Article:
RIva, N., Vella, K., Hickey, K. et al. (2018) Biomarkers for the diagnosis of venous thromboembolism: D-dimer, thrombin generation, procoagulant phospholipid and soluble P-selectin. Journal of Clinical Pathology, 71 (11). pp. 1015-1022. ISSN: 0021-9746

https://doi.org/10.1136/jclinpath-2018-205293

Research article for Journal of Clinical Pathology

Biomarkers for the diagnosis of venous thromboembolism: D-Dimer, Thrombin Generation, Procoagulant Phospholipid and soluble P-selectin

Running title: biomarkers for VTE diagnosis

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Word count

Abstract: 249 – Text: 3100
Tables: 2 – Figures: 4
Detailed methodology reported in the supplement

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ABSTRACT

Background: The diagnostic algorithm for venous thromboembolism (VTE) currently involves a composite of pre-test probability, D-dimer and imaging. Other laboratory tests, however, may assist in the identification of VTE patients.

Aim: To assess the accuracy of different coagulation tests (D-dimer, thrombin generation, phospholipid-dependent [PPL] clotting time, sP-selectin) as biomarkers of acute VTE.

Methods: Random samples arriving at the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) from the Accident and Emergency Department with a request for D-dimer measurement were collected between August 2015 and February 2016. The following tests were performed: Innovance D-dimer (Siemens Healthcare Diagnostics), HemosIL D-dimer HS (Instrumentation Laboratory), thrombin generation (using the CAT), STA Procoag PPL (Diagnostica Stago), sP-selectin (Affymetrix, eBioscience). VTE was objectively confirmed by compression ultrasonography, computed tomography pulmonary angiography or ventilation/perfusion lung scan.

Results: 100 samples were collected (33 with VTE). A strong positive linear correlation was found between the two D-dimer tests ($r=0.97$, $p<0.001$). VTE patients showed significantly higher sP-selectin concentrations compared to patients without VTE (75.7 ng/mL vs 53.0 ng/mL, $p<0.001$). In the random forest plot, the two D-dimer assays showed the highest variable importance, followed by sP-selectin. A sP-selectin cut-off of 74.8 ng/mL was associated with 72.7% sensitivity and 78.2% specificity for acute VTE in our cohort.

Conclusion: Our results confirmed D-dimer as the main biomarker of VTE and speculated a role for sP-selectin. The impact of thrombin generation was limited and no role emerged for the PPL clotting time. These observations need to be confirmed in large management studies.

Keywords: biomarkers, diagnosis, venous thromboembolism

1. INTRODUCTION

Venous thromboembolism (VTE), which encompasses deep vein thrombosis (DVT) and pulmonary embolism (PE), is a common public health problem worldwide, with an incidence of approximately 100 new cases per 100,000 persons every year [1]. The diagnostic algorithm for VTE currently involves a composite of clinical pre-test probability scores (such as the Wells or the Geneva scores [2-4]), laboratory D-dimer and specific imaging tests (such as compression ultrasound for DVT and computed tomography or ventilation/perfusion lung scan for PE).

D-dimer is a very useful diagnostic test to rule out the suspicion of VTE, due to its high sensitivity and negative predictive value [5]. However, the low specificity mandates further diagnostic tests to confirm the diagnosis of VTE. Furthermore, there are other laboratory coagulation tests (such as the thrombin generation, the procoagulant phospholipid-dependent clotting time and the soluble P-selectin) that are currently used only for research purposes but might contribute to identify a pro-thrombotic predisposition. So far, some alterations of the thrombin generation parameters have been reported in VTE patients, although at a small extent [6-8]. Studies evaluating patients with acute DVT showed increased values of soluble P-selectin compared to normal controls [9, 10]. Finally, the procoagulant phospholipid-dependent clotting time can detect the presence of procoagulant microparticles in the plasma [11], but has never been tested in the diagnosis of VTE before.

The aim of this study was to evaluate the accuracy of different laboratory tests (D-dimer, thrombin generation, phospholipid-dependent clotting time and soluble P-selectin), and their relative importance, as biomarkers of acute VTE.

2. MATERIALS AND METHODS

2.1 Study population

From August 2015 to February 2016 we collected random samples arriving at the Coagulation Laboratory at Mater Dei Hospital (in Msida, Malta) from the Accident and Emergency Department with a request for D-dimer measurement. This sample collection was part of a service development

initiative, due to the imminent change in the D-dimer assay in our laboratory and the need to test a certain number of samples with both assays in order to assure reproducible results. Samples were divided into three groups: negative D-dimer without VTE (group 1), positive D-dimer without VTE (group 2), samples from patients with VTE confirmed by compression ultrasonography, computed tomography pulmonary angiography or ventilation/perfusion lung scan (group 3). We planned a sample size of at least 25 patients per group. All samples were taken before any anticoagulants were administered and before a diagnostic test was performed. The decision whether to perform imaging tests was entirely left at the discretion of the attending physicians; however, none of the patients with VTE excluded at the time of D-dimer test had a VTE diagnosis in the following 3 months.

Since this study consisted in an analysis of residual plasma from anonymized samples that were previously used to validate the new D-dimer assay as part of our laboratory standard operating procedure, ethical approval was waived by our University Ethics Committee.

The following tests were performed: Innovance D-dimer (Siemens Healthcare Diagnostics Products GmbH, Germany), HemosIL D-dimer HS (Instrumentation Laboratory, Italy), thrombin generation (using the Calibrated Automated Thrombogram), procoagulant phospholipid-dependent clotting time (STA Procoag-PPL kit, Diagnostica Stago, France), sP-selectin (Affymetrix, eBioscience, Austria). Details regarding sample collection and tests performed are reported in the ***Supplement***. The results of each test were available for the following number of samples: Innovance D-Dimer 82 samples, HemosIL D-Dimer HS 98 samples, thrombin generation 92 samples, sP-selectin 94 samples, procoagulant phospholipid-dependent clotting time 98 samples. The test results were not available for all samples because of technical issues in the performance of the tests or insufficient plasma.

2.2 Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) or as median with interquartile range (IQR), when data did not have a normal distribution (according to the Wilk-

Shapiro test); categorical variables were expressed as counts and percentages. Continuous variables were compared using the Mann-Whitney U test for the comparison of two groups, or the Kruskal-Wallis test for the comparison of three or more groups (the Dunn's test with p values adjusted with the Bonferroni correction was used for the post-hoc analysis). Unadjusted and adjusted median differences (according to age and sex), together with their 95% confidence interval (CI), were also calculated.

Significant results were graphically represented using box and whisker plots, where the line inside the box is the median value, and the bottom and top limits of the box represent the first and third quartiles. The whiskers represent the lower and the upper adjacent values, while the outliers are represented as dots.

The correlation between different laboratory tests was evaluated using the nonparametric Spearman's rank correlation test, according to data distribution, and the correlation coefficients (r) were calculated. In order to evaluate the clinical agreement and to estimate the percentage of D-dimer values which might have resulted in a different clinical management, the D-dimer values were categorized as positive or negative, according to the manufacturers' cut-offs (500 ng/mL FEU for the Innovance D-dimer and 230 ng/mL for the HemosIL D-dimer).

To assess the predictive accuracy of each biomarker, we measured the area under the receiver operating characteristic (ROC) curves, equivalent to the C (concordance)-statistics. The C-statistic represents the concordance between predicted and observed events, with c=0.5 for prediction no better than chance and c=1.0 for perfect discriminative ability [12].

Considering only those samples with all test results available, we applied random forest algorithm to identify the relative importance of each biomarker in VTE prediction (Innovance D-dimer, HemosIL D-dimer, Lag time, Peak thrombin concentration, Time to peak, Endogenous thrombin potential, Velocity index, PPL clotting time and sP-selectin). In this procedure 1000 decision trees were grown to form the random forest and a random subset of variable was used at each split point with 3 out of 9 variables used in each subset. The out of bag error was calculated as an unbiased

estimate of misclassification error of the random forest method. The random forest method was applied to two different models: 1) considering all potential biomarkers of VTE; 2) excluding D-dimers, which are well-known biomarkers of VTE, in order to identify any other emergent biomarker which might be obscured by the D-dimers. To assess the importance of the variables, we evaluated: mean minimal depth (it assumes that variables with high impact on the prediction are those that most frequently split nodes nearest the root of the trees), accuracy decrease (mean decrease of prediction accuracy) and Gini decrease (mean decrease in the Gini index of node impurity). A decisional classification tree algorithm was applied to biomarkers identified by the random forest, to find the cut-offs associated with the highest sensitivity/specificity.

Data analysis was performed using the statistical software STATA SE 12 (StataCorp LP, College Station, TX, USA), SAS v. 9.4 (SAS Institute Inc, Cary, NC, USA) and R software packages (Party and randomForest) [13-15]. Two-tailed p values less than 0.05 were considered statistically significant.

3. RESULTS

3.1 Study population

Overall, 100 samples were collected. Median (IQR) age was 59.0 (41.3-70.2) years, and 47% were males. Thirty-three patients had confirmed VTE: 16 PE, 11 lower limb proximal DVT and three isolated distal DVT, one upper limb proximal DVT and two superficial vein thrombosis of the great saphenous vein. Patients with VTE were significantly older than patients without VTE (median age 69.5 vs 52.0, respectively, $p<0.001$), while sex distribution was not significantly different (males 45.5% vs 47.8%, respectively, $p=0.83$).

3.2 D-dimers

A strong positive linear correlation was found between the two D-dimers ($r = 0.97$, $p<0.001$) and is shown in ***Figure 1***. The clinical agreement between the two D-dimers in the categorization of

patients as positive/negative was 93.8%, since four patients with positive Innovance D-dimer were classified as negative by HemosIL D-dimer HS, and one patient with positive HemosIL D-dimer HS was classified as negative by Innovance D-dimer. None of these five patients had VTE and overall none of VTE patients had a negative D-dimer.

3.3 Thrombin generation

On the thrombin generation curve, patients with VTE showed prolonged lag time (median 5.42 vs 4.5 min, p<0.001) and prolonged time to peak (median 8.59 vs 7.33 min, p=0.004) compared to patients without VTE. After adjustment for age and sex, median lag time, time to peak and also velocity index were significantly different between the two groups. Detailed results are reported in ***Table 1*** and ***Figure 2***.

Results of thrombin generation divided into the three groups of patients are reported in ***Table 2***. Apart from the differences between patients with and without VTE, among patients without VTE we observed that those with positive D-dimer had higher peak thrombin concentration (median 318.2 vs 278.5 nM, p=0.007) and higher velocity index (median 121.2 vs 91.6 nM/min, p=0.003) compared to those with negative D-dimer.

3.4 Procoagulant phospholipid-dependent clotting time

There was no difference in the PPL clotting time between patients with and without VTE (***Table 1***), or between patients with positive vs. negative D-dimer (***Table 2***). These results did not change when the PPL clotting time was expressed as ratio (***Tables 1-2***).

3.5 Soluble P-selectin

The median concentration of sP-selectin was significantly higher in patients with VTE compared to patients without VTE (75.7 ng/mL vs 53.0 ng/mL, p<0.001; adjusted median difference 25.1, 95%

CI 11.7 to 38.5) (***Table 1, Figure 2***). No difference was found between patients with positive vs. negative D-dimer (***Table 2***).

3.6 Correlation between D-dimers and the other biomarkers of VTE

There was a weak or no correlation between the two D-dimers (Innovance D-dimer and HemosIL D-dimer HS) and the other biomarkers of VTE: lag time (r=0.21 p=0.07 and r=0.22 p=0.06, respectively); peak thrombin concentration (r=0.38 p=0.0008 and r=0.38 p=0.0009); time to peak (r= -0.03 p=0.81 and r= -0.02 p=0.84); endogenous thrombin potential (r=0.19 p=0.11 and r=0.19 p=0.11); velocity index (r=0.44 p=0.0001 and r=0.44 p=0.0001); PPL clotting time (r= - 0.18 p=0.11 and r= -0.18 p=0.12); sP-selectin (r=0.38 p=0.0007 and r=0.36 p=0.002).

3.7 Receiver operating characteristic curves

Excluding the D-dimers, which are well-known biomarkers of VTE and which may have been influenced by our sample selection, among the other potential biomarkers the best predictive value for VTE was identified by the sP-selectin concentration (area under the ROC curve [AUC] 0.77; 95% CI, 0.66-0.87); lag time (AUC 0.73; 95% CI, 0.61-0.85) and time to peak (AUC 0.68; 95% CI, 0.56-0.81).

The predictive value was poor for the remaining parameters: peak thrombin concentration (AUC 0.45; 95% CI, 0.31-0.59); endogenous thrombin potential (AUC 0.48; 95% CI, 0.34-0.61); velocity index (AUC 0.45; 95% CI, 0.31-0.58); PPL clotting time (AUC 0.52; 95% CI, 0.39-0.65). The ROC curves are reported in ***Figure 3***.

3.8 Importance of each biomarker in VTE prediction

A random forest plot, generated from data of 69 patients with all test results, showed that the two D-dimer assays had the highest variable importance, followed by sP-selectin concentration, with an out of bag error (prediction error of the random forests algorithm) of 10% (***Figure 4 a,b***). In the

model without D-dimers, sP-selectin concentration was the most relevant biomarker (***Figure 4 c,d***). The classification tree on sP-selectin showed that the best cut-off in our sample was 74.8 ng/mL with a sensitivity of 72.7% and a specificity of 78.2%.

In order to evaluate whether the sP-selectin might improve the predictive ability of the D-dimers, we created two logistic models considering these biomarkers as dichotomous variables (Innovance D-dimer and HemosIL D-dimer HS according to manufacturers' cutoff, sP-selectin positive above our identified cut-off of 74.8 ng/mL). We observed that the addition of sP-selectin improved both the AUC of the HemosIL D-dimer HS (p=0.0004) and the AUC of the Innovance D-dimer (p=0.0006).

4. DISCUSSION

In this study we evaluated several tests, namely two D-dimers, the thrombin generation, the PPL clotting time and the sP-selectin, as potential biomarkers of VTE. Our results suggest that the sP-selectin concentration has a good predictive value for the diagnosis of VTE and can also improve the predictive ability of the D-dimer tests.

D-dimers are well-known biomarkers of VTE with small differences in sensitivity and specificity reported with the use of different assays [16]. The Innovance D-dimer and the HemosIL D-dimer HS are two latex-enhanced turbidimetric immunoassays, and are classified among those with the highest sensitivity for VTE (93-95%), although at the price of lower specificity (50-53%) [5]. We found a strong positive linear correlation between these two D-dimers (r=0.97) and a very good clinical agreement, with 93.8% of samples equally classified as negative or positive. Despite the design of our study not allowing calculation of specificity and sensitivity, we observed less false positive results with the HemosIL D-dimer HS, in line with the very high negative predictive value reported by the manufacturer.

In our study two thrombin generation parameters emerged as potentially associated with the diagnosis of VTE: prolonged lag time and time to peak. Although in hypercoagulable states the lag

time (time until the initiation of thrombin generation) and the time to peak (time to reach the peak thrombin concentration) are usually shortened [17], several previous studies analysing patients with suspected VTE reported prolonged lag time and time to peak in those with confirmed VTE [6-8, 18]. These findings suggested that the thrombin generation on the CAT is delayed and prolonged in patients with acute VTE and, therefore, several authors have hypothesized that the increased thrombin generation *in vivo* is associated with consumption of coagulation factors and reduced thrombin generation potential *ex vivo* [7, 8]. In contrast, the endogenous thrombin potential (the total amount of thrombin generated), which is considered to be the parameter that best reflects the actual generation of thrombin, has been reported to be increased in acute VTE in some studies [6, 7]; while in others [8, 18], including our study, no statistically significant difference emerged between the two groups. However, the diagnostic accuracy and the relevance of the thrombin generation parameters in our study cohort was inferior compared to other biomarkers, suggesting a limited application of this test to VTE diagnosis.

Our study, for the first time, evaluated the PPL clotting time in the setting of VTE diagnosis. The PPL coagulation time is a functional test that measures a clotting time dependent on procoagulant phospholipid and is based on the principle that procoagulant microparticles will shorten the activated factor X clotting time [11, 19]. Microparticles are emerging biomarkers of venous thrombosis, being increased in patients with acute VTE, although it is still unclear whether microparticles themselves are a cause or a consequence of the thrombosis [20]. Microparticles are traditionally measured by flow cytometry, which provides information about their absolute number and cellular origin, but can not detect their functional activity [19]. Considering the great correlation between the PPL clotting time and flow cytometry [11, 19], we hypothesised that the PPL clotting time could be useful also in the management of patients with suspected VTE. However, our results showed that this assay does not appear to be correlated with the diagnosis of VTE.

Finally we investigated P-selectin, a cell adhesion molecule, expressed on the surface of activated platelets and endothelial cells, that can be released in soluble form into the plasma [21]. Recent

evidence suggest that P-selectin might have a role also in thrombosis and haemostasis, since it can mediate platelet rolling, generate procoagulant microparticles and enhance fibrin deposition [22, 23]. In our study sP-selectin showed a good predictive value and was the most relevant biomarker of VTE, after the D-dimers. Furthermore, we identified a sP-selectin cut-off of 74.8 ng/mL, which showed high sensitivity (72.7%) and high specificity (78.2%) in our cohort.

Our results are in line with previous findings. Rectenwald et al. [9] in a pilot study of patients diagnosed with DVT reported higher mean concentrations of sP-selectin in 22 patients with acute DVT (0.98 ± 2.03 ng/mg of total protein), compared to 21 symptomatic patients without DVT (0.55 ± 0.08) and 30 controls (0.34 ± 0.05). They also identified a threshold of sP-selectin (0.68 ng/mg of total protein) which provided the highest sensitivity and the highest specificity (68% and 81%, respectively). Furthermore, combining this threshold of sP-selectin with total microparticles (> 125% of controls) and D-dimer (> 3 mg/L), they obtained a sensitivity of 73% and a specificity of 81% [9]. Ramacciotti et al. [10] reported higher levels of sP-selectin in 62 patients with DVT vs 116 patients without DVT (87.3 ± 44 ng/L vs 53.4 ± 24 ng/mL, $p<0.0001$). A combination of sP-selectin cut-off ≥ 90 ng/mL combined with Wells score ≥ 2 resulted in a specificity of 95%, a sensitivity of 33% and a positive predictive value of 100% for the diagnosis of DVT. In contrast a combination of sP-selectin cut-off ≤ 60 ng/mL combined with Wells score < 2 resulted in a sensitivity of 99%, a specificity of 33% and a negative predictive value of 96% for the exclusion of DVT [10]. More recently Torres et al. [24] reported a trend towards increased value of sP-selectin in 15 patients with previous VTE vs. 20 normal individuals (median concentration 90 vs. 72 ng/mL, respectively, $p=0.099$). However, in this study blood samples were collected after the acute phase (at least one month after the last VTE).

The main strength of our study is the simultaneous comparison of different biomarkers in suspected VTE with objectively confirmed VTE at imaging tests. However, there are also some limitations that need to be acknowledged. First, not all test results were available for all samples, due to technical errors in the tests or insufficient plasma available. Furthermore, being a collection of

anonymised samples, sex and age were the only available demographics characteristics and we could not apply a posteriori the clinical prediction rules for VTE. Second, the median time to storage of VTE samples was longer than the other patients groups (approximately 4.5 vs 1.5 hours). While there is some evidence that changes in D-dimer are low after storage at room temperature for up to 24 hours [25, 26], data regarding plasma stability for the other tests are scarce. However, our results are in line with previous studies addressing the role of thrombin generation, on samples stored within 1-2 hours from collection, which reported a prolongation of lag time and time to peak in VTE patients [7, 8]. On the other hand, it is unknown whether the delayed freezing time could have influenced the PPL clotting time or the sP-selectin concentration. Third, our sample size was relatively small, but similar to previous studies assessing the role of sP-selectin in patients with suspected VTE [9, 24].

In conclusion, our study confirmed the D-dimer as the main biomarker of VTE and hypothesised a role for sP-selectin. The impact of thrombin generation is limited, while it seems that there is no role for PPL clotting time. However, this data should be confirmed in large management studies.

FOOTNOTES

Contributors: N.Riva and A.Gatt contributed to the conception and design of the study, analysis and interpretation of data and drafted the article. K.Vella, K.Hickey, D.Zammit, and S.Spiteri contributed to acquisition and interpretation of data. L.Bertù contributed to analysis and interpretation of data. S.Kitchen, M.Makris and W.Ageno contributed to interpretation of data and critical revision of the manuscript. All authors provided final approval of the manuscript.

Funding: This study was supported by a research grant from the University of Malta.

Competing Interests: None declared.

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- The Innovance D-dimer and the HemosIL D-dimer HS showed a strong positive linear correlation
- The two D-dimer assays showed the highest variable importance, in patients with suspected venous thromboembolism, followed by sP-selectin concentrations
- A sP-selectin cut-off of 74.8 ng/mL was associated with 72.7% sensitivity and 78.2% specificity for acute venous thromboembolism
- Thrombin generation had a limited impact as biomarker of venous thromboembolism, while no role emerged for the phospholipid-dependent clotting time

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Table 1. Results of thrombin generation, procoagulant phospholipid-dependent clotting time and soluble P-selectin in patients with and without venous thromboembolism

	Patients without VTE (n=67)	Patients with VTE (n=33)	Unadjusted median difference (95% CI)	Adjusted median difference (95% CI)
Thrombin generation				
Samples with available results, n	60	32		
• Lag time (min)	4.5 (4-5)	5.42 (4.75-6.25)	0.84 (0.34 to 1.34)	0.84 (0.30 to 1.38)
• Peak thrombin concentration (nM)	288.1 (257.3-329.8)	276.8 (224-339.2)	-8.5 (-49.9 to 33.0)	-17.8 (-62.5 to 26.91)
• Time to peak (min)	7.33 (6.67-8.17)	8.59 (7.25-9.92)	1.17 (0.36 to 1.98)	1.32 (0.38 to 2.26)
• Endogenous thrombin potential (nM/min)	1609.8 (1465.8-1966.8)	1743 (1269.3-1934.3)	73.5 (-202.1 to 349.0)	100.9 (-176.72 to 378.6)
• Velocity index (nM/min)	106.8 (78.5-134.8)	98.4 (62.3-127.0)	-5.1 (-30.4 to 20.2)	-32.6 (-57.9 to -7.4)
Procoagulant phospholipid-dependent clotting time				
Samples with available results, n	67	31		
• PPL clotting time (sec)	35.5 (31.6-38.9)	35.7 (31.5-41.2)	0.20 (-3.9 to 4.3)	-0.88 (-3.3 to 5.1)
• PPL clotting time (ratio)	0.83 (0.74-0.91)	0.83 (0.74-0.96)	0.0005 (-0.09 to 0.10)	0.02 (-0.08 to 0.12)
Soluble P selectin				
Samples with available results, n	63	31		
• sP-selectin concentration (ng/mL)	53.0 (41.9-63.2)	75.7 (51.6-93.6)	22.7 (6.66 to 38.7)	25.1 (11.7 to 38.5)

All results are reported as median (IQR). Median difference is reported between the VTE positive and the VTE negative patients, unadjusted and adjusted for age and sex.

Legend: CI=confidence interval, IQR=interquartile range, PPL= procoagulant phospholipid, VTE=venous thromboembolism

Table 2. Results of thrombin generation, procoagulant phospholipid-dependent clotting time and soluble P selectin in the 3 subgroups of patients: negative D-dimer without VTE (group 1), positive D-dimer without VTE (group 2) and patients with VTE (group 3)

	Group 1: negative DD (Innovance DD < 500 ng/mL FEU) (n=32)	**Group 2: positive DD (Innovance DD ≥ 500 ng/mL FEU) (n=35)**	**Group 3: VTE (n=33)**
Thrombin generation			
Samples with available results, n	30	30	32
• Lag time (min)	4.33 (3.83-4.67) #	4.59 (4.17-5.17) §	5.42 (4.75-6.25) # §
• Peak thrombin concentration (nM)	278.5 (232.9-292.2) °	318.2 (279.8-345.9) ° §	276.8 (224-339.2) §
• Time to peak (min)	7.25 (6.67-8.67) *	7.5 (6.67-7.83) †	8.59 (7.25-9.92) * †
• Endogenous thrombin potential (nM/min)	1552.3 (1409.5-1826)	1688.5 (1559-1998)	1743 (1269.3-1934.3)
• Velocity index (nM/min)	91.6 (64.7-108.9) °	121.2 (99.1-139.4) ° §	98.4 (62.3-127.0) §
Procoagulant phospholipid-dependent clotting time			
Samples with available results, n	32	35	31
• PPL clotting time (sec)	36.8 (32.4-38.8)	33.4 (31.6-40.2)	35.7 (31.5-41.2)
• PPL clotting time (ratio)	0.86 (0.76-0.91)	0.78 (0.74-0.94)	0.83 (0.74-0.96)
Soluble P selectin			
Samples with available results, n	30	33	31
• sP-selectin concentration (ng/mL)	47.9 (38.4-61.8) #	55.5 (42.1-66.4) †	75.7 (51.6-93.6) # †
Time to storage (hh:mm)	01:21 (01:05-01:41) #	01:31 (01:22-01:50) †	04:37 (02:14-07:29) #†

All results are reported as median (IQR). Classification into negative DD and positive DD was based on the Innovance DD cut-off 500 ng/mL FEU. When the Kruskal-Wallis test was significant, the differences were further analysed with the Dunn's test and reported as follows:

° $p \leq 0.01$ for the comparison group 1 vs group 2

* $p < 0.05$ for the comparison group 1 vs group 3

# $p < 0.01$ for the comparison group 1 vs group 3

§ $p < 0.05$ for the comparison group 2 vs group 3

† $p < 0.01$ for the comparison group 2 vs group 3

Legend: DD=D-dimer, FEU=fibrinogen equivalent units, IQR=interquartile range, PPL= procoagulant phospholipid, VTE=venous thromboembolism

LEGEND TO FIGURES

Figure 1. Correlation between the Innovance D-dimer and the HemosIL D-dimer HS
The vertical dashed line represents the cut-off for the Innovance D-Dimer (500 ng/mL FEU), while the horizontal dashed line represents the cut-off for the HemosIL D-dimer HS (230 ng/mL).
D-dimer values above the upper limits (>4400 ng/mL FEU for the Innovance D-dimer and >3610 ng/mL for the HemosIL D-dimer HS) have been displayed as one unit above the limit.

Figure 2. Biomarkers significantly different in patients with and without venous thromboembolism: lag time (a) and time to peak (b) on the thrombin generation curve, soluble P-selectin concentration (c)
Legend: VTE = venous thromboembolism

Figure 3. Receiver operating characteristics curve for different biomarkers of venous thromboembolism: Lag time (a), Peak thrombin concentration (b), Time to peak (c), Endogenous thrombin potential (d), Velocity index (e), PPL clotting time (f), sP-selectin (g)

Figure 4. Random forest and multi-way importance plot for the model including all potential biomarkers of VTE (a, b) and for the model excluding the D-dimers (c, d)
In the random forest method, 1000 decision trees were grown to form the random forest. A random subset of variable was used at each split point (3 random variables out of 9 available were used in each subset). Only variables that were chosen at least in half of the trees are represented. Minimal depth is the distance from the root of the tree and assumes that variables with high impact on the prediction most frequently split nodes near the root of the trees. Accuracy decrease is the mean decrease of prediction accuracy when a certain variable is removed from the model. Gini index decrease is the mean decrease in the index of node impurity (e.g. the variance in a node). The higher decrease of accuracy and Gini index means higher variable importance.