses were performed in (1) patients without suspected cancer at enrolment, (2) patients with cancer detected during the first 6 months of follow-up, (3) patients not using antiplatelet therapy at enrolment, (4) in samples with >1500 RNAs detected and an intersample cross-correlation of >0.5, and (5) by using the SMARTer v3 chemistry in a subset of patients, which is the version used to develop the algorithm. All analyses were conducted with R, version 3.5.1 (R Foundation for Statistical Computing, www.R-project.org).

2.7 | Sample size

Anticipating a 50% sensitivity of limited screening, a 12-month cancer incidence of 5% [24], 5% invalid platelet samples, and 5% loss to follow-up, an enrolment of at least 462 patients was required to have 80% power to demonstrate that the sensitivity of platelet RNA sequencing was higher than that of limited screening, based on McNemar test statistic for paired proportions at a 2-sided alpha of 0.05.

2.8 | Role of the funding source

This investigator-initiated study received a research grant from the Dutch Thrombosis Foundation (nr: 2017-02) and CanVECTOR (pilot trial grant, nr: 2017-01). The funders had no role in the design of the study, nor in the data collection, analyses, writing of the manuscript, or in the decision to submit the article for publication.

3 | RESULTS

3.1 | Patients

A total of 476 patients with a first unprovoked VTE were enrolled in 13 centers in 6 countries between June 2016 and October 2020. Seven (1.5%) participants withdrew consent within the 12-month study period, 11 (2.3%) were lost to follow-up, and 3 (0.6%) died without known cancer (study flowchart provided in Figure 1). The median age of the remaining 452 patients was 65 years (IQR, 56-74); 184 (41%) were women (baseline characteristics provided in Table 1). The index VTE was lower-extremity DVT in 226 (50%) patients, pulmonary embolism in 169 (37%) patients, and both DVT and pulmonary embolism in 57 (13%) patients.

TABLE 1 Patient characteristics.

Characteristic	Total Study group *n* = 452, n (%)[a]	Patients with confirmed cancer *n* = 25, n (%)[a]	Selected control patients without cancer (random sample) *n* = 75, n (%)[a]
Age, y, median (IQR)	65 (56-74)	68 (63-77)	66 (58-73)
Female	184 (40.7)	15 (60.0)	30 (40.0)
Index event			
Deep vein thrombosis only	226 (50.0)	13 (52.0)	41 (54.7)
Pulmonary embolism only	169 (37.4)	6 (24.0)	24 (32.0)
Pulmonary embolism and deep vein thrombosis	57 (12.6)	6 (24.0)	10 (13.3)
Body mass index, median (IQR)	28.1 (25.4-32.1)	27.0 (25.9-30.7)	27.6 (25.2-33.2)
Cancer suspected at enrolment	26 (5.8)	6 (24.0)	1 (1.3)
Smoking			
Current smoker	57 (12.6)	2 (8.0)	10 (13.3)
Former smoker	161 (35.6)	8 (32.0)	25 (33.3)
Never smoked	195 (43.1)	12 (48.0)	35 (46.7)
Unknown	39 (8.6)	3 (12.0)	5 (6.7)
Previous malignancy >5 years prior to enrolment	20 (4.4)	5 (20.0)	2 (2.7)
Previous provoked VTE	31 (6.9)	4 (16.0)	3 (4.0)
Previous cardiovascular disease	47 (10.4)	1 (4.0)	10 (13.3)
Hypertension	197 (43.6)	14 (56.0)	40 (53.3)
Diabetes mellitus	36 (8.0)	3 (12.0)	6 (8.0)
COPD	16 (3.5)	2 (8.0)	2 (2.7)
Autoimmune disease	25 (5.5)	2 (8.0)	7 (9.3)

COPD, chronic obstructive pulmonary disease; VTE, venous thromboembolism.
[a] Unless specified otherwise.

3.2 | Cancer

Thirty-one (6.9%) participants were reported to have cancer during the study period. Two of these potential cancer cases were not confirmed by the adjudication committee. One case was excluded because the cancer was a squamous cell carcinoma (Figure 1). The time to diagnosis for the 28 confirmed cancers is shown in Figure 2. Twenty-five (89%) of 28 patients with cancer had a platelet sample available and were included in the primary analysis. The most frequent cancer types were nonsmall-cell lung (*n* = 4, [16%]) and pancreatic cancers (*n* = 4, [16%]; Table 2) [25,26]. Cancer diagnosis was based on a histological biopsy in 24 (96%) patients and based on imaging findings in combination with a strong clinical suspicion in 1 (4%) patient. Supplementary Table S2 shows details regarding the cancer diagnoses. Median time from VTE to cancer diagnosis was 86 days (IQR, 40-157). For each cancer case, 3 participants without cancer diagnoses were randomly selected from the 424 patients without cancer during follow-up. Baseline characteristics of the 25 patients with cancer and the 75 patients without cancer that were included in the analysis are given in Table 1.

3.3 | Limited screening

In 18 (72%) of the 25 cancer cases, the cancer was detected by limited screening (limited screening true positives), and in 7 (28%) of the cancer cases the cancer was only detected during the 12-month follow-up period (limited screening false negatives). In 7 (9.3%) of the 75 noncancer cases, abnormal findings on limited screening led to targeted testing for cancer (false positives); in 68 (91%) noncancer cases, no targeted testing was performed (true negatives; Table 3). The sensitivity of limited screening was estimated at 72% (95% CI, 52-86) for a specificity of 91% (95% CI, 82-95). Cancer types missed by limited screening were pancreatic cancer (*n* = 3; diagnosed on days 36, 48, and 152), vaginal cancer (*n* = 1; diagnosed on day 98), melanoma (*n* = 2; diagnosed on days 225 and 263), and cutaneous sarcoma (*n* = 1;

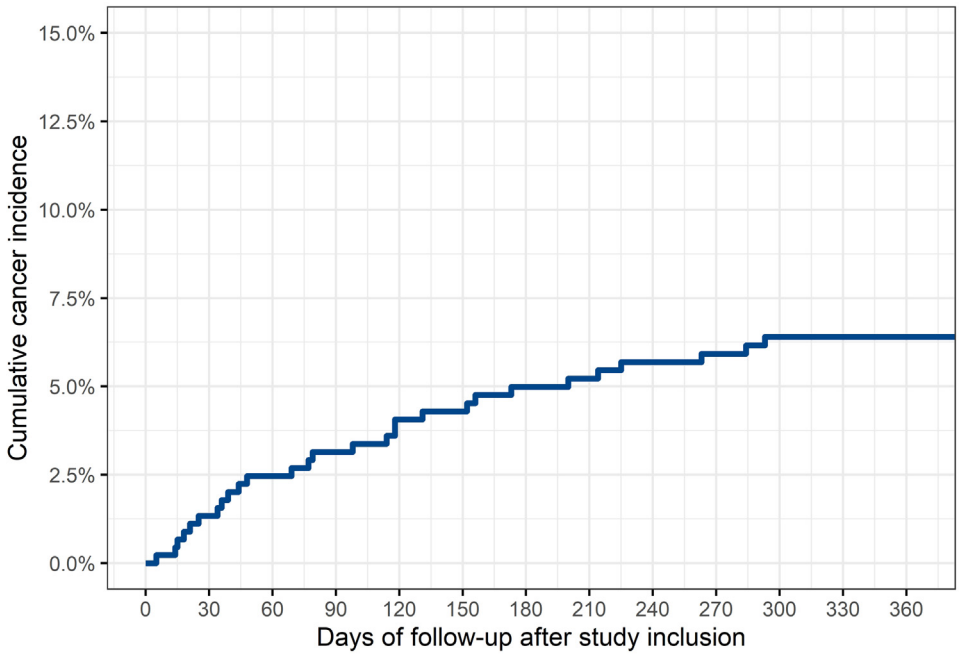

FIGURE 2 Cumulative proportion of patients with a cancer diagnosis during 12-month study period.

diagnosed on day 284). Characteristics of the detected cancers are shown in Table 2.

3.4 | Platelet RNA sequencing

Platelet RNA sequencing yielded unexpectedly high cancer probability scores, and most of the study group was predicted to have cancer (median, 0.94; IQR, 0.85-0.98). The AUROC for platelet RNA sequencing was 0.54 (95% CI, 0.41-0.66; Figure 3). The sensitivity and specificity plotted for all possible platelet RNA sequencing thresholds are shown in Figure 4. Two (2%) of 100 samples did not pass the algorithms quality check (number of RNAs <1500 or intersample correlation <0.5) but were included in the main analyses.

When evaluating platelet RNA sequencing dichotomously at the predefined primary positivity threshold of 0.54, 94 of 100 patients had a positive test result. Twenty-five (100%) of 25 cases had a positive test result (true positives); none of the cases had a negative test result (no false negatives). In 69 (92%) of the 75 noncancer cases, the platelet RNA sequencing test result was above the threshold (false positives), and in 6 (8%) the result was below the threshold (true negatives; Table 3). The estimated sensitivity of platelet RNA sequencing was 100% (95% CI, 87-99), which was statistically significantly higher than the sensitivity of the standard-of-care limited screening approach (72%; p = .008). The specificity of platelet RNA sequencing (8%; 95% CI, 3.7-16.4) was significantly lower than that of limited screening (91%; p < .001).

When evaluating the test at the secondary positivity threshold of 0.89, 17 (68%) of 25 cases had a positive test result (true positives); 8 (32%) cases had a negative test result (false negatives). In 48 (64%) of 75 noncancer cases, the platelet RNA sequencing test result was above the threshold (false positives), and in 27 (36%) the result was below the threshold (true negatives; Table 3). The sensitivity of platelet RNA sequencing was 68% (95% CI, 48-83), which was not statistically significantly different from the sensitivity of the standard-of-care limited screening approach (72%; p = .71). The specificity of platelet RNA sequencing (36%; 95% CI, 26-47) was significantly lower than that of limited screening (91%; p < .001).

The cancer types missed by platelet RNA sequencing at this threshold (0.89) were pancreatic cancer (n = 2; diagnosed on day 21 and 48), nonsmall-cell lung cancer (n = 1; diagnosed on day 77), renal cancer (n = 2; diagnosed on day 200 and 214), melanoma (n = 2; diagnosed on day 225 and 263), and cutaneous sarcoma (n = 1; diagnosed on day 284) (Supplementary Table S2). The ratio between sensitivity and 1 − specificity was 1.06, well below the predefined minimally acceptable ratio of 3.0, which would indicate a net clinical benefit.

3.5 | Additional analyses

The estimated AUROC for platelet RNA sequencing was 0.62 (95% CI, 0.44-0.81) for patients aged <65 years and 0.64 (95% CI, 0.48-0.80) for those aged ≥65 years. The AUROC was comparable to the one from the main analyses when the 2 samples with an insufficient number or RNAs (≤1500) were excluded (0.54; 95% CI, 0.41-0.66). Outcomes from the other sensitivity analyses were also consistent with the main results (Supplementary Table S3).

4 | DISCUSSION

This international prospective cohort study evaluated the performance of platelet RNA sequencing for cancer detection in 476 patients with unprovoked VTE. Approximately 6% of the study group was diagnosed

TABLE 2 Characteristics of detected cancers.

Characteristic	Overall *n* = 25, n (%)[a]	Detected by limited screening *n* = 18, n (%)[a]	Detected during follow-up *n* = 7, n (%)[a]
Days from VTE to cancer diagnosis, median (IQR)	86 (40-157)	81 (32-124)	153 (78-249)
Solid or hematological cancer			
Solid	22 (88.0)	15 (83.3)	7 (100.0)
Hematological	3 (12.0)	3 (16.7)	-
Cancer type			
Pancreatic cancer	4 (16.0)	1 (5.6)	3 (42.9)
Lung cancer (NSCLC)	3 (12.0)	3 (16.7)	-
Melanoma	2 (8.0)	0 (0.0)	2 (28.6)
Ovarian cancer	2 (8.0)	2 (11.1)	-
Prostate cancer	2 (8.0)	2 (11.1)	-
Renal cancer	2 (8.0)	2 (11.1)	-
Breast cancer	1 (4.0)	1 (5.6)	-
Colon cancer	1 (4.0)	1 (5.6)	-
Esophageal cancer	1 (4.0)	1 (5.6)	-
Leiomyosarcoma (pelvis)	1 (4.0)	1 (5.6)	-
Lung cancer (carcinoid)	1 (4.0)	1 (5.6)	-
Dermatofibrosarcoma protuberans	1 (4.0)	0 (0.0)	1 (14.3)
Vaginal cancer	1 (4.0)	0 (0.0)	1 (14.3)
Chronic lymphocytic leukemia	1 (4.0)	1 (5.6)	-
Follicular Lymphoma	1 (4.0)	1 (5.6)	-
Non-Hodgkin lymphoma	1 (4.0)	1 (5.6)	-
Solid cancer stage			
Local	8 (36.4)	5 (33.3)	3 (42.9)
Regional	6 (27.3)	5 (33.3)	1 (14.3)
Distant	8 (36.4)	5 (33.3)	3 (42.9)
Hematological cancer stage			
Stage I (Non-Hodgkin lymphoma)	1 (33.3)	1 (33.3)	-
Stage IV-B (follicular lymphoma)	1 (33.3)	1 (33.3)	-
Unknown (Chronic lymphocytic leukemia)	1 (33.3)	1 (33.3)	-
Method of cancer diagnosis			
Histology/cytology	24 (96.0)	18 (100.0)	6 (85.7)
Radiology	1 (4.0)	0 (0.0)	1 (14.3)
Cancer suspected at enrolment			
Yes	6 (24.0)	6 (33.3)	-
No	19 (76.0)	12 (66.7)	7 (100.0)

Cancer stage for solid cancers was classified according to the prognostic staging system of the American Joint Committee on Cancer criteria [25]. Lymphoma was staged according to Ann Arbor Staging classification and chronic lymphocytic leukemia according to the Rai et al. [26] classification.

NSCLC, nonsmall-cell lung cancer; VTE, venous thromboembolism.

[a] Unless specified otherwise.

TABLE 3 Cross tabulation of platelet RNA sequencing and limited screening for cancer in case-control analysis.

Test ouctome		
	Cancer	No Cancer
Positive test	18	7
Negative test	7	68
	25	75
	Sensitivity 72% (95% CI, 52-86)	Specificity 91% (95% CI, 82-95)
Platelet RNA sequencing (primary threshold 0. 54)		
	Cancer	No Cancer
Positive test	25	69
Negative test	0	6
	25	75
	Sensitivity 100% (95% CI, 87-99)	Specificity 8.0% (95% CI,3.7-16.4)
Platelet RNA sequencing (secondary threshold 0.89)		
	Cancer	No Cancer
Positive test	17	48
Negative test	8	27
	25	75
	Sensitivity 68% (95% CI, 48-83)	Specificity 36% (95% CI, 26-47)

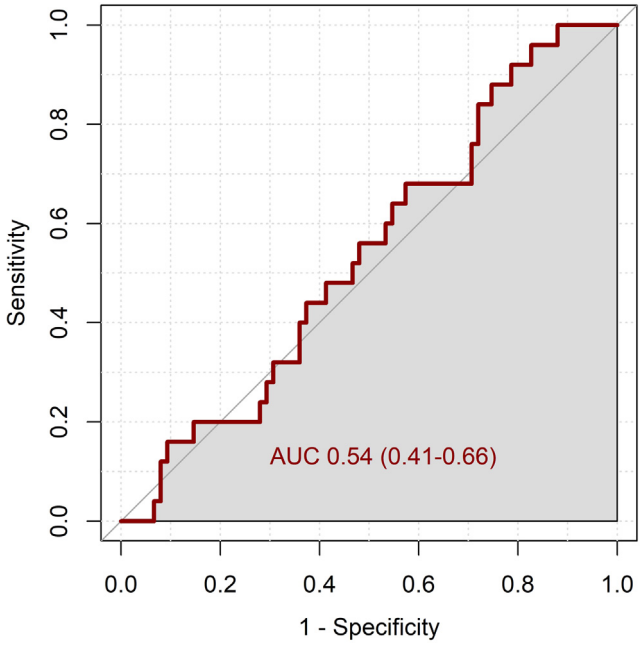

FIGURE 3 Receiver operating characteristic curve of platelet RNA sequencing for cancer diagnosis. AUC, area under the curve.

with cancer over the 12-month follow-up period of which 28% were not identified by the standard-of-care limited screening strategy. Platelet RNA sequencing yielded unexpectedly high cancer probability scores in most patients, resulting in poor diagnostic accuracy which was much lower than that of limited screening.

Platelet RNA sequencing showed promising results in previous studies in which the AUROC indicated excellent diagnostic accuracy (≥0.93) [9,10,27,28]. Reasons for the profound disparity between these studies and the current findings are yet unclear. Besides the poor analytical performance of platelet RNA sequencing itself, several factors could partly explain this observation.

There were substantial differences in patient characteristics between the current study group and the set of individuals used to develop the self-learning pan-cancer screening algorithm. This algorithm was developed with the use of platelet RNA profiles from asymptomatic healthy individuals without acute VTE. The systemic prothrombotic and inflammatory state associated with acute VTE could lead to specific alterations of the platelet RNA profiles, for example, due to (systemic) platelet activation or shifts in platelet subpopulations [29]. These alterations may have interfered with the platelet RNA-based test, resulting in high cancer probability scores for noncancer individuals. The profound effects of VTE on RNA profiles have previously been described for whole-blood RNA [30,31]. Therefore, it may be necessary to develop a dedicated VTE platelet RNA classification algorithm that includes such patients with VTE in the development set.

In one retrospective case-control study of 402 patients with stage IV metastasized nonsmall-cell lung cancer and 377 cancer-free controls, platelet RNA sequencing was associated with good diagnostic performance (AUROC, 0.94; 95% CI, 0.92-0.96) despite including controls with (inflammatory) diseases, such as pulmonary hypertension (n = 34), angina pectoris (n = 11), atherosclerosis (n = 13), multiple sclerosis (n = 58), epilepsy (n = 21), and chronic pancreatitis (n = 6) [10]. Whether delaying sample collection and testing after the acute VTE moment (eg, 3 months after the event) improves diagnostic accuracy is currently unknown. The study group used to develop the pan-cancer screening algorithm was younger than the current study (median age, 51 vs 65 years). In addition, the development studies used for training of the algorithm largely comprised patients with advanced-stage cancer, who had often received cancer treatment at the time of sample collection. Conversely, cancer cases in our study were not diagnosed with cancer yet at the time of blood sample collection, nor did they receive cancer treatment.

Another factor to consider is anticoagulant treatment, which all patients received at the time of blood sample collection. To explore this possible explanation, we evaluated 7 patients, not included in the present study, before the start of anticoagulation for VTE and at approximately 10 days of follow-up, in an explorative analysis. The intra-individual variation in platelet RNA sequencing test results between both time points was high (Supplementary Table S4). The intra-individual variation of platelet RNA sequencing has not been evaluated before, nor have the effects of exercise, diet, or comedication on test performance. Therefore, it is unclear whether the high variability is a unique observation for our study population. Follow-up studies should include such an evaluation and perhaps even require standardized moments of platelet collection.

Preanalytical factors should also be considered when analyzing the poor performance of platelet RNA sequencing. These may include

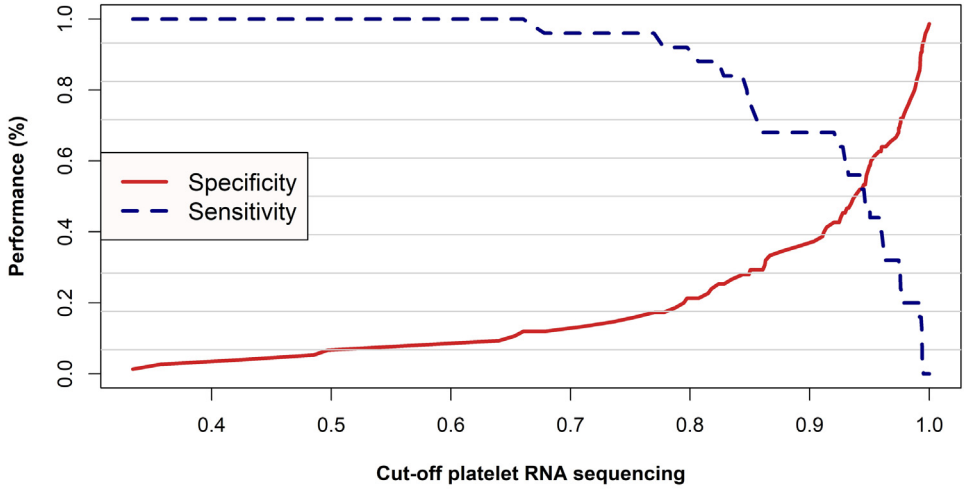

FIGURE 4 Sensitivity and specificity of platelet RNA sequencing at various positivity thresholds.

variations in local blood sample collection, sample processing, platelet isolation, leukodepletion, and storage. However, all centers adhered to the laboratory protocol and only a minority of platelet samples was considered as having insufficient RNA quality (2%). Further optimization and standardization of the platelet RNA isolation, perhaps using automated platelet processing machines, should be investigated.

Unlike the current study, previous studies on platelet RNA sequencing relied on 2-gate designs in which cases and controls were selected from separate study populations [3,9]. These designs have been empirically shown to generate inflated estimates of diagnostic test performance [32–34]. Potential causes explaining the poor performance of platelet RNA sequencing in this validation study are summarized in Table 4.

A major strength of this study is the recruitment of a consecutive series of patients in 13 academic and nonacademic centers from 6 countries, yielding a representative group of patients with unprovoked VTE. Laboratory analyses and collection of outcome events were performed by researchers unaware of the platelet RNA sequencing test result. Researchers who evaluated the cancer probability of participants with the platelet RNA sequencing algorithm were blinded for cancer diagnosis. Samples were collected and processed according to a strict laboratory protocol and checked for RNA quality. Cancer diagnoses were adjudicated centrally to increase internal validity. Limitations include the fact that platelet RNA sequencing was not performed for the total cohort, which precluded calculation of positive and negative predictive values. We used 12-month clinical follow-up as the reference standard, which can lead to an underestimated specificity of platelet RNA sequencing if cancer was present at baseline but did not become clinically overt during this period. An analysis with a longer follow-up period of 24 months is planned.

TABLE 4 Potential causes of the observed poor performance of platelet RNA sequencing.

Potential causes
Differences in patient characteristics between derivation study and validation study
Differences in cancer characteristics and stages between derivation study and validation study
Prothrombotic inflammatory state following acute VTE interferes with platelet RNA
Anticoagulant treatment received by all patients in derivation study interferes with platelet RNA
Preanalytical factors include variations in local blood sample collection, sample processing, platelet isolation, and storage
Inflated test performance in derivation study due to 2-gate design
Poor performance of the pan-cancer algorithm

VTE, venous thromboembolism.

The diagnostic accuracy of platelet RNA sequencing for cancer screening in patients with unprovoked VTE was disappointing in this study. It is well-known that validation studies of new diagnostic or prognostic tests often fail to demonstrate similar performance as in derivation studies. As outlined above, this disparity may relate to patient selection (ie, patients with VTE vs asymptomatic controls and anticoagulation use), study design (ie, prospective cohort vs 2-gate design), preanalytical factors (ie, sample collection, handling, and storage), and analytical factors [33]. Additional analyses may focus on the development of dedicated algorithms for platelet RNA sequencing specifically tailored to the VTE population, potentially using the data from this study. Whether other promising liquid biopsy pan-cancer screening tools, such as circulating tumor DNA or proteomics, can improve the current screening approach will be the topic of investigation in future studies. Until then, the current limited screening approach for occult cancer in patients with unprovoked VTE remains the recommended approach.

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ETHICS STATEMENT

Ethics approval was first granted by the ethics committee of the coordinating center Amsterdam UMC, location AMC on June 9, 2016 (study nr 2016_110), followed by the ethics committees of all participating centers. All patients provided informed consent before participating in the study.

AUTHOR CONTRIBUTIONS

N.v.E., N.K., P.M.M.B., and H.R.B. conceived and designed the study. F.I.M., N.K., N.v.E., P.M.M.B., and H.R.B. drafted the first manuscript. F.I.M., N.v.E., N.A.G. verified underlying data. All authors critically revised the paper for important intellectual content and approved the final version of the paper.

DECLARATION OF COMPETING INTERESTS

N.K., F.I.M., N.A.G., M.t.W., H.-M.B.O., S.G.J.G.I.t.V., E.P., V.S.-L., K.Z., J.R., P.M.M.B., and E.P. report no conflicts of interest. M.C. has received research funding from BMS, Pfizer, and LEO Pharma. He has also received Honoria from Bayer, BMS, Pfizer, Servier, and LEO Pharma. A.D. has received research funding from BMS-Pfizer and Honoria from Bayer, BMS-Pfizer, Servier, and LEO Pharma. T.W. is an inventor on relevant patent applications, received funding from Illumina Inc, and is a shareholder of GRAIL, Inc. L.J.-P. 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. P.W.K. has received research grants from Daiichi Sankyo and Roche Diagnostics M.D.N. has received research funding from LEO Pharma and honoraria and consultancy fees from Daiichi Sankyo, Bayer, BMS-Pfizer, Sanofi, and LEO Pharma outside the submitted work. M.G.B. is an inventor on relevant patent applications. W.A. has received research funding from Bayer and honoraria from Bayer, BMS-Pfizer, Aspen, Sanofi, Janssen, Werfen, LEO Pharma, and Portola. J.B.-W. has received research funding from Bayer, Daiichi Sankyo, Pfizer, and Portola/Alexion. He has also received Honoria from Bayer, Daiichi Sankyo, Pfizer, and Portola/Alexion. T.V. has served as a speaker and/or advisor for Boehringer Ingelheim, Daiichi Sankyo, BMS/Pfizer, Bayer, Sanofi, and LEO Pharma. F.A.K. has received research grants from Bayer, Bristol-Myers Squibb, Boehringer Ingelheim, MSD, Daiichi Sankyo, Actelion, the Dutch thrombosis association, and the Dutch Heart foundation. B.C. has received speakers' fees from Daiichi Sankyo and Sanofi. H.B. reports personal fees from Daiichi Sankyo, Bayer Healthcare, BMS/Pfizer, Boehringer Ingelheim, Portola, Medscape, Eli Lilly, Sanofi Aventis, and Ionis. N.v.E. has received advisory board honoraria from Daiichi Sankyo, Bayer, and LEO Pharma which were transferred to his institute.

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SUPPLEMENTARY MATERIAL

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