
Article:
RIva, N., Vella, K., Hickey, K. et al. (7 more authors) (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

Online supplemental data to: Riva N, Vella K, Hickey K, et al. Biomarkers for the diagnosis of venous thromboembolism: D-Dimer, Thrombin Generation, Procoagulant Phospholipid and soluble P-selectin**.** Journal of Clinical Pathology 2018

Detailed methodology

Sample collection and tests performed

D-dimer

Venous blood sample was collected into vacuum coagulation tubes, containing 2 mL of whole blood and sodium citrate 0.109M/3.2% (Vacuette, Greiner Bio-One). According to the local standard operating procedure, samples arrived at the Coagulation Laboratory using the pneumatic tube system, they were centrifuged for 10 minutes at 2500g (Eppendorf Centrifuge 5810) and plasma was immediately analysed in order to measure D-dimer levels.

The standard D-Dimer test at our laboratory until December 2015 was the Innovance D-Dimer (Siemens Healthcare Diagnostics Products GmbH, Germany), a latex-enhanced turbidimetric immunoassay, performed on the automated coagulation analysers Sysmex CA-1500 or CS-2100i (Siemens Healthcare Diagnostics Products GmbH, Germany) [1]. The cut-off for the Innovance D-Dimer was 500 ng/mL FEU. From January 2016 onwards, the standard D-Dimer test at our laboratory was the HemosIL D-Dimer HS (Instrumentation Laboratory, Italy), another latex-enhanced turbidimetric immunoassay, performed on the automated coagulation analyser ACL TOP 500 (Instrumentation Laboratory, Italy) [2]. The cut-off for the HemosIL D-Dimer was 230 ng/mL. In our laboratory, the inter-assay coefficients of variation (CV) of the D-dimer tests were 5.0% for the Sysmex CA-1500, 7.0% for the Sysmex CS-2100i, and 10.6% for the ACL TOP 500. Samples were tested with both D-Dimers, either as fresh plasma or frozen/thawed plasma. It has previously been demonstrated that freezing plasma does not affect the D-Dimer results [3].

For the purpose of this study, the remaining plasma was separated and centrifuged again for 10 minutes at 2500g, and it was stored in 300 µL aliquots at -80° C within a 2-hour time frame from phlebotomy. However, since we encountered some difficulties in the collection of samples from patients with confirmed VTE, the time window for the second centrifugation process and the freezing of platelet poor plasma (PPP) for this group was subsequently extended up to 14 hours. The first centrifugation process was still performed within 2 hours, according to the standard local practice, and afterwards samples were stored at controlled room temperature. It has previously been demonstrated that the mean percentage of changes for D-Dimer after storage of samples at room temperature up to 24 hours is <10% [4, 5].

Thrombin generation

Thrombin generation was performed using the Calibrated Automated Thrombogram (CAT), according to the method described by Hemker et al [6].

Prior to this analysis, samples were thawed in a water bath at 37° C for 5 minutes. Afterwards, 80 µL of PPP were added to 20 µL of tissue factor trigger at a concentration of 1pM (PP-reagent-LOW, Thrombinoscope BV, Maastricht, the Netherlands) in a 96-well plate. We chose the 1pM concentration, since it is more sensitive to plasma levels of procoagulant factors (such as factors VIII, IX and XI) compared to the 5pM concentration [7]. Although the addition of corn trypsin inhibitor (CTI) can increase the CAT sensitivity at low tissue factor concentrations [8], we decided not to use CTI since a recent study showed its beneficial effect only when the CAT is triggered with tissue factor concentrations below 0.5 pM [9]. All samples were tested in duplicate and one calibrator (Thrombin Calibrator, activity 580 nM) well was run in parallel. Three quality control (QC) plasma samples were tested in each run.

The reaction was initiated after automated dispensing of 20 µL of fluorogenic substrate (FluCa-kit, Thrombinoscope BV, Maastricht, the Netherlands). The fluorescence intensity was measured for 90 minutes using a Fluoroskan Ascent fluorimeter (Thermo Electron Corporation), after the samples

were incubated for 10 minutes at 37°C. Using a dedicated software (Thrombinoscope BV, Maastricht, the Netherlands, version 3.4.0.154), the following parameters were calculated: lag time (LT), peak thrombin concentration (Peak), time to peak thrombin (ttPeak), endogenous thrombin potential (ETP) and velocity index.

In our experiment, the intra-assay coefficient of variation (CV) of the ETP parameter on thrombin generation was 4.3% and the inter-assay CV for the normal QC was 5.5%.

Procoagulant phospholipid-dependent clotting time

The procoagulant phospholipid (PPL)-dependent clotting time was measured using the STA Procoag-PPL kit (Diagnostica Stago, France) on the automated coagulation analyser ACL TOP 500 (Instrumentation Laboratory, Italy), as described by Exner et al [10]. It is a phospholipid-dependent factor Xa-based clotting time. Briefly, 25 µL of thawed PPP was incubated at 37°C with 25 µL of PPL-depleted plasma (provided in the test kit), to replace the coagulation factors. Afterwards, 100 µL of an activating reagent containing factor Xa and calcium was added, therefore triggering the coagulation cascade at the level of factor Xa and eliminating the interference of upstream coagulation factors. Under these conditions, clot formation depends only on PPL present in the plasma sample. The clotting time is recorded and expressed in seconds. A shorten clotting time reflects increased levels of PPL, which is known to correlate with the functional activity of microparticles present in the patient sample [11]. We also tested two kit controls with known clotting time, to check the reproducibility of the assay.

This clotting time was also compared with a reference time, obtained from the median value of PPP from 20 healthy controls, and the results were expressed as a ratio (clotting time of the tested plasma / reference clotting time). The ratio is <1 when the clotting time of the tested plasma is shortened compared to control plasma, meaning increased levels or PPL.

According to manufacturer's instructions, the intra-assay and inter-assay CVs for this assay were <1% and <2.5%, respectively.

Soluble P-selectin

Soluble P-selectin (sP-selectin) was measured using an enzyme-linked immunosorbent assay (ELISA) technique and a commercial kit (Human sP-selectin Platinum ELISA, Affymetrix, eBioscience, Austria). According to manufacturer's instructions, 10 µL of PPP was diluted 10-fold into the sample diluent provided in the test kit, in a 96-microwell plate coated with monoclonal antibodies anti-human sP-selectin to bind the sP-selectin present in the PPP. Afterwards, 50 µL of HRP-conjugated monoclonal antibodies anti-human sP-selectin was added into each well, in order to bind, in turn, the sP-selectin captured by the antibodies. After incubation at room temperature for 2 hours, the plate was washed to remove the unbound antibodies and 100 µL of Substrate Solution (tetramethyl-benzidine) reactive with the HRP was added into each well. The plate was incubated again at room temperature, monitoring the colour development, which is proportional to the concentration of sP-selectin, and when the highest standard developed a dark blue colour the reaction was stopped by adding 100 µL of Stop Solution (1M phosphoric acid). The absorbance of each microwell was measured at 450 nm wavelength using a microplate reader (DS2, Dynex Technologies, Germany). For each plate, a standard curve from 7 standard dilutions with known sP-selectin concentration was generated with the software DS-matrix 1.34 performing a linear regression. Results were converted into sP-selectin concentrations and reported as ng/mL. We also tested two kit controls with known sP-selectin concentration in each run, to check the reproducibility of the assay. All samples and controls were run in duplicates.

According to manufacturer's instructions, the intra-assay and inter-assay CVs for this assay were 7.8% and 5.4%, respectively.

References

1. Innovance D-dimer (version 01/2016) [Package insert], Siemens Healthcare Diagnostics Products GmbH, Germany.
2. HemosIL® D-dimer HS 0020007700 (version 02/2017) [Package insert], Instrumentation Laboratory, Italy.
3. Woodhams B, Girardot O, Blanco MJ, Colesse G, Gourmelin Y. Stability of coagulation proteins in frozen plasma. Blood Coagul Fibrinolysis. 2001;12(4):229-36.
4. Kemkes-Matthes B, Fischer R, Peetz D. Influence of 8 and 24-h storage of whole blood at ambient temperature on prothrombin time, activated partial thromboplastin time, fibrinogen, thrombin time, antithrombin and D-dimer. Blood Coagul Fibrinolysis. 2011;22(3):215-20.
5. Zhao Y, Lv G. Influence of temperature and storage duration on measurement of activated partial thromboplastin time, D-dimers, fibrinogen, prothrombin time and thrombin time, in citrate-anticoagulated whole blood specimens. Int J Lab Hematol. 2013;35(5):566-70.
6. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, Lecompte T, Béguin S. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb. 2003;33(1):4-15.
7. van Veen JJ, Gatt A, Makris M. Thrombin generation testing in routine clinical practice: are we there yet? Br J Haematol. 2008;142(6):889-903.
8. van Veen JJ, Gatt A, Cooper PC, Kitchen S, Bowyer AE, Makris M. Corn trypsin inhibitor in fluorogenic thrombin-generation measurements is only necessary at low tissue factor concentrations and influences the relationship between factor VIII coagulant activity and thrombogram parameters. Blood Coagul Fibrinolysis. 2008;19(3):183-9.
9. Spronk HM, Dielis AW, Panova-Noeva M, van Oerle R, Govers-Riemslag JW, Hamulyák K, Falanga A, Cate HT. Monitoring thrombin generation: is addition of corn trypsin inhibitor needed? Thromb Haemost. 2009;101(6):1156-62.
10. Exner T, Joseph J, Low J, Connor D, Ma D. A new activated factor X-based clotting method with improved specificity for procoagulant phospholipid. Blood Coagul Fibrinolysis. 2003;14(8):773-9.
11. Campello E, Spiezia L, Radu CM, Gavasso S, Woodhams B, Simioni P. Evaluation of a procoagulant phospholipid functional assay as a routine test for measuring circulating microparticle activity. Blood Coagul Fibrinolysis. 2014;25(5):534-7.