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Laboratory and Psycho-social Aspects of Anticoagulation and Venous Thromboembolism

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Nicoletta Riva

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Declaration of Authenticity

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Publications and conferences presentations

The following papers, extracted from this thesis, have been already published in peer-reviewed medical journals:

- Riva, N., Vella, K., Meli, S., Hickey, K., Zammit, D., Calamatta, C., Makris, M., Kitchen, S., Ageno, W., & Gatt, A. (2017). A comparative study using thrombin generation and three different INR methods in patients on Vitamin K antagonist treatment. Int J Lab Hematol, 39(5), 482-488.
- Riva, N., Vella, K., Hickey, K., Bertù, L., Zammit, D., Spiteri, S., Kitchen, S., Makris, M., Ageno, W., & Gatt, A. (2018). Biomarkers for the diagnosis of venous thromboembolism: D-dimer, thrombin generation, procoagulant phospholipid and soluble P-selectin. J Clin Pathol, 71(11), 1015-1022.
- Riva, N., Borg Xuereb, C., Ageno, W., Makris, M., & Gatt, A. (2019). Validation and psychometric properties of the Maltese version of the Duke Anticoagulation Satisfaction Scale (DASS). Psychol Res Behav Manag, 12, 741-752.
- Riva, N., Borg Xuereb, C., Makris, M., Ageno, W., & Gatt, A. (2019). Reliability and validity of the Maltese version of the Perception of Anticoagulant Treatment Questionnaire (PACT-Q). Patient Prefer Adherence, 13, 969-979.

I wrote the first draft of all these papers, which are included in Appendix A.
Data and text from these papers are included in the relevant chapters of this thesis: Chapter 2 “Materials and Methods”, Chapter 3 “Point-of-care coagulometers for VKA monitoring”, Chapter 4 “Biomarkers of venous thromboembolism”, and Chapter 7 “Maltese translations of the DASS and the PACT-Q”.

Furthermore, the following abstracts have been presented at national or international conferences:

- Riva, N., Meli, S., Calamatta, C., Zammit, D., Vella, K., Gatt, A. Correlation between different INR tests in patients with stable anticoagulation control. Discussed poster at the 9th Malta Medical School Conference (MMSC), St. Julian’s (Malta), 3-5th December 2015.
- Riva, N., Vella, K., Meli, S., Calamatta, C., Zammit, D., Makris, M., Ageno, W., Gatt, A. A comparative study using four different clot detection methods for INR monitoring in patients on vitamin K antagonist treatment. Poster at the 62nd Annual Meeting of the Scientific and Standardization Committee (SSC) of the

International Society on Thrombosis and Haemostasis (ISTH), Montpellier (France), 25-28th May 2016.

- Riva, N., Vella, K., Zammit, D., Spiteri, S., Hickey, K., Kitchen, S., Makris, M., Ageno, W., Gatt, A. Biomarkers for the diagnosis of venous thromboembolism: D-Dimers, Thrombin Generation, and Phospholipid-dependent Clotting Time. Poster at the XXVI Congress of the International Society on Thrombosis and Haemostasis (ISTH), Berlin (Germany), 8-13th July 2017.
- Riva, N., Borg Xuereb, C., Ageno, W., Makris, M., Gatt, A. Patient satisfaction associated with warfarin treatment in different settings: a cross-sectional study. Poster at the 64th Annual Meeting of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH), Dublin (Ireland), 18-21st July 2018.
- Riva, N., Borg Xuereb, C., Ageno, W., Makris, M., Gatt, A. Patient satisfaction associated with warfarin treatment in different settings: a cross-sectional study. Discussed poster at the 10th Malta Medical School Conference (MMSC), St. Julian's (Malta), 29th November-1st December 2018.

Abstract

This thesis aimed to provide more evidence in the grey areas of the management of anticoagulation and venous thromboembolism (VTE), from both a laboratory and a clinical perspective. The following gaps were identified from the literature review and addressed in this thesis: 1) The point-of-care (POC) coagulometers represent a simpler and quicker alternative to the standard laboratory monitoring of the international normalised ratio (INR) in patients on vitamin K antagonist (VKA) treatment; however, their accuracy was questioned by several studies. By performing a comparison of the POC INR with several other assays, the accuracy of the POC devices was ascertained. 2) Despite the importance of a timely diagnosis of VTE, the current diagnostic algorithms still require a composite of clinical pre-test probability, laboratory D-dimer and imaging test. From the analysis of several potential biomarkers of acute VTE, the human soluble P-selectin was found to improve the diagnostic accuracy of the D-dimers. 3) Thromboelastography and thrombin generation are global coagulation assays, which can assess all the phases of coagulation; however, variable sensitivity of these assays has been reported to ongoing anticoagulant treatment. The thrombin generation assay appeared to be more sensitive to the presence of the direct oral anticoagulants (DOAC) than the routine coagulation assays and the specific tests. The thromboelastography appeared to be sensitive to the presence of edoxaban, rivaroxaban and dabigatran. 4) Different strategies have been proposed to reverse the effect of the anticoagulant drugs in patients with treatment-related bleeding complications; however, no clear superiority of one agent over the others has been demonstrated. Fresh frozen plasma, when used for VKA reversal, did not obtain a complete normalisation of the haemostatic balance *ex vivo*. For DOAC reversal *in vitro*, different concentrations of prothrombin complex concentrates or recombinant factor VIIa were needed to normalise the coagulation profile, based on the initial DOAC plasma concentrations. The neutralising effect of DOAC Stop® *in vitro* on basic coagulation assays was confirmed, but there might be the risk of reduction of the plasma levels of several coagulation factors. 5) There is a known correlation between patients' satisfaction and adherence to chronic treatment, but there was no validated questionnaire available in the Maltese language specifically assessing the satisfaction of anticoagulated patients. Two psychometric questionnaires (the DASS and the PACT-Q) were translated into the Maltese language and validated, by assessing their reliability and validity. 6) The use of the POC coagulometers by healthcare professionals can simplify the management of VKA patients; however, it was not clear whether they were also associated with an improvement in the quality of life. Through a comparison of patients' satisfaction associated with the POC INR vs. the laboratory INR, the POC devices were found to be associated with increased convenience and decreased hassles and burdens. 7) While the use of the POC coagulometers for self-testing has the potential of improving the time within the INR therapeutic range (TTR) and reduce the risk of thromboembolic events, contradictory findings were reported for the POC devices used by healthcare professionals. By analysing the TTR in different time frames, it emerged that the POC devices correlated better with anticoagulation control. Based on these results, recommendations for future research and clinical practice were proposed.

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A30	Amplitude at 30 minutes after MA on the TEG
A60	Amplitude at 60 minutes after MA on the TEG
ACCP	American College of Chest Physicians
ACT	Activated clotting time
ACTS	Anti-Clot Treatment Scale
AF	Atrial fibrillation
AGFI	Adjusted goodness-of-fit index
APS	Antiphospholipid syndrome
APTT	Activated partial thromboplastin time
AT	Antithrombin
AUC	Area under the ROC curve
BID	Twice daily
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAT	Calibrated automated thrombin generation assay
CFI	Comparative fit index
CFT	Clot formation time on the ROTEM
CI	Confidence interval
CIx	Coagulation index on the TEG
CL30	Clot lysis at 30 minutes after MA on the TEG
CL60	Clot lysis at 60 minutes after MA on the TEG
CT	Clotting time on the ROTEM
CV	Coefficient of variation
CWB	Citrated whole blood

DASS	Duke Anticoagulation Satisfaction Scale
DMSO	Dimethyl sulfoxide
DOAC	Direct oral anticoagulant
dRVVT	Dilute Russell's Viper Venom time
DTT	Diluted thrombin time
DVT	Deep vein thrombosis
ELFA	Enzyme-Linked Fluorescence Assays
ELISA	Enzyme-Linked ImmunoSorbent Assays
EMA	European Medicines Agency
ESC	European Society of Cardiology
ETP	Endogenous thrombin potential
FDA	US Food and Drug Administration
FEIBA	Factor VIII Inhibitor By-passing Activity
FEU	Fibrinogen Equivalent Units
FFP	Fresh frozen plasma
GFI	Goodness-of fit index
HEPES	Hydroxyethyl-piperazine ethane-sulfonic acid buffer
HIT	Heparin induced thrombocytopenia
HR	Hazard ratio
HRP	Enzyme horseradish peroxidase
ICC	Intraclass correlation coefficient
INR	International normalised ratio
IQR	Interquartile range
ISI	International sensitivity index
K time	Coagulation time on the TEG

LA	Lupus anticoagulant
LMWH	Low molecular weight heparin
LTE	Lysis time estimate on the TEG
LY30	Percent lysis at 30 minutes after MA on the TEG
LY60	Percent lysis at 60 minutes after MA on the TEG
MA	Maximum amplitude on the TEG
MCF	Maximum clot firmness on the ROTEM
NA	Not available
NC	Not computable
NEQAS	UK National External Quality Assessment Scheme
NICE	UK National Institute for Health and Clinical Excellence
OD	Once daily
OR	Odds ratio
PACT-Q	Perception of Anticoagulation Treatment Questionnaire
PBS	Phosphate buffered saline
PCC	Prothrombin complex concentrate
PCI	Percutaneous coronary intervention
PE	Pulmonary embolism
POC	Point of care
PPP	Platelet poor plasma
PPL	Procoagulant phospholipid-dependent coagulation time
PRP	Platelet rich plasma
PSM	Patient self-management
PST	Patient self-testing
PT	Prothrombin time

QC	Quality control
QoL	Quality of life
R time	Reaction time on the TEG
RCT	Randomized controlled trial
rFVIIa	Recombinant factor VII activated
RMSEA	Root mean square error of approximation
ROC	Receiver operating characteristics curve
ROTEM	Rotational thromboelastometry
SD	Standard deviation
SF-12	12-item short form
SF-36	36-item short form
SP	Split point on the TEG
sP-selectin	Soluble P-selectin
SRMR	Standardized root mean squared residual
TAFI	Thrombin activatable fibrinolysis inhibitor
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TMA	Time to MA on the TEG
TMB	Tetramethyl-benzidine
TPA	Tissue plasminogen activator
TPI	Thrombodynamic potential index on the TEG
TRALI	Transfusion-related acute lung injury
TT	Thrombin time

TTR	Time within the therapeutic range
UFH	Unfractionated heparin
VKA	Vitamin K antagonist
VTE	Venous thromboembolism
WHO	World Health Organization

Chapter 1 :

Introduction and Literature Review

1.1 Introduction

Anticoagulation is one of the most common therapies prescribed nowadays. It has been reported that the anticoagulant drugs generated sales of approximately US$ 12 billion worldwide in 2013, with a projected increase to approximately US$ 18 billion by 2018 (Chaudhari et al., 2014). Anticoagulation can be used for different clinical indications, the most common being the treatment of venous thromboembolism (VTE) and the prevention of stroke in patients with atrial fibrillation (AF). VTE is the third most common cardiovascular disease, after acute coronary syndrome and stroke (S. Z. Goldhaber, 2012), with an estimated annual incidence in the United States of 300,000-600,000 events (Benjamin et al., 2018). AF is the most common cardiac arrhythmia, with an estimated prevalence in the United States of 2.7-6.1 million (Benjamin et al., 2018).

There are several anticoagulant drugs available on the market. Heparin was the first anticoagulant to be used in clinical practice at the beginning of the 20th century (McLean, 1916). From the initial molecule, several modifications have been performed in order to produce the low molecular weight heparins (LMWHs). LMWHs are still currently used in specific situations, although requiring parenteral administration (Garcia et al., 2012). The effect of coumarins was initially described in the 1920s (Roderick, 1929), while the synthesis of warfarin dates to the late 1940s (Pirmohamed, 2006). Vitamin K antagonists (VKAs) have been the only available oral anticoagulant drugs for more than 60 years and they are still widely used for the prevention and treatment of arterial and venous thrombosis (Ageno et al., 2012). Due to their high intra- and inter-individual variability in dose-response, VKAs require periodic laboratory monitoring in order to keep an adequate anticoagulation control. The coagulation laboratory still has a crucial role in managing VKA patients, but the

recent introduction of the point-of-care (POC) coagulometers had the potential to simplify their management, by allowing the patients to perform self-monitoring of the international normalised ratio (INR) (Barcellona et al., 2017). However, the accuracy of the POC coagulometers has been recently questioned (Biedermann et al., 2015; Hur et al., 2013; Lawrie et al., 2012). Over the past two decades several novel direct oral anticoagulants (DOAC) have been produced (Husted et al., 2014). The DOACs have a more predictable anticoagulant response and more favourable pharmacokinetics properties than the VKAs, thus not requiring routine laboratory monitoring. However, specifically calibrated assays should be used, if monitoring is needed (Patel, Byrne, et al., 2019), and specific antidotes should be administered if rapid reversal is required (Garcia & Crowther, 2019).

Patients' psycho-social perspectives should also be considered, such as the adherence and the persistence with chronic anticoagulant therapies. In fact, it is well-known that "drugs don't work in patients who don't take them", as C. Everett Koop said (Osterberg & Blaschke, 2005, p.487). It is important to evaluate the level of satisfaction associated with chronic treatments, since there is a well-known correlation between dissatisfaction, decreased adherence and worse clinical outcomes (A. T. Hirsh et al., 2005; Ho et al., 2009). Nevertheless, there was no validated questionnaire, specifically assessing the quality of life and the satisfaction associated with the anticoagulant treatment, that was available in the Maltese language.

Thus, the aim of this thesis was to address some of the current gaps in the international literature and in the actual management of the anticoagulant therapy in the Maltese context. This chapter provides an overview of the most accredited models of blood coagulation, the available laboratory coagulation assays, the different anticoagulant drugs and their reversal strategies, and the different instruments for assessing patients'

perspectives on the anticoagulant treatment. The literature review provides the background for the rationale of this thesis, which is outlined in the final section (Paragraph 1.7).

1.2 Blood coagulation models

The coagulation cascade, together with the platelets and the vascular wall, constitute the three main components of the haemostatic process (Kumar et al., 2010). The term "haemostasis" refers to the normal physiological mechanism which aims to maintain the blood in a liquid state in intact vessels, but also aims to stop the blood loss from an injured vessel (Kumar et al., 2010). The physiological haemostasis leads to the formation of a plug of platelets and fibrin at the site of vessel injury, but necessitates that the pro-coagulant process remains localized at that site.

Several models of coagulation have been proposed over time. The "classic theory" of coagulation was described by Schmidt in 1892 and Morawitz in 1905 and included only four coagulation factors: calcium and thromboplastin contributed to the conversion of prothrombin into thrombin, which in turn could convert fibrinogen into fibrin (Ackroyd, 1954).

Several coagulation factors were discovered afterwards, leading to the "cascade" and "waterfall" models of coagulation in the 1960s (Davie & Ratnoff, 1964; Macfarlane, 1964). In these models, most of the coagulation factors exist in the blood as inactive precursors of the actual enzymes (zymogens) and there are several steps in which one activated coagulation factor can catalyse the activation of the next one. Schematically, the coagulation cascade was divided into two initial pathways, the intrinsic and the extrinsic pathways, which converge into a common pathway (Figure 1.1). The final product is a burst of thrombin generation.

The extrinsic pathway (or tissue factor pathway) is initiated by the exposure of circulating factor VIIa to tissue factor (TF), a transmembrane protein expressed on non-vascular cells, also known as factor III or tissue thromboplastin. The formation of the complex TF/factor VIIa catalyses the conversion of factor X to Xa (Riddel et al., 2007).

In the intrinsic pathway (or contact activation pathway), the reactions are initiated by components already present in the blood. In the presence of a negatively charged surface (e.g. the membrane of activated platelets or, *in vitro*, the glass container) and high molecular weight kininogen as cofactor, factor XII is converted to XIIa. Factor XIIa converts prekallikrein into kallikrein, which can accelerate the activation of factor XII through a positive feedback mechanism. Furthermore, factor XIIa activates factor XI to XIa. In turn, factor XIa converts factor IX to IXa. Factor IXa, along with factor VIIIa and calcium, form the "tenase complex" which catalyses the conversion of factor X into Xa (Riddel et al., 2007).

The common pathway begins when factor X is activated, by either the extrinsic or intrinsic pathways. Factor Xa, along with factor Va and calcium, form the "prothrombinase complex" which converts prothrombin (factor II) into thrombin (factor IIa). In turn, thrombin converts fibrinogen (factor I) into fibrin (factor Ia). Fibrin is initially produced as fibrin monomers, which subsequently become fibrin polymers. Furthermore, thrombin activates factor XIII (the fibrin stabilizing factor) to XIIIa, which creates the covalent crosslinks between the polymerised fibrin chains, leading to the formation of the stabilised fibrin clot (Riddel et al., 2007).

Current laboratory tests reflect the waterfall/cascade model of coagulation: the prothrombin time (PT) assesses the extrinsic pathway, the activated partial

thromboplastin time (APTT) the intrinsic pathway and the thrombin time (TT) the common pathway.

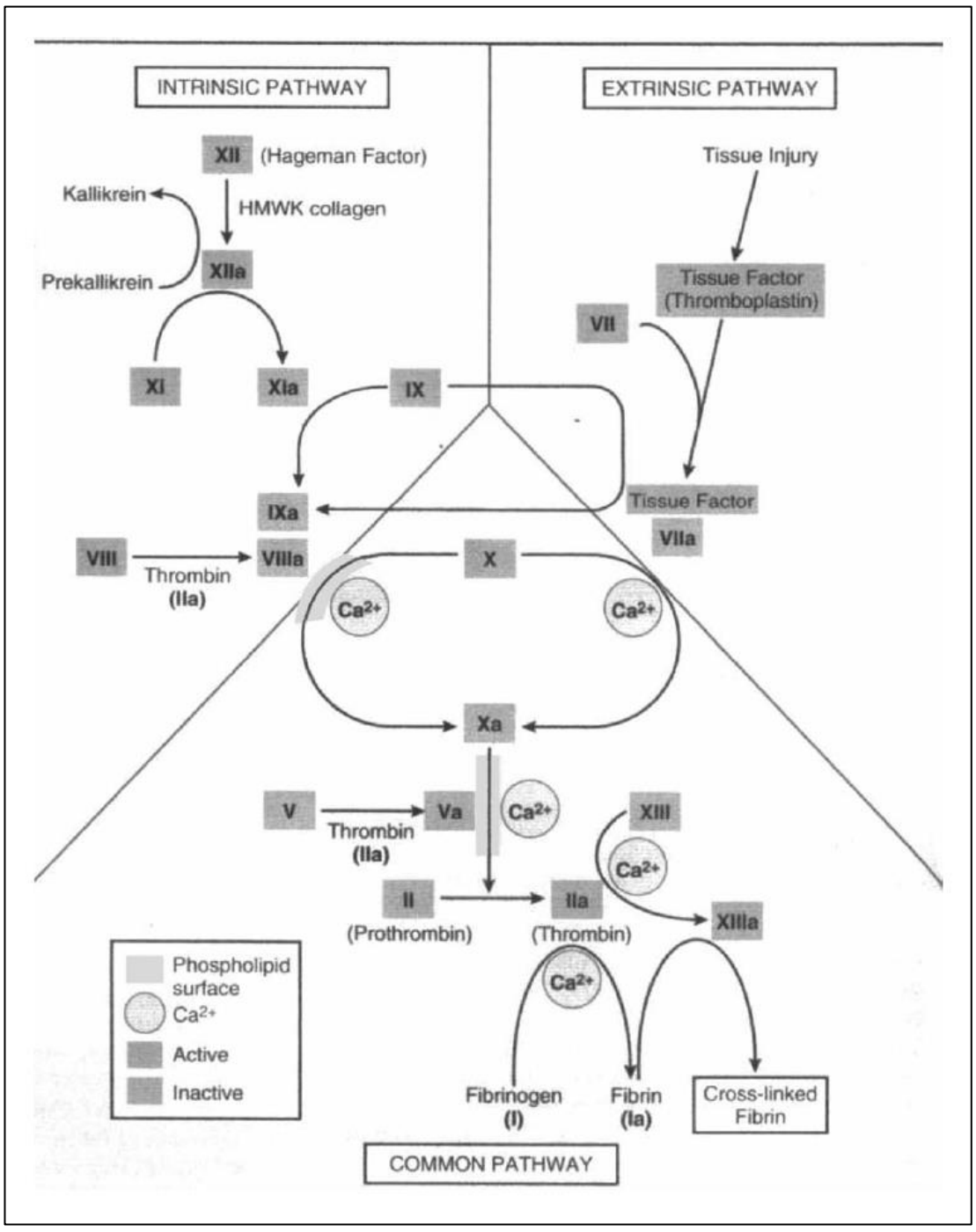


Figure 1.1 The cascade model of blood coagulation (Riddel et al., 2007) (Reproduced with permission)

Nevertheless, the waterfall/cascade model of coagulation showed several flaws. For example, it does not explain why deficiencies in factors VIII and IX are not compensated by the extrinsic TF pathway but present clinically with severe bleeding diathesis, whereas deficiencies in the initial steps of the intrinsic pathway (factor XII, high molecular weight kininogen or prekallikrein) show no bleeding tendency *in vivo*. More recently, a "cell-based" coagulation model was proposed (Hoffman, 2003). This model recognized the essential role of platelets and TF-bearing cells, such as smooth muscle cells and fibroblasts. In fact, cell surfaces not only host the reactions but also serve to localise the coagulation process at the site of injury and to prevent spreading throughout the vascular system. Furthermore, in the cell-based model the two above-mentioned pathways are not completely independent.

The three main coagulation phases have been identified as initiation, amplification and propagation (Figure 1.2). In the initiation phase, at the site of vascular injury, the TF expressed on the surface of extravascular cells binds circulating factor VIIa and they activate factors IX and X. The prothrombinase complex is formed on the phospholipid surface of cell membranes and leads to thrombin generation. However, in this phase only a small amount of thrombin is produced. The initiation phase can be terminated by the tissue factor pathway inhibitor (TFPI) secreted by the endothelium. Therefore, *in vivo* coagulation is initiated by the TF pathway (Hoffman, 2003).

In the amplification phase, the small amount of thrombin previously generated activates platelets, which in turn release factor V from their granules and expose phospholipids on their membrane surfaces. Thrombin then activates factors V and VIII, which are the cofactors involved in the formation of the tenase and prothrombinase complexes on the negatively-charged phospholipid surface of the activated platelets (Hoffman, 2003).

In the propagation phase, a considerable number of platelets are recruited at the site of injury. The tenase and prothrombinase complexes on the platelet surface lead to the generation of a consistent amount of thrombin. Thrombin converts fibrinogen into fibrin and, through the activation of factor XIII, the stabilized fibrin/platelet clot is produced. The thrombin activatable fibrinolysis inhibitor (TAFI) protects the clot from premature plasmin-mediated fibrinolysis (Hoffman, 2003).

In the physiological haemostasis, the coagulation process is limited by several counteracting anticoagulant pathways, in order to avoid the formation of thrombi in the normal undamaged vasculature. The intact endothelial cells express two transmembrane proteins, thrombomodulin (TM) and the endothelial protein C receptor (EPCR). TM can bind to circulating thrombin to form the thrombin-TM-EPCR ternary complex, which in turn activates the anticoagulant protein C (Navarro et al., 2011). Protein C binds to its cofactor protein S and they can inactivate both factors V and VIII. Endothelial cells also express TFPI, which can inhibit the TF pathway, and antithrombin (AT), which in turn can inhibit the activity of thrombin (Riddel et al., 2007). In normal haemostasis there is indeed a perfect balance between the procoagulant and the anticoagulant elements of the coagulation system.

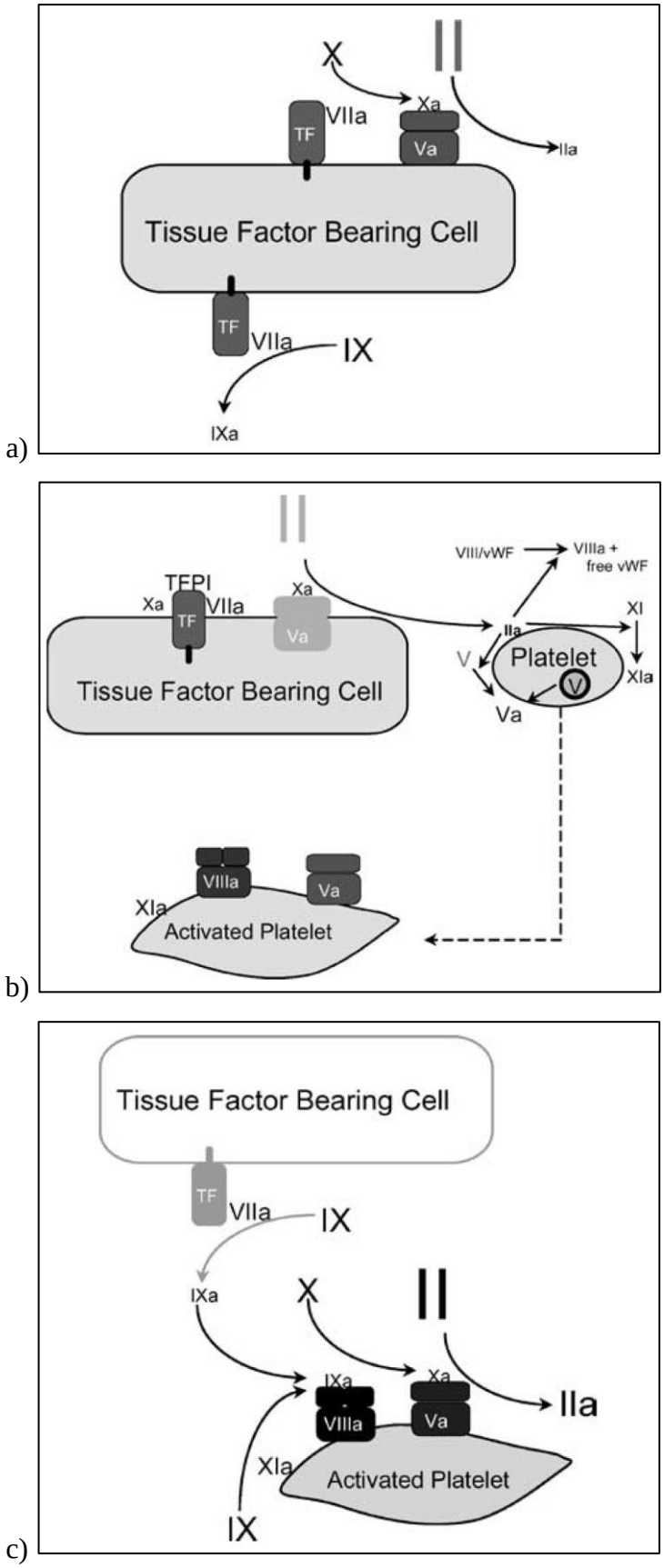


Figure 1.2 The cell-based model of coagulation representing the initiation (a), the amplification (b) and the propagation phase (b) (Hoffman, 2003) (Reproduced with permission, copyright licence no: 4645360583541)

Thrombin is considered the main coagulation factor, due to its central role in blood coagulation:

- Thrombin can activate platelets;
- Thrombin can catalyse the formation of the cofactors Va and VIIIa;
- Thrombin can convert fibrinogen into fibrin;
- Thrombin can activate factor XIII, which stabilises the fibrin clot;
- Thrombin can activate TAFI, which protects the clot from fibrinolysis;
- When bound to TM, thrombin can also initiate the protein C anticoagulant pathway, a phenomenon known as "thrombin paradox" (Griffin, 1995).

Furthermore, in the coagulation cascade there are no pathways that can bypass thrombin. For this reason, the thrombin generation capacity of the plasma can accurately reflect the balance between haemostasis and thrombosis.

1.3 Laboratory coagulation assays

1.3.1 The prothrombin time and the international normalized ratio

The VKAs were initially monitored using the PT, originally described in the 1930s (Quick, 1935). The PT assesses the time to clot formation of citrated plasma, following recalcification and addition of thromboplastin in order to trigger the coagulation cascade. Thromboplastin is a combination of phospholipids and TF, which can be recombinant or extracted from different tissues (e.g. brain, lung or placenta) of different species (e.g. human, rabbit or bovine). The PT reflects the activation of factor X by the complex TF/factor VIIa, therefore assessing the extrinsic pathway of the coagulation cascade. During warfarin treatment, a prolongation of the PT can be due to a reduction of three vitamin-K dependent coagulation factors: II, VII and X. Among

these, factor VII is the one with the shortest half-life. Therefore, in the first few days of VKA treatment, PT prolongation is mainly caused by the reduction of factor VII, afterwards accounting also for the reduction of factors II and X (J. Hirsh et al., 2001). The PT is reported in seconds or as a ratio of the patient's value over the normal control value. However, different thromboplastins have variable responsiveness to VKA, depending on their species of origin, method of preparation and phospholipid content. A responsive thromboplastin produces a slow activation of factor X by the complex TF/factor VIIa, resulting in a great prolongation of PT. Conversely, for the same reduction of vitamin K-dependent clotting factors, a less responsive thromboplastin rapidly activates factor X and results in a less prolonged PT (S. Kitchen & Preston, 1999).

In order to standardise the reporting of results and to guarantee reproducibility among different laboratories, the PT was subsequently expressed as INR, which takes into consideration the local test system and the sensitivity of the specific thromboplastin used in each laboratory. The INR is calculated dividing the patient's PT by the geometric mean of the PT range for adult normal subjects under that reagent/analyser combination, at the power of the thromboplastin's International Sensitivity Index (ISI) (Poller, 2004). The ISI is specific for each thromboplastin used in association with a particular coagulometer (test system); therefore, the same reagent can have different ISI according to the different instruments. There is an inverse relationship between sensitivity and ISI: thromboplastins with low ISI are very sensitive and provide more accurate INR results. The Guidelines of the World Health Organization (WHO) state that thromboplastins with an ISI between 0.9 and 1.7 are acceptable, but they suggest that the ISI should be in the lower part of this scale (World Health Organization, 1999). Calibration of the thromboplastin is crucial and the WHO recommends to calibrate

each thromboplastin against an international reference preparation from the same species (World Health Organization, 1999).

Although the gold standard for PT testing is the manual tilt-tube technique (Estridge & Reynolds, 2012), PT assays are currently performed using automated coagulation analysers and clotting can be detected by either photo-optical or electro-mechanical methodologies (Bennett et al., 2015). In the photo-optical coagulometers, a monochromatic light passes through the specimen and its intensity is recorded. The plasma optical density increases with fibrin formation, thus reducing the intensity of the recorded light. When the recorded light reaches a predetermined variance from baseline, it means that clot formation is obtained and the timer is stopped. This method therefore detects the change in turbidity during clotting (Bennett et al., 2015). However, there are coloured substances (e.g. haemoglobin, bilirubin) and suspended particles (e.g. lipoproteins) that might alter the plasma optical density and interfere with this assay. In order to minimize the interference of lipemic, haemolysed or icteric specimens, the plasma optical density is also measured at baseline. Examples of photo-optical analysers include the Sysmex series (Siemens Healthcare Diagnostics) and the ACL series (Instrumentation Laboratories) (Bennett et al., 2015).

In the electro-mechanical coagulometers, there is a moving steel ball within the test plasma and a magnetic sensor. The movement slows down progressively as the fibrin strands form, and the timer is stopped when a predefined rate is reached. There is a variation in which the steel ball is located on an inclined well and changes position as fibrin forms. Mechanical methods therefore measure the increase in viscosity during clotting and are unaffected by icterus or lipemia. Examples of mechanical analysers include the STA series (Diagnostica Stago) (Bennett et al., 2015).

However, data show that there is no advantage in one methodology over the other. A large study, testing more than 2,000 samples with both photo-optical (Sysmex CA-1500, Dade Behring) and electro-mechanical (STA, Diagnostica Stago) coagulation analysers, showed an excellent correlation in the PT measured by the two methodologies (r=0.99) (Bai et al., 2008). Furthermore, 26.5% of samples had visual interferences, due to haemolysis, lipemia or icterus, and the excellent correlation was confirmed also in this subgroup of samples (r=0.98) (Bai et al., 2008). Similar results emerged from another smaller study which tested more than 400 samples with different photo-optical (MTX II, TrinityBiotech) and photo-mechanical (AMAX 200, TrinityBiotech) coagulation analysers (Tekkesin & Kılınc, 2012). A very high correlation between the two different methodologies in measuring the PT was reported in the overall samples and also in the subgroup of turbid samples (r=0.97 for both analyses) (Tekkesin & Kılınc, 2012).

1.3.2 Point-of-care coagulometers for INR measurement

1.3.2.1 Patient self-testing and self-management

In the last 20 years several portable coagulometers, commonly known as POC, were developed for the self-care of patients taking VKA. The use of the POC indeed allows the patients to self-monitor the VKA treatment, which can involve patient self-testing (PST), when patients perform the INR tests by themselves and communicate the results to healthcare professionals for dose adjustment, and eventually patient self-management (PSM), when patients also interpret the INR result and adjust the VKA dosage accordingly (National Institute for Health and Care Excellence, 2014a). The use of POC devices has become more common recently: for instance, in 2006 only

28.3% of chronic warfarin users in New York State were monitored using POC, and this percentage increased to 37.6% in 2011 (Triller et al., 2015).

1.3.2.2 Advantages and disadvantages of the point-of-care coagulometers

There are several advantages associated with the use of POC devices:

- More practical use: the POC devices are easy to handle and can be used in different settings, such as in the hospital anticoagulation clinics, at the doctor's office, at health centres, at pharmacies, or by patients themselves at home, after adequate training. With the use of POC devices, patients do not need to perform frequent long trips, in order to reach the nearest laboratory, reducing the costs and the waste of time.
- Less invasive procedure: the INR test can be performed on a very small sample of capillary blood (usually 10-30 µl) from a fingertip. Therefore, the traditional venepuncture is not needed, resulting in a procedure which is less traumatic and less painful. This aspect is particularly advantageous for monitoring children or frail elderly people with small or difficult venous access.
- Immediate results: the POC devices can directly analyse whole blood, eliminating the time necessary to transport the samples to the laboratory and to prepare the plasma for testing (through procedures like centrifugation and recalcification). The INR result is therefore available within few minutes, allowing prompt VKA dose adjustment and immediate medical attention, in case of extremely out of range values. The elimination of the delay between blood sample collection and INR results is an advantage for both the patients, who can avoid going back to the hospital to collect the VKA prescription, but also for doctors working in

ambulatory surgery centres or emergency department, who can speed up the management of their patients according to the results.

Nevertheless, there are also several disadvantages associated with the use of POC devices:

- The need for educational programs: healthcare professionals using the POC devices have to be trained, supported and monitored. This can result in the need for a large amount of resources, since they are often located in remote areas. In order to reduce the costs associated with this issue, the Australian Point-of Care Practitioner's Network has developed a website offering online training for POC operators, focused webinars and a specific telephone helpline (St John et al., 2015). Furthermore, patients willing to perform self-management also need to be adequately selected and trained. For instance, in the Netherlands the National Thrombosis Service provides medical supervision, training and extensive support (e-learning, medical help desk, motivational support, newsletter, reminders) (Brouwer et al., 2014). In this context, potential POC users have to show their ability to perform self-testing, need to undergo a specific training and obtain a mandatory certificate, in order to become eligible for home-monitoring (Brouwer et al., 2014).
- The need for internal and external quality assessment: although the POC testing is often conducted outside the hospital laboratory setting, it is important to follow an adequate quality management. An internal quality assessment can be performed by analysing a quality control (QC) material before patient samples, in order to check that the system is working properly (Briggs et al., 2008). Some devices have also an embedded QC, which checks each test strip while performing the INR, in order to test the functionality of the device. An external quality assessment can be

performed by comparing the POC coagulometer results with plasma samples with known but undisclosed INR values, received from an accredited external laboratory, as developed by the UK National External Quality Assessment Scheme (NEQAS) (D. P. Kitchen et al., 2012). Otherwise the patient's POC device can be compared with a certified calibrated POC coagulometer using five sets of plasma, as endorsed by the European Action on Anticoagulation (Poller et al., 2006).

- The cost: while the laboratory INR is quite cheap, the POC system is certainly more expensive, when considering the costs related to the coagulometers and the test strips and the cost related to staff training. However, these costs can be balanced by the reduced amount of time spent by doctors in the anticoagulation clinics and the reduced transport costs for the patients.

1.3.2.3 Different point-of-care coagulometers

There are different POC coagulometers available on the market, with similar functions but some technical differences. The most commonly used are part of the CoaguChek system, including the CoaguChek S, the CoaguChek XS and the CoaguChek XS Plus (Roche Diagnostics). The CoaguChek S was the first model to be developed by Roche and it was subsequently implemented into the CoaguChek XS system, which includes CoaguChek XS and CoaguChek XS Plus. The CoaguChek XS is designed for patients' self-testing, while the CoaguChek XS Plus is designed for healthcare professionals' use in primary and secondary care. In fact, the latter has a touch-screen user interface and includes additional characteristics such as a bar-code reader, extended capacity for data memory and the possibility of operator and sample identification.

The CoaguChek S uses a mechanical clot detection method and the test strips contain dry rabbit brain thromboplastin (lot-specific ISI ranging from 1.6 to 2.2) (Marzinotto

et al., 2000; Medicines and Healthcare Products Regulatory Agency, 2004). On the test strip there are also tiny iron particles, which are moved by an electromagnetic field. When the blood is mixed with the thromboplastin on the test strip, the coagulation cascade is activated. Coagulation is effective when the clot stops the iron particles from moving (Greenway et al., 2009; Marzinotto et al., 2000).

The CoaguChek XS systems uses an electrochemical clot detection method and the test strips contain human recombinant thromboplastin (ISI = 1.01). On the test strip there is also a peptide substrate, which is split by the newly generated thrombin. It generates an electrochemical signal which is subsequently converted into INR. The measuring range of the CoaguChek XS system is 0.8-8.0 INR. The time required for obtaining the results is about one minute (Leichsenring et al., 2007; Plesch & Wolf, 2006).

The other POC coagulometers available on the market include: the Alere INRatio monitoring system (HemoSense, USA); the i-STAT (Abbott Point of Care Inc., USA); the Coag-Sense (CoaguSense, USA); the ProTime microcoagulation system (International Technidyne Corporation, USA); and the SmartCheck INR (Unipath, UK).

1.3.2.4 Current guidelines on the use of point-of-care coagulometers

Several guidelines from scientific societies provide specific recommendations on the use of the POC coagulometers (Fitzmaurice et al., 2005; Holbrook et al., 2012; National Institute for Health and Care Excellence, 2012, 2014a). For instance, the UK National Institute for Health and Clinical Excellence (NICE) published a diagnostics guidance where they recommended the use of the POC devices “for self-monitoring coagulation status in adults and children on long-term vitamin K antagonist therapy

who have atrial fibrillation or heart valve disease if: the person prefers this form of testing and the person or their carer is both physically and cognitively able to self-monitor effectively" (National Institute for Health and Care Excellence, 2014a, p.3). Conversely, another NICE guideline stated "Do not routinely offer self-management or self-monitoring of INR to patients who have had DVT or PE and are having treatment with a VKA" (National Institute for Health and Care Excellence, 2012, p.21). This difference in management is probably due to the fact that some VTE patients only need a limited anticoagulant treatment and 2-3 months might be required before a patient becomes accustomed to the POC monitoring. In fact, the guidelines of the British Society of Haematology suggested that "Only patients with long-term (>1 year) indications for warfarin therapy should be considered for self-testing or -management" (Fitzmaurice et al., 2005, p.161). The British Society of Haematology also stated that a difference of ± 0.5 INR units between different systems is considered clinically acceptable (Fitzmaurice et al., 2005). The American College of Chest Physicians (ACCP) guidelines on the management of the anticoagulant therapy, stated "For patients treated with VKAs who are motivated and can demonstrate competency in self-management strategies, including the self-testing equipment, we suggest patient self-management (PSM) rather than usual outpatient INR monitoring (Grade 2B)", where grade 2B means a weak recommendation of moderate-quality evidence (Holbrook et al., 2012, p.e153S).

1.3.2.5 Precision and accuracy of the point-of-care coagulometers

In order to estimate the quality of an assay, it is important to evaluate the precision (degree of reproducibility) and the accuracy (degree of veracity). A systematic review reported that the POC coagulometers have an adequate precision and a variable

accuracy among studies (Christensen & Larsen, 2012). The CoaguChek XS was the most studied device, evaluated in 14 out of 22 included studies, and showed a coefficient of variation (CV) ranging between 1.4-5.9% (imprecision) and a coefficient of correlation ranging between 0.81-0.98 (accuracy) (Christensen & Larsen, 2012). Furthermore, in a recent study comparing the performance of different POC devices, the CoaguChek XS showed the best precision, with CV < 5% (Bonar et al., 2015). In another study, the within-subject variation in the INR values, in patients receiving a stable dose of VKA and using the CoaguChek XS, was 10.2% in those prescribed with acenocoumarol and 8.6% in those prescribed with phenprocoumon (van den Besselaar, Biedermann, et al., 2015).

There are some reports that the POC devices tend to overestimate the INR results; in fact, a positive bias ranging between 0.01-0.26 has been reported in studies evaluating the CoaguChek XS (Beynon et al., 2015; Greenway et al., 2009; Kalçık et al., 2015) and between 0.13-0.27 in studies evaluating the CoaguChek XS Plus, compared to the different laboratory INR (Donaldson et al., 2010; Hur et al., 2013; Meneghelo et al., 2015). However, it should be noted that different laboratory reagent-coagulometer combinations also display some variability.

So far, the largest study on the accuracy of POC devices analysed 3257 adult patients on VKA treatment for various indications and compared the INR values obtained by the CoaguChek XS with the laboratory INR measured by the STA-R Evolution coagulometer (Biedermann et al., 2015). The overall correlation between the two instruments was very strong ($r = 0.90$, $p<0.001$). Separate analyses according to different INR cut-off levels showed a mean difference of -0.13 at sub-therapeutic INR levels (defined as POC INR < 2.0); -0.13 at therapeutic INR levels (POC INR 2.0-4.0); and +0.72 at supra-therapeutic INR levels (POC INR > 4.0). The clinical

agreement, defined according to the patient's therapeutic range, was 88.3% (Biedermann et al., 2015).

Although this data suggests that at high INR values there is increasing discordance between the different methods, a study specifically evaluating over-anticoagulated patients showed acceptable correlation and slight variations in warfarin dosing (Lawrie et al., 2012). In 168 patients with POC values between 4.5 and 8.0, the CoaguChek XS Plus showed correlation coefficients of 0.87 and 0.75, when compared with two different laboratory assays (CA-7000 analyser, Sysmex, using Innovin, Siemens Healthcare Diagnostics, and CA-1500 analyser, Sysmex, using HemosIL PT-Fibrinogen HS Plus, Instrumentation Laboratory). Only 63.6% and 50% of the results, respectively, were within 0.5 INR units; however, from a clinical point of view, they would have resulted in the same warfarin dosing in more than 70% of patients (Lawrie et al., 2012). A subgroup of these samples was also tested with the calibrated automated thrombogram (CAT); however, no clear association was found between the parameters of the thrombin generation curve and INR values > 4.5 (Lawrie et al., 2012).

Another study compared the INR obtained from the POC devices (CoaguChek S or CoaguChek XS) with the standard laboratory INR (STA-R Evolution coagulometer, Stago), but also with the CAT (Thrombinoscope BV, Maastricht) and with the clotting activity of the coagulation factors II, VII, IX and X (Christensen et al., 2009). They included 24 adult patients on VKA for AF or mechanical aortic valve replacement and stable oral anticoagulant therapy, defined as unchanged weekly dose of warfarin during the previous six weeks. Each patient had blood samples collected at three time points (week 1, 3 and 6). The parameters of the thrombin generation curve and the activity of the coagulation factors were significantly correlated with the INR levels,

regardless of the assay used (laboratory INR, CoaguChek S or CoaguChek XS). However, the thrombin generation showed wide variability in patients with the same INR.

Few studies directly compared the POC devices with the manual tilt-tube technique (Kaatz et al., 1995; Vacas et al., 1998; Vacas et al., 2003; van den Besselaar, van der Meer, et al., 2015). A Spanish group of researchers compared two portable POC monitors (the CoaguChek PT in 80 anticoagulated patients and the CoaguChek S in 60 anticoagulated patients) with the manual tilt-tube technique (using Thromboplastin Bilbao, a high-sensitivity rabbit thromboplastin) (Vacas et al., 2003). The same two sets of plasma were also analysed with two automated coagulometers (STA, using Neoplastin Plus, Diagnostics Stago, and ACL7000, using PT-FibrinogenHsPlus, Instrumental Laboratories). The coefficients of correlation of the two POC devices compared with the manual technique were 0.81 and 0.74, for CoaguChek PT and CoaguChek S respectively, and were lower than those obtained in the comparison between the automated coagulometers and the manual technique (ranging from 0.89 to 0.92) (Vacas et al., 2003). A Dutch group compared four different POC devices (the microINR system, the ProTime InRhythm System, the INRatio2 system and the CoaguChek XS system) with the manual tilt-tube technique (van den Besselaar, van der Meer, et al., 2015). The mean bias was -13.7%, -9.3%, 10.1% and -0.9%, for the four POC devices respectively, with the CoaguChek XS showing the lowest absolute bias (van den Besselaar, van der Meer, et al., 2015).

1.3.2.6 Clinical implications of the point-of-care INR monitoring

Several trials evaluated the impact of PST and PSM on clinical outcomes during VKA treatment. The largest trial, The Home International Normalized Ratio Study

(THINRS), randomized 2922 patients, on VKA treatment due to AF or mechanical heart valve, to weekly PST at home or monthly testing in several anticoagulation clinics (Matchar et al., 2010). During a minimum follow-up of 2.0 years, the rate of clinical outcomes included in the primary endpoint (stroke, major bleeding, death) was comparable in the two groups (19% vs. 20%; hazard ratio [HR] 0.88, 95% CI 0.75-1.04, p=0.14). The time within the therapeutic range (TTR) was greater in the self-testing group than in the clinic-testing group (66.2 ± 14.2% vs. 62.4 ± 17.1%, p<0.001) and patients' satisfaction, measured using the Duke Anticoagulation Satisfaction Scale (DASS) in which lower scores represent higher satisfaction, was also greater in the self-testing group (46.8 ± 16.3 points vs. 49.2 ± 18.0 points, p=0.002) (Matchar et al., 2010). In a sub-study of the THINRS, some centres also randomized the patients to home testing twice a week, once a week or once every four weeks, in order to evaluate the effect of more frequent home self-testing on the TTR (Matchar et al., 2015). The TTR at 1-year after randomization was higher in the group tested more often, being 66.8% (± 13.2), 63.3% (± 14.3) and 59.9% (± 16.7), respectively (Matchar et al., 2015). A pre-post study confirmed that the use of portable devices and home self-testing, in patients with unstable laboratory INR, allows a more frequent monitoring (median interval from 15 days to 11 days, p<0.0001) and increases the TTR (from 63% to 68%, p=0.001) (Barcellona et al., 2013).

A systematic review and meta-analysis, which pooled the results of 22 randomized clinical trials conducted in adult patients, including the THINRS, found a 42% relative risk reduction of major thromboembolic events (2.5% vs. 4.0%; odds ratio [OR] 0.58, 95% CI 0.45-0.75, p<0.001) and a 26% reduction of mortality (9.2% vs. 12%; OR 0.74, 95% CI 0.63-0.87, p<0.001), with PST alone or PST in combination with PSM, compared to the usual care (Bloomfield et al., 2011). No difference emerged in major

bleeding events (7.0% vs. 7.9%; OR 0.89, 95% CI 0.75-1.05, p=0.169) and in the TTR (66.1% vs. 61.9%; p=0.168) (Bloomfield et al., 2011). A subsequent individual patient meta-analysis showed that the reduction in thromboembolic events in the self-monitoring group was especially evident in some categories of patients, such as those younger than 55 years old (HR 0.33, 95% CI 0.17-0.66) or having mechanical heart valves (HR 0.52, 95% CI 0.35-0.77) (Heneghan et al., 2012). However, it has been reported that only approximately 80% of patients are able to perform PST and only approximately 50% are able to perform PSM (Garcia-Alamino et al., 2010).

A recent study evaluated the effect of the POC used by the healthcare professionals, but obtained contrasting results (Biedermann et al., 2016). This retrospective analysis of more than 1900 patients showed that the median TTR was significantly reduced during POC INR monitoring compared to laboratory INR monitoring (77.9% vs. 81.0%, p<0.001). However, no difference emerged in major clinical events, thus confirming the POC as a safe and effective alternative to the standard laboratory INR.

Several studies have also been conducted in the paediatric population, in which the use of POC devices is particularly appealing because of the frequent INR fluctuations and the difficulties in performing the venepuncture. A systematic review of 11 trials, mainly observational studies, found that PST or PSM are good treatment options also in highly selected children on VKA treatment (Christensen et al., 2011). However, there is still limited evidence to establish whether these two ways of monitoring are superior to conventional management. Only one randomized controlled trial (RCT) has been conducted in the paediatric population (the EMPoWarMENT, Edmonton Pediatric Warfarin Self-Management Pilot Study) (Bauman et al., 2010). In this small pilot trial 28 children, after performing PST for at least three months, were randomized to continue with PST or to commence PSM. No significant differences were observed

in the TTR, which was 83.9% pre- and 83.9% post-randomization in the PST group vs. 77.7% and 83.0% in the PSM group (p=0.312). However, the quality of life was higher in families undergoing PSM, since they more frequently reported satisfaction with the intervention and positive changes in their lives (Bauman et al., 2010). A larger cohort study was subsequently conducted in the same Canadian setting (the EMPoWARed, Edmonton pediatric warfarin self-management study) (Bauman et al., 2015). This cohort consisted of 42 patient-family units who performed PSM for a median of 2.7 years. The study was divided into phase 1 (the first six months of independent PSM) and phase 2 (the last six months of PSM). The TTR was stable throughout the study (from 90.0% to 92.9%, p=0.30), despite less frequent monitoring (from one INR test every 10.0 days to 17.1 days, p<0.0001). There were no clinically relevant adverse events. Therefore, the authors concluded that the sustainability of PSM is maintained during long-term VKA treatment (Bauman et al., 2015).

Finally, patients with antiphospholipid syndrome (APS) represent a particular category, since the presence of lupus anticoagulants can lead to a prolonged PT on some assays, and therefore to a false INR value. Few studies evaluated whether the use of POC devices can be reliable in these patients. A German study included 140 patients with APS and 100 controls and compared the INR results obtained from the CoaguChek XS with four laboratory assays using different thromboplastins (Isert et al., 2015). The authors found that the concordance between the CoaguChek XS and the Neoplastin Plus, a rabbit brain thromboplastin, was lower for APS patients compared to controls (values within 0.5 INR units 81.5% vs. 91.4%, p=0.028). Conversely, the agreement was higher when compared to the human recombinant thromboplastins RecombiPlasTin (83.9% vs. 72.0%, p=0.017) and Innovin (87.7% vs. 76.3%, p=0.013). No difference emerged when the CoaguChek was compared to the

Thromborel S, a thromboplastin extracted from human placenta (84.4% vs. 83.9%, p=0.914) (Isert et al., 2015). However, since the sensibility of different thromboplastins to antiphospholipid antibodies varies, the authors concluded that they could not estimate which was the best method for VKA monitoring in these patients. Another study showed that the concordance between the CoaguChek XS and the RecombiPlasTin was acceptable for INR values below the therapeutic range (values within 0.5 INR units 97.3% vs. 97.6%) (Barcellona et al., 2012). However, the disagreement rose with increasing INR values (83.4% vs. 92.7% for INR within the therapeutic range 2.0-3.0; 58.4% vs. 83.0% for INR values 3.1-4.0; and 40.0% vs. 61.2% for INR values > 4.0) and was more pronounced in patients with triple positivity (Barcellona et al., 2012). These results suggest that, so far, the VKA treatment in patients with APS is better monitored using the laboratory automated coagulometers.

1.3.3 D-dimer

1.3.3.1 D-dimer formation

D-dimers are fibrin degradation products, obtained through a stepwise process which is represented in Figure 1.3 (Adam et al., 2009). In the first step, thrombin cleaves the fibrinopeptides A and B from the fibrinogen molecule, producing the fibrin monomers. There is exposure of a previously hidden polymerisation site, which can bind another fibrin monomer, in order to form the fibrin polymers. Fibrinogen has also three peptide chains, one central node (E domain) and two outer nodes (D domains). At this stage, the fibrin polymers are connected by non-covalent links between end-to-end D-D domains and side-to-side D-E domains (Bates, 2012). In the second step, thrombin activates factor XIII to factor XIIIa which, in turn, catalyses the formation

of covalent links between the end-to-end D-D domains of the fibrin polymers (cross-linked fibrin). The covalent links are resistant to human proteases (Righini et al., 2008). In the third step, plasmin cleaves the fibrin molecules at sites of non-covalent links, with the release of fibrin degradation products constituted by E fragments and D-D fragments. The latter are the D-dimers (Adam et al., 2009). Therefore, D-dimer is a marker of coagulation and fibrinolysis activation and the measurement of D-dimer can identify a disturbance in the haemostatic balance.

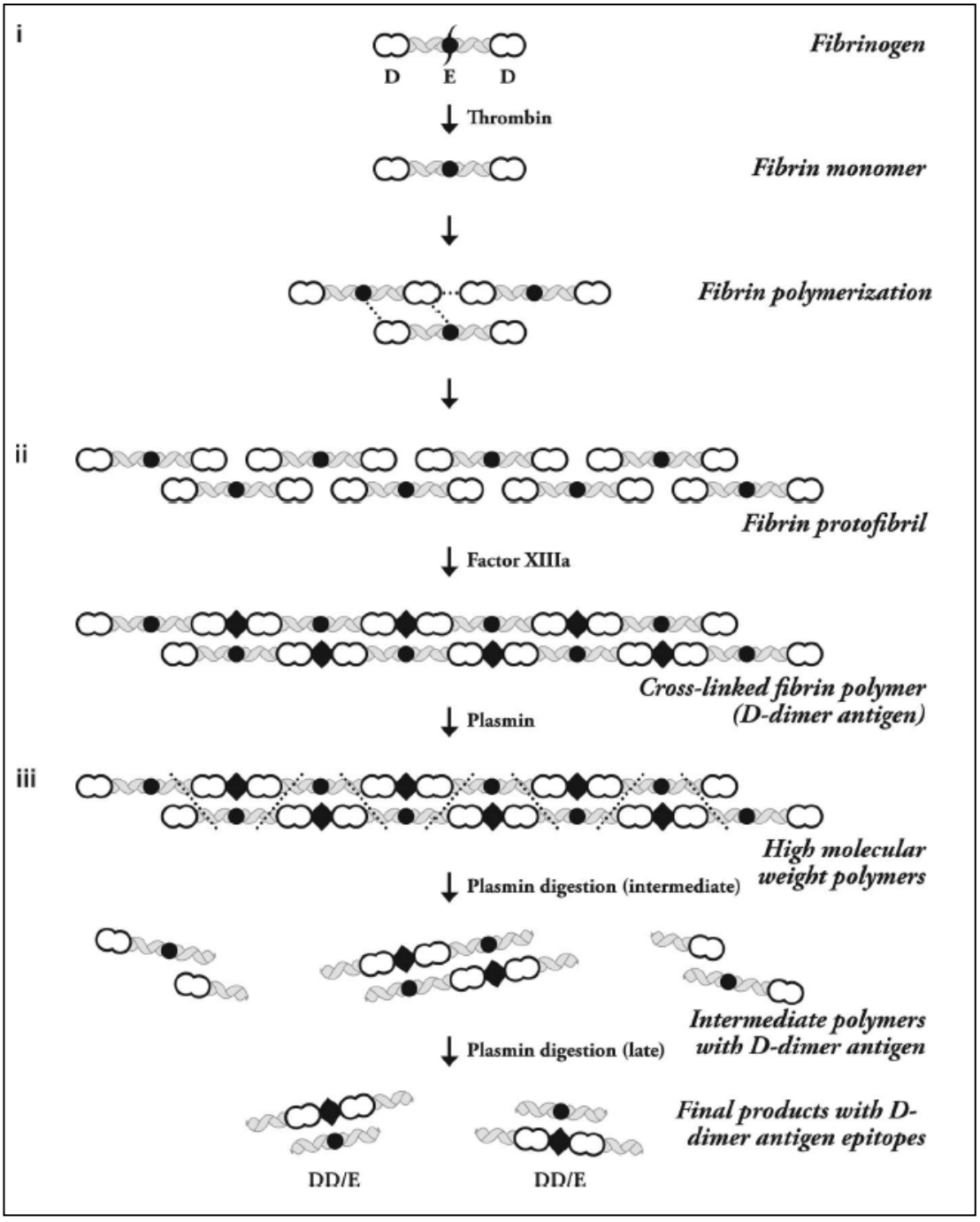


Figure 1.3 The stepwise process of D-dimer formation (Adam et al., 2009) (Reproduced with permission, copyright licence no: 4650190198007)

1.3.3.2 Different D-dimer assays

All D-dimer assays utilise monoclonal antibodies able to detect epitopes that are found on the cross-linked D-domains of the fibrin molecule (D-dimer antigen), but that are

absent on fibrinogen or non-cross-linked fibrin (Adam et al., 2009). However, the assays are not identical because the D-dimer antigen can have diverse sizes and the antibodies can detect different epitopes. In fact, the D-dimer antigens detected by the assays can be derived from fibrin polymers before their proteolysis by plasmin or after plasmin cleavage of the fibrin clot (Righini et al., 2008). The D-dimer fragment has a plasma half-life of approximately eight hours and is cleared through the kidneys and the reticuloendothelial system (Righini et al., 2008). The D-dimer is a very sensitive but not specific assay. It can be raised in patients with thrombotic diseases (e.g. VTE, disseminated intravascular coagulation) but also other pathological and physiological states (e.g. surgery, trauma, infection, cancer, pregnancy) (Lippi et al., 2014).

There are several D-Dimer assays, which can be classified as follows (Heim et al., 2004; Righini et al., 2008):

- Enzyme-Linked ImmunoSorbent Assays (ELISA):
    - The microplate ELISA is a quantitative test. It was used in early clinical studies and is considered the gold standard. It needs to be run in batches and requires a long procedural time of approximately 2-4 hours to produce the results. An example is the Asserachrom (Diagnostica Stago).
    - The membrane ELISA is a rapid but not quantitative test. The monoclonal antibody is chemically marked in order to produce a visible colour change when the concentration of D-dimer is raised. It can provide results within 20 minutes; however, it needs to be interpreted manually. Examples are Instant I.A. (Diagnostica Stago) and NycoCard (Nycomed Pharma AS).
- Enzyme-Linked Fluorescence Assays (ELFA): these are quantitative tests which combine the ELISA technique with a final detection by fluorescence. They can

analyse single samples and produce the results within 35 minutes. Examples are the VIDAS D-Dimer (BioMerieux) and the Stratus D-dimer (Dade-Behring).

- Latex agglutination assays:
    - The latex qualitative assays were the first generation of latex assays. The antibodies against the D-dimer antigen are coated with latex particles, which can provide visible agglutination. The amount of agglutination is proportional to the concentration of the D-dimer. When the sample is positive, serial dilutions can be performed in order to provide a semiquantitative estimation of D-Dimer plasma concentration (latex semiquantitative assays). Examples are D-dimer test (Diagnostica Stago) and Dimertest (Agen Biomedical).
    - The latex quantitative assays are the second generation of latex assays, which use either photometric or turbidimetric methods. They are fully automated agglutination assays which can be assayed on routine coagulation analysers and can provide results within 15 minutes. Examples are Liatest (Diagnostica Stago), Tinaquant (Roche Diagnostics), Auto Dimertest (Agen Biomedical), Innovance D-dimer (Dade-Behring) and HemosIL Dimertest HS (Instrumentation Laboratories).
- Whole-blood D-dimer assays: these tests evaluate erythrocyte agglutination, which needs to be determined manually, therefore being qualitative assays. Since they do not require plasma separation, the result is ready in less than two minutes and can be performed as a POC test. An example was the SimpliRED (Agen Biomedical), which has now been discontinued.

Regardless of the type of assay, samples with very high (or very low) concentrations of D-dimer are usually correctly identified as positive (or negative) by all methods.

However, there are some discrepancies among tests when the D-dimer concentrations are only slightly increased (Heim et al., 2004), with potential dangerous consequences in real-life clinical practice.

The Innovance® D-dimer was compared with two ELFA assays (VIDAS D-Dimer, Stratus D-Dimer) (Coen Herak et al., 2009; de Moerloose et al., 2008; Elf et al., 2009; Mullier et al., 2014; Salvagno et al., 2009) and with two Latex agglutination assays (Liatest, Autodimer) (Elf et al., 2009; Park et al., 2011), showing very good correlation ($r \geq 0.90$). The HemosIL® was compared to an ELFA assay (VIDAS D-dimer) (Mullier et al., 2014; Salvagno et al., 2009; Salvagno et al., 2008) and with one ELISA assay (AcuStar D-Dimer) (Lippi et al., 2012), showing good correlation ($r \geq 0.88$). There was only one study which reported low coefficients of correlation ($r \sim 0.70$) when the Innovance® D-dimer and the HemosIL® D-Dimer HS were compared to the VIDAS D-dimer (Oude Elferink et al., 2015). These contrasting results are probably due to the different range of D-dimer values, since correlation was calculated for D-dimers up to 1000 ng/mL, which is twice the diagnostic cut-off level.

1.3.3.3 Clinical applications of the D-dimer

The main clinical application of D-dimer is in the diagnostic algorithm for VTE. The accuracy of different D-dimer tests in this context has been evaluated in a systematic review and meta-analysis (Di Nisio et al., 2007). The authors reported that the ELFA, the microplate ELISA and the latex quantitative assay had significantly better sensitivity (96-97%, 94-95%, 93-95%), but lower specificity (43-46%, 50-53%, 50-53%) than the other D-dimer assays (Di Nisio et al., 2007). Due to its high sensitivity and negative predictive value, but low specificity, the D-dimer must be integrated in a comprehensive approach in which patients with suspected VTE are assessed using

pre-test clinical probability scores (also known as clinical prediction rules), D-dimer and imaging tests.

The most commonly used algorithm for patients with suspected deep vein thrombosis (DVT) uses the Wells DVT score (Wells et al., 2003). The Wells DVT score has 10 clinical variables: active cancer, recent immobilization of the lower limbs, recent bedridden, tenderness localized along the course of the deep veins, entire leg swollen, calf swelling at least three cm larger than the other side, pitting oedema in the symptomatic leg only, collateral non-varicose superficial veins, previous DVT (scored one point each); presence of an alternative diagnosis (scored -2 points). Patients with a score < 2 points were considered to have an unlikely pre-test probability of DVT, while patients with a score ≥ 2 points were considered to have a likely pre-test probability of DVT. Patients with an unlikely probability of DVT were evaluated with the D-dimer: if the D-dimer was below the cut-off level, DVT was ruled out; if the D-dimer was above the cut-off level, they underwent a lower limb venous compression ultrasonography to confirm or exclude the diagnosis. Patients with a likely DVT underwent a lower limb venous compression ultrasonography, without the need for D-dimer test. In these patients, if the first ultrasound was negative a second ultrasound was performed within a week. This diagnostic algorithm showed low rates of thromboembolic events at 3-month follow-up (0.4%) in those patients for whom DVT was ruled out using this combined strategy (Wells et al., 2003). Furthermore, it avoided the need of a compression ultrasound in 39% of patients.

For patients with suspected pulmonary embolism (PE), two clinical prediction rules are available: the Wells PE score and the revised Geneva score. The Wells PE score has seven clinical variables: sign and symptoms of DVT, lack of alternative diagnosis (scored 3 points each); heart rate ≥ 100 bpm, recent immobilization or surgery,

previous VTE (scored 1.5 points each); haemoptysis, malignancy (scored 1 point each). Patients can be classified in a three-level score (low probability if < 2 points, intermediate if 2-6 points, high if > 6 points) or in a two-level score (PE unlikely if ≤ 4.0 points, PE likely if > 4.0 points) (Wells et al., 2000). The revised Geneva score has nine clinical variables: heart rate ≥ 95 bpm (5 points); pain on lower-limb deep venous palpation and unilateral oedema (4 points); previous VTE, unilateral lower limb pain, heart rate 75-94 bpm (3 points); recent surgery or fracture, active malignancy, haemoptysis (2 points each); age > 65 years (1 point) (Le Gal et al., 2006). Patients can be classified in a three-level score (low probability if < 4 points, intermediate if 4-10 points, high if > 10 points) or in a two-level score (PE unlikely if < 6 points, PE likely if ≥ 6 points) (Konstantinides et al., 2014; Le Gal et al., 2006).

The Guidelines of the European Society of Cardiology (ESC) recommend the following algorithm for patients with suspected acute PE (Konstantinides et al., 2014). The first step is the assessment of shock or hypotension, since this category of patients (haemodynamically unstable or high-risk PE) has a very high-risk of early mortality and therefore requires an immediate diagnosis. Patients without shock or hypotension (non-high risk PE) should be evaluated using one of these two clinical prediction rules. If low/intermediate clinical probability or PE unlikely, a D-dimer test should be performed: if the D-dimer is below the cut-off level, the PE is ruled out; if the D-dimer is above the cut-off level, imaging tests are needed (computed tomographic angiography or ventilation–perfusion scintigraphy) to confirm or exclude the diagnosis. Patients with a high clinical probability or PE likely should be evaluated with imaging tests, without the need for D-dimer (Konstantinides et al., 2014). The advantage of using the D-dimer is that, in patients with low clinical pre-test probability, a negative result can avoid unnecessary exposure to radiations or contrast.

Using this algorithm the PE can be ruled out in about 30% of patients without further testing, with a very low risk of thromboembolic events at 3-month follow-up (< 1%) (Konstantinides et al., 2014).

Since D-dimer concentrations can increase with aging, age adjusted cut-off for the D-dimer have been recently proposed, in order to further increase its specificity, but without reducing its high sensitivity. For instance, a cut-off of age x 10 µg/l has been proposed for patients aged > 50 years with suspected DVT or PE. The specificity increased from 57.6% to 62.3% in patients 51-60 years old, from 39.4% to 49.5% in patients 61-70 years old, from 24.5% to 44.2% in patients 71-80 years old, and from 14.7% to 35.2% in patients > 80 years old. The sensitivity remained > 97% in all the age categories (Schouten et al., 2013). Furthermore, it was recently demonstrated that the combination of the Wells score and the age-adjusted D-dimer can rise by 5% the proportion of patients with suspected PE (van Es et al., 2016) or suspected DVT (Riva, Camporese, et al., 2018), who can be managed without imaging.

While the role of D-dimer in the diagnostic algorithm for DVT and PE is clearly established, there are still areas of uncertainty in the diagnosis of isolated distal DVT (Sartori et al., 2012), upper extremities DVT (Di Nisio et al., 2010), cerebral vein thrombosis (Dentali, Poli, et al., 2012) or splanchnic vein thrombosis (J. Dai et al., 2015). There is some evidence that values of D-dimer measured at the time of DVT diagnosis might also predict the development of post-thrombotic syndrome (Roberts et al., 2013).

Another clinical application of D-dimer is the prediction of recurrent VTE, with the aim to identify patients with a low risk of recurrence who can safely discontinue the anticoagulant therapy. D-dimer has been included in several prognostic rules: the Canadian model “Men continue and HERDOO2” (all men continued the anticoagulant

treatment; for the female sex the following variables were considered: signs of post-thrombotic syndrome, D-dimers level ≥ 250 μg/L during anticoagulation, obesity, age ≥ 65 years) (Rodger et al., 2008); the Vienna Prediction Model (Sex, location of first VTE, D-dimer after anticoagulation) (Eichinger et al., 2010); the DASH score (abnormal D-dimer after anticoagulation, Age < 50 years, male Sex, Hormonal therapy) (Tosetto et al., 2012). Furthermore, three recent management studies (the PROLONG, the PROLONG II and the DULCIS) have evaluated the utility of D-dimer tests after anticoagulant suspension in VTE patients. Although using slightly different algorithms, resumption of the anticoagulant treatment was recommended in those patients whose D-dimer became positive during follow-up (Cosmi et al., 2010; Palareti et al., 2014; Palareti et al., 2006).

1.3.4 Thrombin generation assay

1.3.4.1 Principles of thrombin generation by Calibrated Automated Thrombography

Due to the central role of thrombin in the coagulation system, several laboratory tests have been proposed to measure thrombin generation as a surrogate of the blood coagulation capacity. Different markers of thrombin generation *in vivo* are available: fibrin degradation products (e.g. the D-dimer), activation peptides (e.g. the prothrombin fragment F1+2) or enzyme-inhibitor complexes (e.g. the thrombin anti-thrombin complex). However, these tests do not depend only on thrombin generation, but are also influenced by the specific clearance time and the constitutional fibrinolytic activity (Baglin, 2005).

Conversely, measurement of the thrombin generation *in vitro* can provide a global evaluation of coagulation. Thrombin generation was firstly described in the 1950s (Macfarlane & Biggs, 1953; Pitney & Dacie, 1953). However, this assay was performed manually and required regular subsampling and quantification of thrombin in each subsample, resulting in the production of one curve per man-hour and very high variability. Afterwards, Hemker et al. (2002) developed an automated method able to measure the thrombin generation in multiple samples simultaneously and to produce approximately 100 curves per man-hour.

The following materials and reagents are needed in order to perform the thrombin generation assay (Baglin, 2005; Hemker et al., 2006; Hemker & Béguin, 2000; Hemker et al., 2002; van Veen et al., 2008):

- A thrombin sensitive substrate is used to estimate the concentration of thrombin from the velocity of its cleavage. The substrate can be chromogenic or fluorogenic. The chromogenic assay requires defibrinated plasma, since turbidity changes associated with clot formation can interfere and cancel the signal from the chromophore. The fluorogenic assay does not require defibrination, since fluorescence is not influenced by turbidity, and therefore enables a continuous measurement of free and fibrin bound thrombin. It can be assayed on platelet poor plasma (PPP), platelet rich plasma (PRP) or citrated whole blood (CWB). It is the most commonly used assay nowadays, since there are two fluorogenic thrombin generation analysers currently available: the Calibrated Automated Thrombin Generation Assay or CAT (Thrombinoscope B.V., Maastricht, The Netherlands) and the Technothrombin TGA Assay (Technoclone, Vienna, Austria).
- A thrombin calibrator is needed in order to correct for the quenching of the fluorescence signal by the plasma colour and by the substrate consumption. The

calibrator consists of a known amount of thrombin bound to α_2-macroglobulin, which cannot be inhibited by plasma protease inhibitors and therefore can convert the fluorogenic substrate at a constant rate.

- TF is needed to trigger the assay. However, different concentrations of TF can result in different sensitivity of the assay. For the CAT method, high concentrations of TF (> 10 pM/l) start the classical extrinsic pathway of the coagulation cascade, bypassing factor VIII, IX and XI-dependent reactions. At intermediate concentrations of TF (5 pM/l), the thrombin generation assay is sensitive to the concentration of factors VIII and IX. At low concentrations of TF (1 pM/l), the thrombin generation assay becomes sensitive also to factor XI.
- Procoagulant phospholipids, usually at a concentration of 4 µM/l, should be added to PPP in order to obtain thrombin generation, so that the phospholipid concentration is not rate-limiting. In PRP procoagulant phospholipids are provided by the platelet surface, after scrambling of the platelet membrane. Therefore, thrombin generation is dependent on platelet phospholipids.
- Additional reagents are: corn tripsin inhibitor (a factor XIIa inhibitor that can be added in order to inhibit the contact pathway activation); TM, activated protein C or protein C sensitizing agents (such as the snake venom Protac, in order to better assess the protein C anticoagulant pathway).

The CAT assay is performed in a microtiter plate with up to 96 wells. Each plasma sample is analysed at least twice: in a measurement well and in a calibrator well. In the measurement well, the fluorescence tracing is produced by the amount of thrombin produced after the initiation of coagulation. In the calibrator well, the fluorescence tracing is produced by a known amount of thrombin. The calibrator well's result is used to correct the fluorescence registered in the measurement well. The analyser is

connected to a computer and a specific software produces a graph (the thrombin generation curve or thrombogram) and a summary of quantitative parameters. The following parameters are reported for the fluorogenic method (Figure 1.4) (Hemker et al., 2006; Hemker & Béguin, 2000; van Veen et al., 2008):

- The lag time represents the initiation phase of coagulation. During the lag phase only a small amount of thrombin is formed, afterwards there is a sudden burst of thrombin. Clotting occurs at the end of the lag time, which therefore represents the time to clot formation;
- The peak thrombin represents the maximum concentration of thrombin generated (thrombin burst), which corresponds to the propagation phase of coagulation;
- The time to peak represents the time necessary to form the maximum concentration of thrombin;
- The endogenous thrombin potential (ETP) represents the area under the thrombogram curve, which corresponds to the total enzymatic work done by thrombin and is the most predictive parameter of thrombotic and/or bleeding risk.
- The velocity index is calculated from the other parameters (lag time, time to peak and peak thrombin).

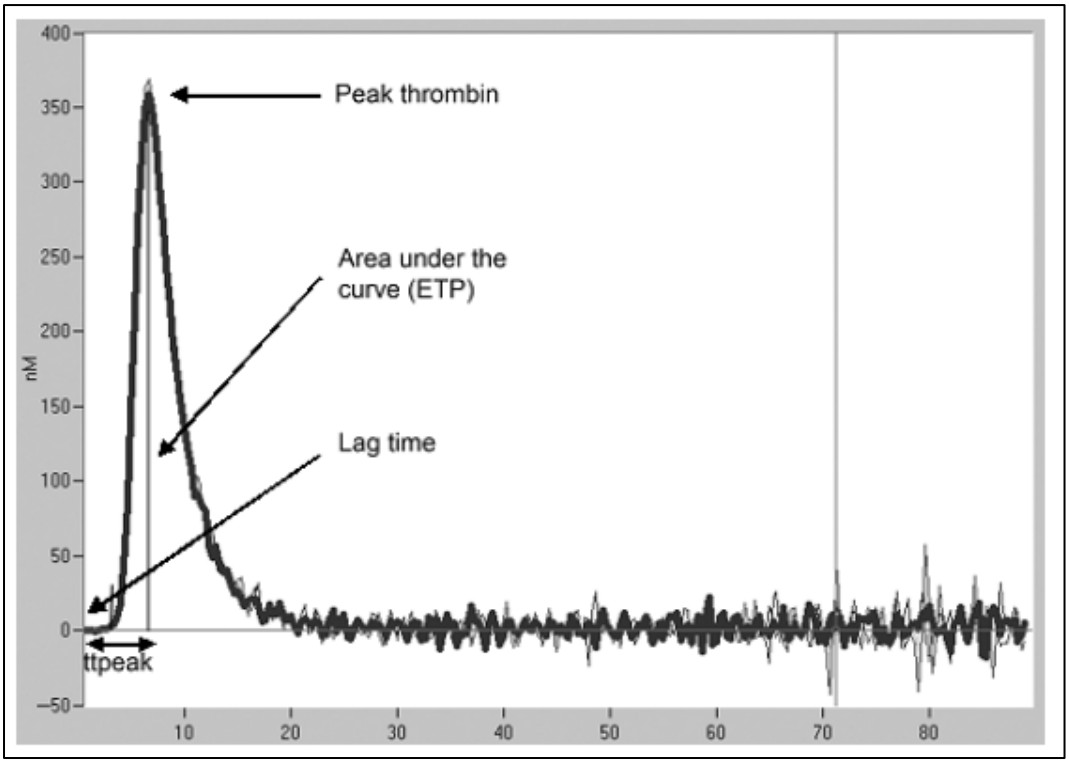


Figure 1.4 Typical thrombin generation curve obtained using the calibrated automated thrombogram (van Veen et al., 2008) (Reproduced with permission, copyright licence no: 4645950546456)

The thrombin generation assay has the advantage of being a global haemostatic test, since it allows the evaluation of all three phases of coagulation (initiation, propagation and termination). It is affected by different biological variables, such as age, sex, body weight, genetic factors or acquired conditions. However, it is mainly used as a research test, since the lack of standardization resulted in high inter-laboratory variation and prevented its widespread use in clinical practice. Variability can be due to pre-analytical variables (such as blood collection or plasma preparation) or analytical variables (such as different reagents and calibrators, temperature). The inter-laboratory variability can be reduced by normalizing the results, for instance expressing them as ratio to a reference plasma measured in parallel (Dargaud et al., 2007; Perrin et al., 2015).

1.3.4.2 Clinical applications of the thrombin generation assay

The thrombin generation assay has been widely studied in numerous conditions, such as inherited bleeding disorders, arterial and venous thrombosis, and anticoagulant treatment monitoring (van Veen et al., 2008).

A few studies evaluated the use of thrombin generation in the diagnosis of VTE. For instance, 443 patients with suspected DVT were enrolled in a study evaluating a chromogenic assay with high concentration of TF (approximately 300 pM/l) (F. J. Haas et al., 2011). The authors found only small differences in thrombin generation results between DVT-positive and DVT-negative patients: ETP, peak and lag time were significantly higher ($p<0.001$), while the time to peak was slightly longer ($p=0.15$). However, the thrombin generation parameters alone showed a low discriminating power for DVT. In the subgroup of elderly patients (age ≥ 75 years), with Wells' pre-test probability unlikely (< 2 points) and positive Innovance D-dimer (≥ 500 μg/L), the addition of a short lag time (using a cut-off of 23 seconds) improved the diagnostic accuracy, maintaining very high sensitivity and negative predictive value (both 100%) and increasing the specificity up to 96% (F. J. Haas et al., 2011). The predictive value of the CAT (TF 5pM) in 591 patients with suspected VTE has been recently evaluated (Wexels et al., 2017). The authors found that patients with confirmed VTE had a significantly prolonged lag time and time to peak ($p<0.001$). After adjusting for age, sex, smoking and comorbidities, VTE patients had also a significantly higher ETP ($p=0.041$) (Wexels et al., 2017).

Several studies evaluated whether the thrombin generation can predict recurrent VTE; with contrasting findings. Furthermore, differences in assays, reagents and populations, make difficult to summarize the results. A large study analysed 914 patients with a first spontaneous VTE treated with VKA for at least three months

(Hron et al., 2006). Thrombin generation was measured after a median of 13 months from VKA discontinuation, using a fluorogenic assay (Technothrombin TGA, Technoclone, Vienna, Austria). Peak thrombin concentration < 400 nM was associated with a 60% lower risk of recurrent events, which occurred in 6.5% of patients below this cut-off and in 20.0% of patients above this cut-off, at 4-year follow-up ($p<0.001$) (Hron et al., 2006). Another study focused on the ETP, measured using a chromogenic assay and expressed as percentage of normal, in 861 patients with a first episode of unprovoked VTE (Eichinger et al., 2008). ETP values ≥ 100% were associated with a 1.6-fold higher risk of recurrent VTE, which occurred in 11% of patients below this cut-off and in 19% of patients above this cut-off. The association of high ETP and high Innovance® D-dimer (defined as ≥ 500 mg/L) increased the risk of recurrence by 2.8 times (Eichinger et al., 2008). In The Thrombophilia, Hypercoagulability and Environmental Risks in Venous Thromboembolism (THE-VTE) study, the area under the thrombin generation curve was significantly increased in patients with a first event, compared to healthy control, but it was not associated with an increased risk of recurrence (van Hylckama Vlieg et al., 2015). In this study, blood samples were collected 2-3 months after suspension of the anticoagulant treatment and two different thrombin generation assays were used, the CAT (Thrombinoscope BV, Maastricht, the Netherlands) and the Technoclone TGA (Technoclone, Vienna, Austria), although with high concentrations of TF recurrence (van Hylckama Vlieg et al., 2015). Another group of researchers evaluated the CAT, performed with a low concentration of TF (1pM) and addition of TM (4 nM), on blood samples collected one month after discontinuation of VKA in 254 patients with a first unprovoked VTE enrolled in the PROLONG study (Tripodi et al., 2008). Patients with recurrent VTE had higher levels of ETP and peak thrombin and shorter lag time, compared to patients without recurrent

VTE. In the presence of TM, the amount of generated thrombin was smaller, but the correlation with the risk of recurrent VTE was stronger, although the lag time was not significantly different (Tripodi et al., 2008). However, another study reported that patients with all three abnormal parameters (ETP, peak thrombin and lag time) had an even higher risk of recurrence (Tripodi et al., 2009).

Some studies evaluated the role of the thrombin generation in patients taking VKA. A progressive decrease of thrombin generation parameters (peak thrombin and ETP) was reported with increasing INR values, measured using different thromboplastins (rabbit brain and human recombinant thromboplastins), but the statistical correlation was modest (Altman et al., 2007). Another study evaluated 143 patients with AF, aged ≥ 60 years, without any inflammatory disease, active malignancy, valvular AF or recent thrombotic events, on warfarin for at least six months and with a stable INR (defined as two consecutive INRs in the range 2.0-3.0) (Gatt, van Veen, Bowyer, et al., 2008). Compared to normal controls, warfarin treatment decreased the peak thrombin and the ETP and prolonged the lag time and the time to peak. However, wide variation was observed in patients with identical INR values (Gatt, van Veen, Bowyer, et al., 2008). Similarly, it has been reported that the parameters of the thrombin generation curve and the coagulation factors activity were significantly associated with the INR values (including also the POC INR), but the thrombin generation showed wide variability in patients with the same INR value (Christensen et al., 2009). Another group of researchers showed that the thrombin generation parameters were lower in patients with bleeding complications (Dargaud et al., 2013). The authors evaluated 341 patients on warfarin and INR between 2.0-3.0, presenting to the emergency department because of a major or clinically relevant non major haemorrhage (group 1, n=28), arterial or venous thrombosis (group 2, n=13) and reasons unrelated to haemostasis

(group 3, n=300). Despite similar INR results, the ETP (evaluated using the CAT with TF 1pM) was significantly lower in patients with bleeding complications, being 333 ± 89 nM*min in group 1, 441 ± 159 nM*min in group 2 (p=0.037), 436 ± 207 nM*min in group 3 (p<0.001) (Dargaud et al., 2013).

Several studies evaluated the use of the thrombin generation assay applied to the DOACs; however, variable sensitivity has been reported. For instance, Wong et al. (2013) spiked *in vitro* normal pooled plasma with increasing concentrations of apixaban, rivaroxaban or dabigatran. The direct factor Xa inhibitors apixaban and rivaroxaban delayed and reduced the rate of thrombin generation (they decreased the peak thrombin and the ETP, and prolonged the lag time and the time to peak) in a concentration-dependent manner. Their effect was more evident on the lag time, the time to peak and the peak thrombin rather than the ETP, although apixaban appeared to be 2-fold less potent than rivaroxaban. Conversely, dabigatran showed a different profile, affecting more the lag time and the time to peak than the peak thrombin. No significant active thrombin was detected at apixaban concentration 10 µM, rivaroxaban 3-10 µM and dabigatran 3-10 µM (Wong et al., 2013). There are other reports in which dabigatran showed a lower effect on the thrombin generation curves. For example, some authors found that the prolongation of the lag time with dabigatran was similar to warfarin at therapeutic range, while the reduction of the peak thrombin and the ETP were more evident with warfarin (Dale et al., 2013).

The thrombin generation has also been applied to the reversal of different anticoagulant drugs. A study evaluated normal plasma spiked *in vitro* with different concentrations of five heparinoids (unfractionated heparin, tinzaparin, enoxaparin, danaparoid and fondaparinux) and four reversal agents: protamine sulphate, recombinant factor VIIa (rVIIa), a concentrate of activated and non-activated clotting

factors (Factor VIII Inhibitor By-passing Activity or FEIBA), and fresh frozen plasma (FFP) (Gatt, van Veen, Woolley, et al., 2008). The CAT was useful to assess the level of anticoagulation and was also superior to traditional coagulation tests (aPTT and anti-factor Xa assay) in monitoring the reversal. In another study, Gatt et al. (2009) evaluated the effect of three reversal agents (FFP, rVIIa, and prothrombin complex concentrates [PCC]) to reverse warfarinised plasma *ex vivo*. The CAT assay was more sensitive than the INR assay in monitoring the reversal of anticoagulation and showed some differences among the three reversal agents: FFP and rVIIa improved only the lag time and time to peak, which represent the initial stages of coagulation, while PCC reversed all parameters of the thrombin generation curve. The authors also reported that PCC concentration equivalent to *in vivo* dose of 20 U/Kg was enough to reverse coagulation in patients with INR 2.0-3.9, while concentration equivalent to *in vivo* dose of 30 U/Kg were required to reverse samples with INR ≥ 4.0 (Gatt et al., 2009). Herrmann et al. (2014) analysed and compared the plasma of 17 patients on dabigatran 150 mg twice daily (BID) for non-valvular AF and 15 patients on rivaroxaban 10 mg once daily (OD) for VTE prevention in orthopaedic surgery. Rivaroxaban significantly increased the lag time and reduce the peak height and the ETP, while dabigatran showed similar modification on the thrombin generation curve, although the effect on the peak height was not statistically significant. They also tested different reversal agents *ex vivo*: a 3-factor PCC, FEIBA and rVIIa. PCC and FEIBA normalized the thrombin generation parameters, while rVIIa showed less efficacy (Herrmann et al., 2014). These results were confirmed by an *in vivo* study in which 35 healthy volunteers received rivaroxaban 20 mg BID for four days and afterwards they were randomized to receive 3- or 4-factor PCC at a dose of 50 U/kg or placebo (M. Levi et al., 2014). At steady state rivaroxaban concentration, the same modifications were observed on

the thrombogram: reduced ETP and maximum thrombin generation, and increased lag time and time to peak. However, the successive administration of PCC reversed the ETP, showed a trend towards the reversal of maximum thrombin and time to peak, but did not reverse the lag time (M. Levi et al., 2014).

1.3.5 Thromboelastography

1.3.5.1 Principles of thromboelastography

The thromboelastography (TEG) was originally described in the 1940s (Hartert, 1948). It is a global haemostatic assay which assesses clot formation/dissolution kinetics and clot strength. Data is obtained by measuring the viscoelastic changes of a small sample of clotting whole blood (about 300 µL), subjected to a constant rotational force (Whiting & DiNardo, 2014).

There are two systems: the TEG and the rotational thromboelastometry (ROTEM), which has evolved from the TEG. In the TEG (Haemonetics Corp, Braintree, MA, USA) there are two heated sample cups, which oscillate through 4° 45’ every 5 seconds, and there is a pin suspended by a torsion wire in each cup (Figure 1.5). When the clot starts forming, there is a physical connection between the cup and the pin, therefore the torque of the cup is transmitted to the pin and to the torsion wire. In the ROTEM (Tem International GmbH, Munich, Germany) there are four sample cups, which remain fixed, and a pin, which oscillates through 4° 75’ every six seconds and is connected to an optical detector. When the clot starts forming, the rotation of the pin is obstructed. In both systems, although to a different extent, the oscillation of the pin is affected by the rate of clot development and its elastic strength. The systems are connected to a computer software which produces a graph (the thromboelastogram)

and a summary of quantitative parameters. However, the two systems have different operating characteristics. The TEG can analyse up to two samples simultaneously, is very sensitive to vibration and therefore should be kept on a firm surface. The ROTEM can analyse up to four samples simultaneously, and can mix the reagents through automatic pipetting (Whiting & DiNardo, 2014).

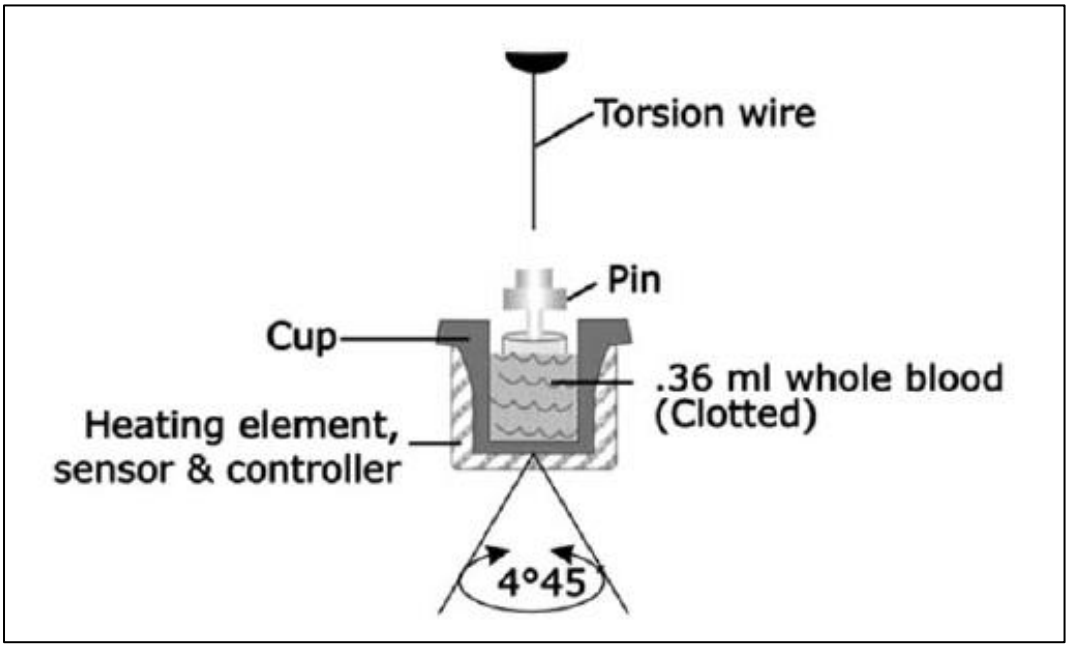


Figure 1.5 Picture of a thromboelastography device (Whiting & DiNardo, 2014) (Reproduced with permission, copyright licence no: 4646410988290)

Samples for TEG and ROTEM are usually collected in Vacutainer tubes containing sodium citrate, they are left standing at room temperature for 30 minutes to equilibrate and they are recalcificated when the whole blood is pipetted into the cup. Non-citrated samples can also be used; however, in this case, the assay should be started within 5 minutes of blood collection, in order to avoid clot formation before testing.

Coagulation can be started by the contact pathway, through the contact between the whole blood and the cup (also known as native TEG). However, since native TEG is quite long and impractical, specific activators can be added in order to reduce the time to clot formation. The intrinsic pathway on the TEG can be activated by adding kaolin; the extrinsic pathways can be activated by adding TF, while corn trypsin inhibitor can be used to inhibit the contact system. For the ROTEM, there are specific reagents (such as INTEM and EXTEM) (Chitlur et al., 2014).

The Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis recommends, when the TEG or ROTEM are used for clinical purposes, the use of intrinsic pathway activation with kaolin or INTEM, respectively. No recommendation is made for extrinsic pathway activation, since additional research is needed with regards to TF and corn trypsin inhibitor (Chitlur et al., 2014).

Several different TEG assays can be performed (Hans & Besser, 2016; Whiting & DiNardo, 2014):

- Native TEG: the whole blood is analysed following only recalcification;
- K-TEG: addition of a reagent containing kaolin, in order to promote contact activation;
- Rapid TEG: addition of a reagent containing kaolin and TF, in order to accelerate both the intrinsic and extrinsic pathways;
- H-TEG: addition of a reagent containing lyophilized heparinase, in order to neutralize the effect of unfractionated heparin (UFH);
- Functional Fibrinogen: addition of a reagent containing TF and abciximab, in order to block the platelet receptor Gp IIb/IIIa and to eliminate platelet contribution to clot firmness;

- TEG-Platelet Mapping: addition of a reagent containing heparin, factor XIII and reptilase, in order to inhibit thrombin production and to induce fibrin polymerization without thrombin generation. This method allows to assess platelet function. Clopidogrel and aspirin-induced platelet dysfunctions can also be evaluated, but they require adenosine diphosphate and arachidonic acid or thromboxane, respectively.

TEG and ROTEM are connected to a computer software which produces a graph. At the beginning of the trace, there are two superimposed flat lines, because there is no connection between the cup and the pin. When the clot starts forming, the two lines progressively diverge up to the maximal clot firmness, then they start converging again due to clot lysis (Figure 1.6). Five parameters are derived from this graph, although using different nomenclature for TEG and ROTEM (Hans & Besser, 2016):

- Reaction time (R) for TEG or clotting time (CT) for ROTEM: represent the latent period between the placement of the blood into the cup and the initial fibrin formation. It therefore provides information about coagulation factor levels and thrombin generation, prior to the formation of fibrin strands. This time is prolonged by clotting factors deficiencies and anticoagulant treatment. If prolonged, it can be normalized by transfusion of plasma containing coagulation factors or by protamine sulphate, in case of UFH;
- Coagulation time (K) for TEG or clot formation time (CFT) for ROTEM: represent the time necessary for the clot to reach a certain level of firmness (amplitude 20 mm). It provides information about the clot kinetics and depends on thrombin, fibrinogen and platelets.
- α-Angle: represents the angle between the baseline and the slope of the developing trace. It provides information about the rapidity of fibrin build-up and cross-

linking. It depends on fibrinogen levels; therefore, a reduced α-Angle can be corrected by fibrinogen concentrates or transfusion of plasma;

- Maximum amplitude (MA) for TEG or maximum clot firmness (MCF) for ROTEM: represent the maximum strength of the fibrin clot. It depends on platelet level, platelet function and platelet-fibrin interaction via GpIIb/IIIa. If reduced, it can be normalized by transfusion of platelet concentrates or by desmopressin;
- Clot lysis (Ly30 and Ly60) for TEG or lysis index (LI30 and LI60) for ROTEM: correspond to the reduction of the area under the curve at 30 and 60 minutes after MA or MCF. It represents the clot lysis and is used to quantify fibrinolysis.

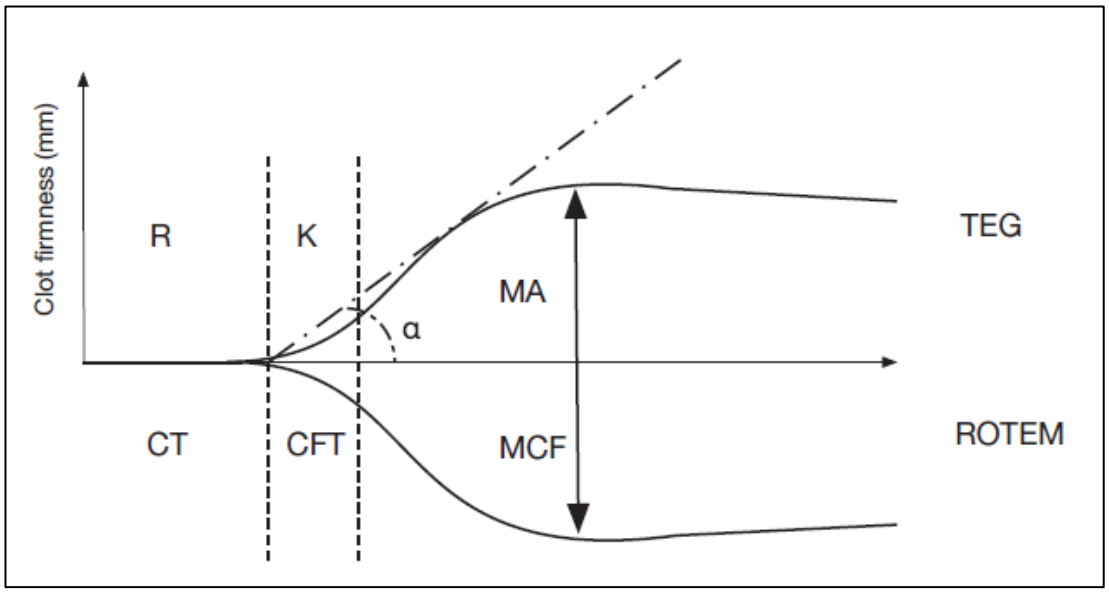


Figure 1.6 Typical trace obtained by thromboelastography and thromboelastometry (Hans & Besser, 2016) (Reproduced with permission, copyright licence no: 4646411156543)

Several variables can interfere with the TEG trace, and they are divided into preanalytical and analytical variables (Chitlur et al., 2014; MacDonald & Luddington, 2010).

Examples of preanalytical variables are the followings:

- Blood collection, either native whole blood or citrated whole blood, collected into tubes containing sodium citrate 3.2%. However, there are also collection tubes prefilled with corn trypsin inhibitor. Native whole blood should be tested within 5 minutes, while CWB should rest at room temperature at least 30 minutes and should be analysed within two hours from blood collection. A recently published study showed that results can differ according to the time elapsed (Durila et al., 2015);
- Use of tourniquet during venous sampling is usually not recommended because it can provoke venous stasis and alterations in coagulation test results;
- The recommended needle size is 21 gauge or larger, in order to avoid the activation of platelets;
- Multiple sampling from the same tube is discouraged because it can activate platelets and coagulation factors;
- Samples obtained from indwelling catheters can be contaminated with heparin;
- Alterations of blood count can affect the results, especially if low haematocrit and low platelet count.

Examples of analytical variables are the followings:

- Normal ranges should be established locally, better if adjusted for age (neonates, children, adults) and sex (male, female);
- Tests performed under different conditions, such as pH and temperature, can provide markedly different results;
- An adequate system of quality control, preferably with external quality assurance, is fundamental.

Examples of possible changes in the TEG trace in different clinical conditions are reported in Figure 1.7. For instance, anticoagulant treatment, coagulation factor deficiency and haemophilia can increase the R-time; conversely, the administration of FFP, PCC or rVIIa can decrease the R-time. Antiplatelet therapy, thrombocytopenia or thrombocytopathies can decrease the MA; conversely, platelet transfusion or fibrinogen supplementation can increase the MA.

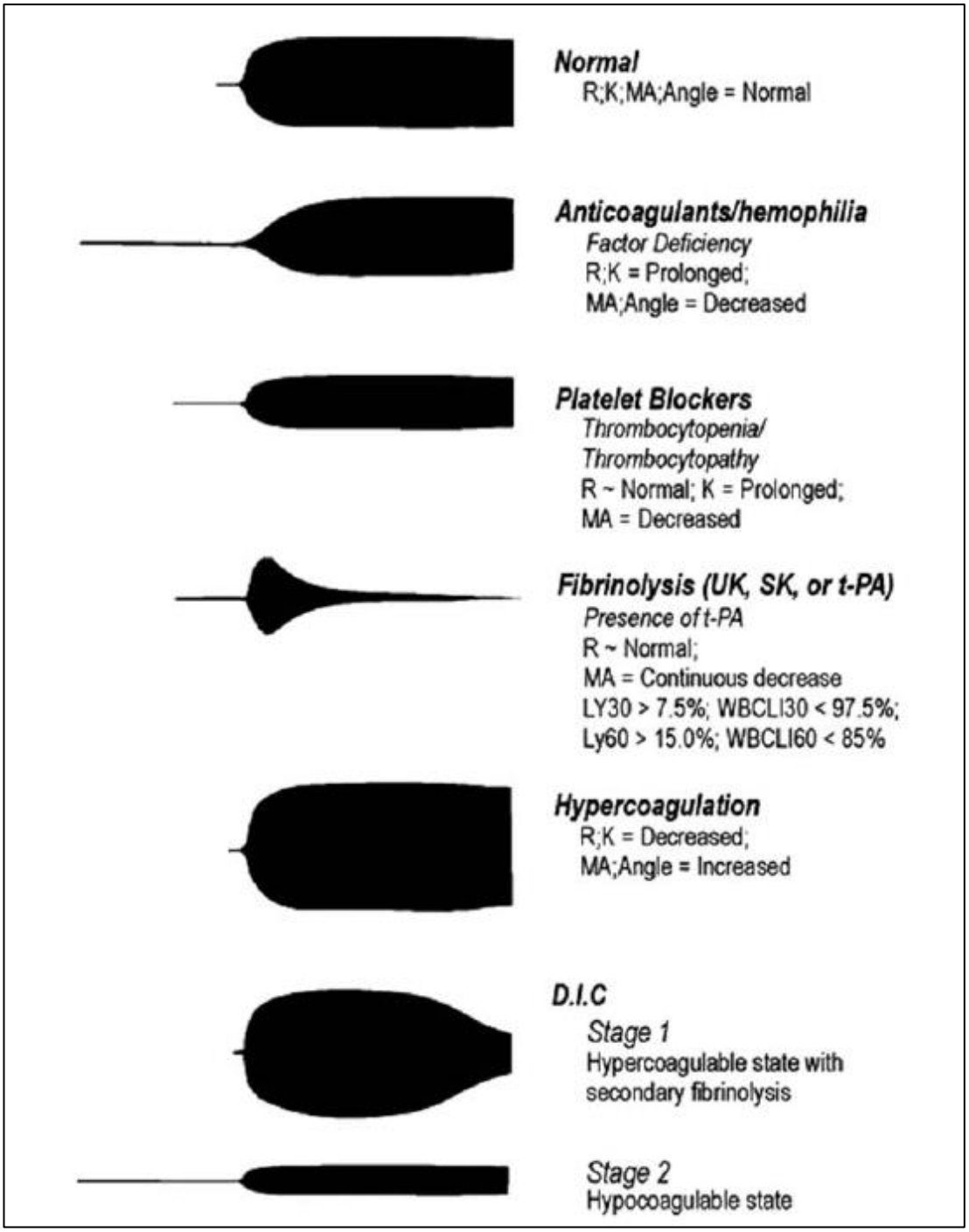


Figure 1.7 Thromboelastography output in different diseases (Whiting & DiNardo, 2014) (Reproduced with permission, copyright licence no: 4646410988290)

The TEG has the advantages of being a global haemostatic assay which can be performed rapidly at the patients' bedside, as a POC testing. However, it has also some limitations. First, the technique is not fully standardized yet and there are still differences among laboratories. Second, a normal curve does not exclude all

haemostatic defects and additional tests might be needed. For example, the TEG cannot detect surgical bleeding, defects in platelet adhesion or von Willebrand disease, deficiencies of factor VII or deficiencies of natural anticoagulants. It can identify, but not monitor, the vitamin K antagonist treatment. Finally, in order to detect the antiplatelet therapy, specific agonists are required (Reikvam et al., 2009).

1.3.5.2 Clinical applications of the thromboelastography

The TEG has limited applications in clinical practice, due to the paucity of studies demonstrating its reliability (H. Hunt et al., 2015; National Institute for Health and Care Excellence, 2014c). The NICE guidelines stated that "The ROTEM system and the TEG system are recommended to help detect, manage and monitor haemostasis during and after cardiac surgery", but also that "There is currently insufficient evidence to recommend the routine adoption of viscoelastometric point-of-care testing […] to help detect, manage and monitor haemostasis in the emergency control of bleeding after trauma and during postpartum haemorrhage" (National Institute for Health and Care Excellence, 2014c, p.4). However, the TEG has been widely studied in a number of conditions, such as cardiac surgery, trauma-induced coagulopathy, liver transplantation, obstetrics and bleeding disorders (Gatt et al., 2014; Hans & Besser, 2016; Whiting & DiNardo, 2014).

Some studies analysed the use of TEG to identify hypercoagulable states and to predict post-operative arterial or venous thromboembolic events. A systematic review of 10 studies published up to 2008 reported wide heterogeneity in test characteristics and reference ranges (Y. Dai et al., 2009). Furthermore, no universal definition of hypercoagulability was provided in these studies. The accuracy of the TEG was represented by a sensitivity of 0-100%, a specificity of 62-92% and a diagnostic odds

ratio of 1.5-27.7. Overall, it seemed that the MA was the most accurate parameter for hypercoagulable states, since several studies found a correlation with post-operative thrombosis (Y. Dai et al., 2009). It has been reported that hypercoagulability on the TEG (defined as MA > 69 mm preoperatively) in patients undergoing coronary artery bypass was associated with a higher 30-day rate of the combined outcomes myocardial infarction, stroke and mortality (Rafiq et al., 2012). Another study found that patients who developed a venous or arterial thromboembolic complication after major non-cardiac surgery showed lower CFT and higher α-angle or MCF at the pre-operative ROTEM (Hincker et al., 2014).

Some authors analysed whether the use of TEG can predict thromboembolic events in patients with cancer or trauma, although with contrasting conclusions. A study reported that patients with prostate cancer showed more hypercoagulability on the TEG, compared to age-matched normal control (Toukh et al., 2014). Among those hypercoagulable patients (defined as at least three abnormal TEG parameters), 31.8% subsequently developed thromboembolic complications during a 12-month follow-up, suggesting that TEG may be helpful in thrombotic risk stratification (Toukh et al., 2014). Conversely, another study found that patients with lung cancer, compared to age-matched healthy controls, had hypercoagulability on the ROTEM, as shown by reduced CT and increased MCF and α-angle, using both INTEM and EXTEM, however most of these results still fell within the established normal ranges (Davies et al., 2015). Only six patients developed VTE during a 12-month follow-up and most of them showed ROTEM parameters outside the normal range, but the group size was too small to show statistically significant differences (Davies et al., 2015).

Some authors found that elevated maximal amplitude on rapid TEG in trauma patients was an independent predictor for the development of PE (for mA > 65 mm the odd

ratio was 3.5, for mA > 72 mm the odd ratio was 5.8) (Cotton et al., 2012). Conversely, others reported that the TEG did not help in stratifying trauma patients at high-risk for VTE, but other variables were instead associated with the risk of developing thrombosis, such as longer stay in the intensive care unit, more operations and more severe abdominal injuries (Van Haren et al., 2014).

Some studies evaluated whether the TEG can be affected by ongoing anticoagulant treatment, with variable reports on its sensitivity. For instance, Coppell et al. (2006) evaluated standard and heparinase-TEG with the addition *in vitro* of UFH, LMWH and danaparoid at different concentrations. They found that the standard TEG parameters were already out of the reference ranges at concentrations of UFH, LMWH and danaparoid that have no effect on conventional coagulation tests (PT, APTT, TT), showing that the TEG is more sensitive than these assays. An exception was represented by the anti-factor Xa activity which was more sensitive than the standard TEG alone. When using the heparinase coated cuvettes, all TEG parameters were within the normal ranges, suggesting a successful neutralization of the anticoagulant effect (Coppell et al., 2006). However, in another study the ROTEM detected the anticoagulant effect of dalteparin only at over-therapeutic levels of anti-factor Xa, suggesting a low sensitivity of this assay (Feuring et al., 2011).

A recently published trial randomized 87 patients to standard enoxaparin dosage (30 mg twice daily) or TEG-adjusted enoxaparin dosage (daily titration in order to obtain ΔR, difference between standard and heparinise TEG, between 1-2 minutes) (Louis et al., 2014). The experimental group achieved a higher daily dosage of enoxaparin (median 50 mg vs. 30 mg BID, $p<0.001$), however no benefit was seen and similar rates of VTE developed in both groups (14% vs. 16%, $p=0.732$) (Louis et al., 2014).

In recent years, several authors tried to evaluate whether the TEG or the ROTEM can be used to evaluate the concentrations of the DOACs. There are some reports that they can detect the presence of rivaroxaban, while in others these assays were not sensitive enough to exclude the presence of rivaroxaban. However, there is some heterogeneity in the population enrolled, dosages of rivaroxaban and time of sample collection, which makes it difficult to summarize the results. For instance, in patients with stroke taking rivaroxaban, an increased time to clot formation and a decreased clot strength have been reported, as represented by prolonged R and K time and reduced MA and α-angle (Bowry et al., 2014). Modifications in K, MA and α-angle were evident up to six hours after rivaroxaban administration, while the R time remained prolonged up to 18 hours (Bowry et al., 2014). In patients undergoing orthopaedic surgery and receiving rivaroxaban 10 mg OD, the EXTEM CT was significantly prolonged on post-operative day 4 at rivaroxaban trough levels, compared to baseline, while the INTEM CT remained within the normal range (Oswald et al., 2015). In patients taking rivaroxaban 20 mg OD for VTE, the INTEM and EXTEM CT at peak concentration were prolonged, especially the CT ratio for EXTEM, while at trough concentration there was no difference with healthy volunteers (Chojnowski et al., 2015). In healthy volunteers after administration of rivaroxaban 10 mg, the INTEM and EXTEM CT were significantly prolonged compared to baseline, however there was a wide overlap of values and therefore the ROTEM parameters did not discriminate the presence of rivaroxaban (Casutt et al., 2012).

A group of researchers spiked *in vitro* different concentrations of dabigatran and reported a prolonged R time on the TEG trace (Solbeck et al., 2016). The other parameters were not influenced, since they remained unchanged across different concentrations. The R time showed also a strong correlation with the diluted thrombin

time (DTT) and the ecarin clotting time, the gold standard tests for monitoring dabigatran concentrations (Solbeck et al., 2016). Thus, the TEG can potentially be useful to rapidly detected dabigatran presence in emergency patients. In another *in vitro* study, different concentrations of dabigatran, rivaroxaban and apixaban were spiked and analysed using the TEG with kaolin and the rapid-TEG (Dias et al., 2015). The authors found that both assays showed prolonged enzymatic phase of coagulation, as expressed by prolonged clotting time.

1.3.6 Procoagulant phospholipid-dependent coagulation time

The procoagulant phospholipid-dependent coagulation time (STA Procoag PPL, Diagnostica Stago) is a functional test that assesses a clotting time based on procoagulant phospholipids. It can be used to detect the presence of microparticles in the plasma.

Microparticles are small phospholipid vesicles (0.1-1 μm diameter), derived from membranes of activated or apoptotic cells (Lacroix, Judicone, et al., 2013). Microparticles that express on their surface phosphatidylserine or TF have a procoagulant potential and, therefore, might have a role in cardiovascular diseases and VTE (Lacroix, Dubois, et al., 2013).

Microparticles are emerging biomarkers of arterial and venous thrombosis (Lacroix, Dubois, et al., 2013). They are increased in patients with transient ischemic attack or coronary artery diseases, but they might also be an independent predictor of cardiovascular complications and mortality. Microparticles are also increased in cancer patients with VTE, in patients with APS and in patient with acute VTE. Some studies reported a correlation between microparticles level and the risk of VTE. However, it is still a matter of debate whether microparticles are a cause or a

consequence of the thrombotic event (Lacroix, Dubois, et al., 2013). Elevated levels of microparticles have also been reported in other conditions, such as women with recurrent miscarriage (Patil et al., 2013), patients with transfusion-related complications (Jy et al., 2011) and patients with inflammatory rheumatic diseases (Distler et al., 2005).

Due to their small size, microparticles are not detected by traditional blood counters, but they are usually measured using flow cytometry. Flow cytometry provides the absolute number of microparticles and their cellular origin; however, it is a time consuming and expensive process, standardization of the method is required and it cannot detect the functional activity of microparticles (Patil et al., 2016).

The STA Procoag PPL has the advantage of being a simple and quick test, which can be tested on different substrates (PPP, PRP or CWB). The original method was described by Exner et al. (2003). It is a functional test, based on the fact that procoagulant microparticles can shorten the factor Xa clotting time. Therefore, the shorter the coagulation time the greater the level and activity of procoagulant phospholipids (Laresche et al., 2014; Patil et al., 2016). It can be performed on a semi-automated or automated coagulometers and it requires only a small amount of patient plasma (25 µl). The activated factor X clotting time showed very good correlation with flow cytometry for measuring microparticles (Exner et al., 2003; Patil et al., 2016).

The STA Procoag PPL has been evaluated in patients with different malignant solid (Chaari et al., 2014; Laresche et al., 2014) or haematological neoplasms (Marchetti et al., 2014; Mignon et al., 2013; Schneider et al., 2010); in patients with sickle cell disease (Noubouossie et al., 2012) or thalassemia major (Agouti et al., 2015); in carriers of factor V Leiden mutation (Campello et al., 2014); in women with recurrent miscarriage (Patil et al., 2013) or pre-eclampsia (Campello et al., 2015); and in other

conditions, such as obesity (Campello et al., 2014; Siklar et al., 2011), obstructive sleep apnoea (Ayers et al., 2014), intracerebral haemorrhage (Van Dreden et al., 2014) or organ failure (Van Dreden et al., 2013). However, so far, the STA Procoag PPL has never been assessed in patients with VTE.

1.3.7 Soluble P-selectin

P-selectin is a cell adhesion molecule usually stored in platelet, within the α-granules, and in endothelial cells, within the Weibel-Palade bodies (Furie & Furie, 2004). P-selectin is found on the surface of activated platelets and endothelial cells and its soluble form can be released into the plasma. P-selectin interacts with the P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed on leukocytes and on platelets (Furie & Furie, 2004). P-selectin not only is involved in the inflammatory response, but recent evidence suggest its role in thrombosis and haemostasis, by mediating platelet rolling, generating procoagulant microparticles and enhancing fibrin deposition (Cambien & Wagner, 2004).

Soluble P-selectin is measured using an ELISA technique. Soluble P-selectin has been reported to be increased in patients with acute DVT (Rectenwald et al., 2005) and also in patients with recurrent VTE (Ay et al., 2007; Kyrle et al., 2007). For instance, a study evaluated the D-dimer (Advanced D-dimer, Dade-Behring) and soluble P-selectin in three groups of patients: 30 normal controls (group 1), 22 patients with confirmed DVT (group 2) and 21 symptomatic patients but without DVT (group 3) (Rectenwald et al., 2005). DVT patients had significantly higher levels of D-dimer (7.57 mg/L vs. 1.53 mg/L in group 1 and vs. 3.19 mg/L in group 3); soluble P-selectin (0.98 ng/mg total protein vs. 0.34 ng/mg in group 1 and vs. 0.55 ng/mg in group 3). Total microparticles were also increased in DVT patients, although this result was not

statistically significant. Furthermore, the authors identified some threshold values (soluble P-selectin > 0.68 ng/mg total protein, total microparticles > 125% of control, D-dimer > 3.0 mg/L) that combined provided sensitivity of 73%, specificity of 81%, and accuracy of 77% for the diagnosis of DVT (Rectenwald et al., 2005).

Kyrle et al. (2007) followed 544 patients with a first unprovoked VTE, of whom 63 (12%) had recurrent VTE during a mean follow-up of 35 months. Levels of P-selectin were higher in patients with recurrent events (45.8 mg/dL vs. 40.1 mg/dL, p=0.006). Furthermore, patients with P-selectin levels above the 75th percentile had a greater probability of VTE recurrence (20.6% vs. 10.8%, p=0.046) (Kyrle et al., 2007).

Finally, a recently published systematic review and meta-analysis of 11 studies reported that mean levels of soluble P-selectin were 2.89 times higher in VTE patients compared to controls (Antonopoulos et al., 2014).

1.4 Anticoagulant drugs

Several anticoagulant drugs are currently available. They can be classified by their route of administration (parenteral vs. oral) and by their mechanism of action (indirect factor Xa inhibitors, direct factor Xa inhibitors, direct thrombin inhibitors, VKA). However, these classifications are sometimes mixed (e.g. the DOAC).

1.4.1 Pharmacology of the parenteral anticoagulants

1.4.1.1 The indirect factor Xa inhibitors

The parenteral indirect factor Xa inhibitors include UFH, LMWH, fondaparinux and danaparoid. The anticoagulant properties of the heparins were discovered at the beginning of the XX century (McLean, 1916). UFH is a mixture of sulfated

glycosaminoglycans, with a mean molecular weight of approximately 15 kDa (around 45 saccharide units) (Garcia et al., 2012). LMWHs are obtained from UFH by depolymerization and they are a group of molecules with slightly different structures. They have a mean molecular weight of approximately 4-5 kDa (around 15 saccharide units), with a range 2-9 kDa. Fondaparinux is a synthetic drug, which consists only in a pentasaccharide fragment with a molecular weight of ~2 kDa (Garcia et al., 2012). Danaparoid is a mixture of glycosaminoglycans, including heparin sulfate (around 84%), dermatan sulfate (around 12%) and chondroitin sulfate (around 4%), and has a mean molecular weight of approximately 5-6 kDa (Greinacher et al., 2013).

From a pharmacodynamics point of view, the action of the indirect factor Xa inhibitors is mediated by their interaction with a plasma cofactor, the natural anticoagulant AT (Garcia et al., 2012). All the indirect factor Xa inhibitors have a specific pentasaccharide sequence with high affinity for AT (Figure 1.8). AT can inactivate not only thrombin, but also the coagulation factors IXa, Xa, XIa, and XIIa. Among all these factors, thrombin and Xa are the most sensitive to the inhibition by AT. Heparins can also block the cascade effects of thrombin, such as the activation of platelets and cofactors V and VIII. However, in order to inactivate thrombin, heparins have to form a ternary complex heparin/AT/thrombin (Garcia et al., 2012). Conversely, for the inactivation of factor Xa, the binding between heparin and AT is sufficient. However, only the free factor Xa can be blocked by the indirect factor Xa inhibitors (Rupprecht & Blank, 2010). LMWHs which contains less than 18 saccharide units are too short to form the ternary complex and cannot inhibit thrombin. Therefore, UFH has an anti-factor Xa:IIa ratio of 1:1, while LMWH have an anti-factor Xa:IIa ratio of 2:1-4:1. Fondaparinux has only anti-Xa activity since it contains only the five saccharide units

with high affinity for AT (Garcia et al., 2012). Danaparoid has mainly anti-Xa activity and only minimal anti-IIa activity (Greinacher et al., 2013).

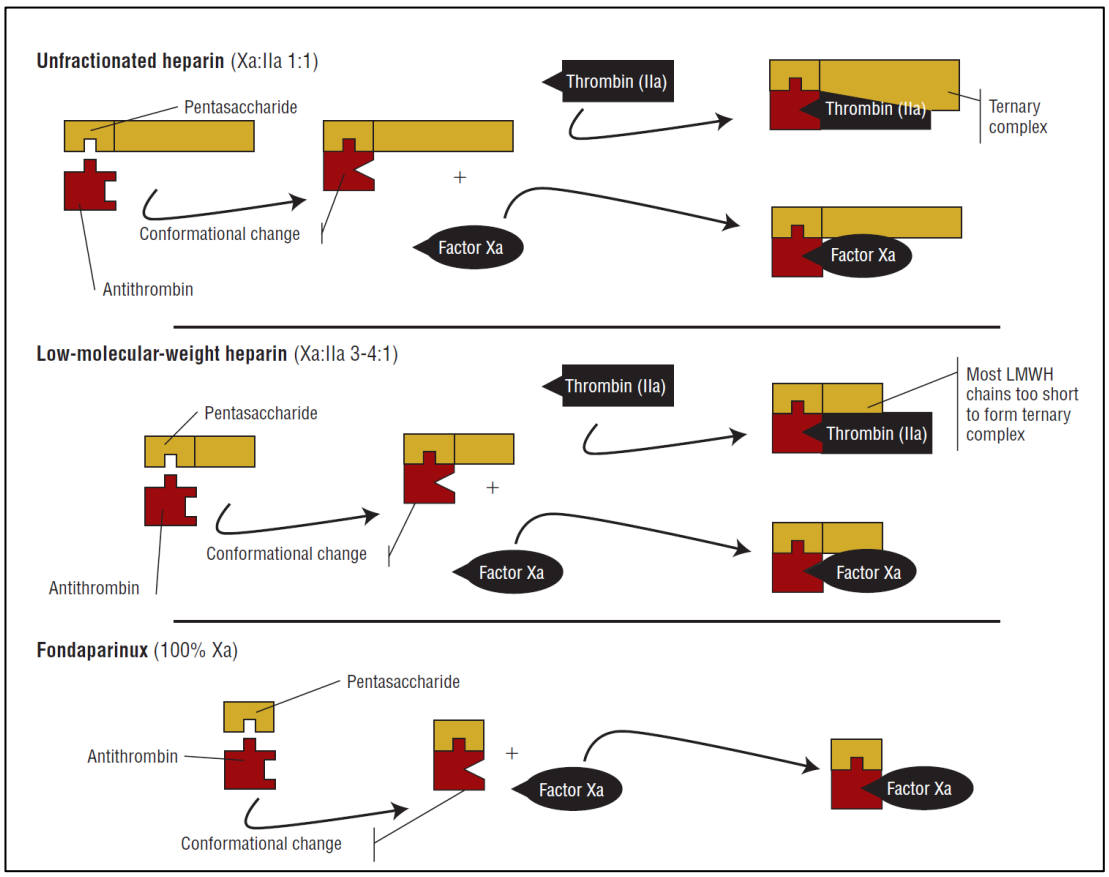


Figure 1.8 Pharmacologic activity of unfractionated heparin, low-molecular-weight heparin and fondaparinux (Haines et al., 2008) (Reproduced with permission, copyright licence no: 4647561231969)

From a pharmacokinetics point of view, when administered subcutaneously UFH has limited and dose-dependent bioavailability (ranging from 30% at low dosages to 70% at high dosages). When administered intravenously is usually given as a continuous infusion. UFH has also high binding to plasma protein, therefore producing a variable anticoagulant response. The clearance is mainly through the reticuloendothelial system; however, this is a saturable mechanism. UFH is also excreted renally, which

is the predominant route at high dosages. Therefore, the half-life is dose-dependent, ranging from 30 to 150 minutes (Haines et al., 2008). UFH is monitored with the APTT ratio, aiming to a target range 1.5-2.5, which corresponds to 0.3-0.7 U/ml on the anti-Xa assay (Garcia et al., 2012).

LMWHs are administered subcutaneously in fixed doses (for prophylaxis) or weight-adjusted doses (for therapy). Bioavailability is approximately 90% and the peak anticoagulant effect is reached at 3-5 hours. Clearance is mainly renal, therefore the half-life of LMWH is prolonged in patients with renal insufficiency. Laboratory monitoring of LMWH is needed only in patients with extreme body weight, pregnancy or severe renal failure. The anti-Xa assays is performed at peak concentration, e.g. four hours after administration (Garcia et al., 2012).

Fondaparinux is administered subcutaneously. It has an almost complete bioavailability, a predictable anticoagulant effect and a long half-life of 17 hours. It can be administered OD without the need for monitoring. Fondaparinux has renal clearance, therefore being contraindicated in severe renal failure (Garcia et al., 2012).

Danaparoid can be administered subcutaneously, with an almost complete bioavailability, or by intravenous infusion. It has a long half-life of 25 hours and is cleared by the kidneys. Laboratory monitoring is recommended only in case of heparin induced thrombocytopenia (HIT) and severe thrombosis, and the therapeutic range is 0.5-0.8 anti-Xa U/ml (Greinacher et al., 2013).

1.4.1.2 The direct thrombin inhibitors

The parenteral direct thrombin inhibitors include hirudin and its recombinant forms (lepirudin, desirudin), argatroban and bivalirudin. They can directly bind and inactivate thrombin, without the need for plasma cofactors (Garcia et al., 2012). The

direct thrombin inhibitor can bind both free and fibrin-bound thrombin (Greinacher et al., 2013). The affinity for thrombin is high for hirudin, intermediate for bivalirudin, and low for argatroban (Warkentin et al., 2008). The parenteral direct thrombin inhibitors are licensed for use in patients with HIT, except bivalirudin which was withdrawn from the European market in 2018 due to commercial reasons.

Hirudin has a molecular weight of 7 kDa. It is usually administered by intravenous infusion, even though the recombinant forms can be also given subcutaneously, and has a predominant renal clearance. The half-life is approximately 1-2 hours. It is monitored with the APTT (target range 1.5-2.5) (Greinacher et al., 2013).

Bivalirudin has a molecular weight of 4 kDa. The route of administration is by intravenous infusion. Bivalirudin has a dual clearance: renal (20%) and non-organ proteolysis (80%). The half-life is approximately 25 minutes. It is monitored with the activated clotting time (ACT) when used during cardiac surgery, otherwise using the APTT (target range 1.5-2.5) (Warkentin et al., 2008).

Argatroban is a small drug with a molecular weight of 0.5 kDa. It is administered by intravenous infusion and has hepatobiliary clearance. The half-life is approximately 45 minutes. It is monitored with the APTT (target range 1.5-2.5) (Greinacher et al., 2013).

1.4.2 Pharmacology of the oral anticoagulants

1.4.2.1 The vitamin K antagonists

The VKAs include warfarin, acenocoumarol and phenprocoumon, and fluindione. Warfarin is the most common VKA used in clinical practice. They have been the only available oral anticoagulant drugs for more than 60 years and they are still widely used

nowadays. From a pharmacodynamics point of view, VKAs block the cycle of vitamin K (interconversion between vitamin K and vitamin K epoxide), therefore inhibiting the carboxylation of some procoagulant factors (II, VII, IX and X) and natural anticoagulant factors (proteins C and S) which are dependent on vitamin K (Whitlon et al., 1978). Due to their effect on the natural anticoagulants, a transient procoagulant effect is described during the first few days of VKA treatment, but afterwards the anticoagulant effect is prevalent (Ageno et al., 2012).

From a pharmacokinetics point of view, warfarin has high bioavailability and is rapidly absorbed. Maximum plasma concentrations are reached in approximately 90 minutes (J. Hirsh, 1991). Warfarin is metabolized in the liver by several enzymes of the cytochrome P450 system (principally CYP2C9, CYP3A4 and CYP1A2) (Miners & Birkett, 1998), circulates bound to albumin and has a plasma half-life of 36-42 hours. The other VKA have different half-lives: 8-9 hours for acenocoumarol, 31 hours for fluindione, and 5.5 days for phenprocoumon (Godbillon et al., 1981). All VKA have an essentially hepatic metabolism and reach their peak of action after 4-5 days (Ageno et al., 2012).

VKAs have high intra- and inter-individual variability in dose-response, and therefore routine laboratory monitoring is required to maintain an adequate anticoagulant level. Several variables can influence the clearance of the drug or the synthesis of the vitamin-K dependent coagulation factors: endogenous variables (such as age, gender, body weight, genetic factors, comorbidities) or environmental variables (such as concomitant medications, food and dietary level of vitamin K, alcohol consumption) (Ageno et al., 2012). VKAs are monitored using the INR and their dose is adjusted in order to maintain the INR within an established therapeutic range. The INR target range is 2.0-3.0 for the majority of patients with AF, VTE and bileaflet mechanical

aortic valves, while a higher target range 2.5-3.5 is required for mechanical mitral valves and old generation aortic valves (Kearon et al., 2016; Lip et al., 2018; Nishimura et al., 2017). Under-therapeutic anticoagulation is associated with an increased risk of thromboembolic events, while supra-therapeutic anticoagulation carries an increased risk of haemorrhagic complications (Fuster et al., 2006; Wan et al., 2008), as shown in Figure 1.9.

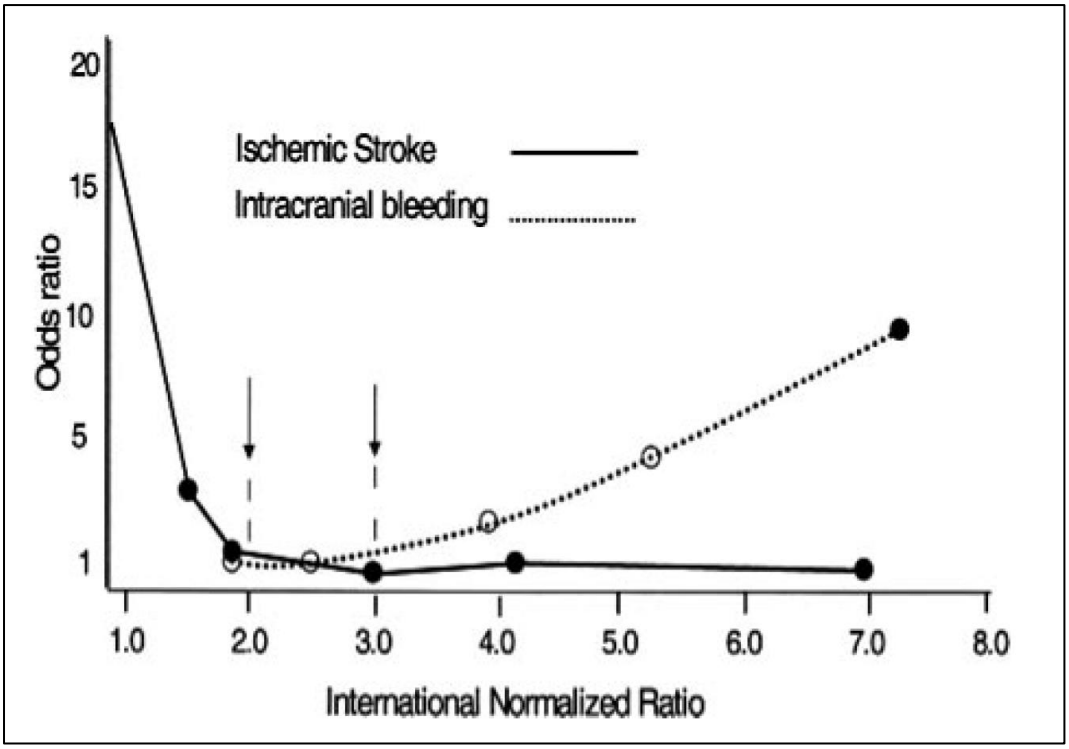


Figure 1.9 Risk of ischemic stroke and intracranial haemorrhage in relation to INR values (Fuster et al., 2006) (Reproduced with permission, copyright licence no: 4663641372409)

The TTR is an indirect measurement of the anticoagulation control. There are different ways of measuring the TTR, the most common being the percentage of INR values within the therapeutic range and the Rosendaal's method. The latter assumes that there is a linear relationship between the INR values and therefore gives a specific INR

value to each day between two tests (Rosendaal et al., 1993). Studies showed a strong relationship between the TTR and the incidence rate of thromboembolic and bleeding complications (Björck et al., 2016; S. Haas et al., 2016; Wan et al., 2008). The benefit of VKA has been reported to be particularly evident in patients with a TTR of at least 65-70% (Connolly et al., 2008; Gallagher et al., 2011).

1.4.2.2 The direct oral anticoagulants

In the last 20 years, several novel DOAC (also called NOAC for Non-vitamin K antagonist Oral AntiCoagulants or TSOAC for Target-Specific Oral AntiCoagulants) were developed (Husted et al., 2014). These novel anticoagulant drugs include the direct thrombin inhibitor (e.g. dabigatran etexilate) and the direct factor Xa inhibitors (e.g. rivaroxaban, apixaban, edoxaban) (Ahrens et al., 2010).

From a pharmacodynamics point of view, dabigatran binds directly to the active site of thrombin, therefore inactivating both free and fibrin-bound thrombin. Fibrin-bound thrombin is a peculiar target of dabigatran, since it cannot be inactivated by the indirect inhibitors, such as UFH or LMWH (Hankey & Eikelboom, 2011). Rivaroxaban, apixaban and edoxaban are direct inhibitors of the factor Xa. They can inhibit free FXa, clot-bound FXa and FXa within the prothrombinase complex. They can directly bind FXa, while the indirect inhibitors, such as fondaparinux, require AT as cofactor (Furugohri et al., 2008; Perzborn et al., 2010; Wong et al., 2011).

From a pharmacokinetics point of view, the DOACs have a quick onset of action and the peak plasma concentrations are reached within two hours. The half-lives are shorter than the VKA and trough concentrations are reached within 12-24 hours (Bounameaux & Camm, 2014; Hankey & Eikelboom, 2011; Kubitza et al., 2005; Wong et al., 2011). They have a low potential for food and drug interactions and a

more predictable anticoagulant response. The DOACs have also a predominantly renal clearance, although some differences have been reported. For instance, dabigatran is the DOAC with the highest rate of renal clearance (Hankey & Eikelboom, 2011), while rivaroxaban can accumulate in patients with moderate liver insufficiency (Kubitza et al., 2013).

The DOACs have several advantages compared to the VKAs. The rapid onset of action and the short half-lives allow short interruption in case of surgery or interventional procedures, without the need for routine heparin bridging therapy. The predictable anticoagulant response allows the administration of fixed doses, without the need for routine laboratory monitoring.

However, the DOACs also carry some disadvantages. Commonly used laboratory tests (such as the PT or APTT) do not accurately reflect the DOAC plasma concentrations. Therefore, in case of bleeding/thrombotic complications, emergency surgery or extreme body weights, more specific and expensive tests should be used (such as the dilute thrombin time for dabigatran or the chromogenic anti-Xa assays for the factor Xa inhibitors) (Patel, Byrne, et al., 2019). The lack of routine monitoring can have a negative impact on patient adherence, making it important to perform accurate patient selection and to establish continuous education and follow-up.

1.4.3 Clinical indications to the anticoagulant therapy

The anticoagulant therapy is currently used for the prevention and treatment of VTE; for the prevention of stroke and systemic embolism in patients with AF, prosthetic heart valves or valvular heart diseases; and for other less common indications, such as patients with acute coronary syndromes or acute limb ischemia.

VTE, which encompass DVT and PE, is the third most common cardiovascular disorder, after acute coronary syndrome and stroke (S. Z. Goldhaber, 2012). The estimated incidence rate of VTE is 1-2 per 1,000 person-years, with PE accounting for one-third of the events and DVT for the remaining two-thirds (Naess et al., 2007). VTE is a potentially fatal disease, with the in-hospital case fatality rate associated with PE being approximately 10%. The risk of short- and long-term recurrent VTE or chronic sequelae, such as the post-thrombotic syndrome and the post-embolic pulmonary hypertension, is also not negligible (S. Z. Goldhaber & Bounameaux, 2012).

Anticoagulation is used for the prevention of VTE in patients undergoing major surgery, but also in some categories of non-surgical patients, such as acutely ill medical inpatients (Falck-Ytter et al., 2012; Gould et al., 2012; Kahn et al., 2012). The anticoagulant treatment after a diagnosis of VTE is usually divided into three phases: the "initial therapy" (from 0 to 7 days) is performed with parenteral anticoagulation, thrombolytic therapy, or some DOAC; the "long-term therapy" (from 7 days to 3 months) is performed with oral anticoagulant drugs (either VKA or DOAC), or LMWH in specific circumstances; the "extended therapy" (from 3 month to indefinite time) is usually performed with the same agents used for the long-term phase (Kearon et al., 2012). The minimum recommended treatment duration is three months, since shorter periods have been correlated with an increased risk of VTE recurrences. Extended treatment duration should be considered in patients with cancer-associated VTE, recurrent VTE and unprovoked VTE, if not contraindicated by an excessively high risk of bleeding. According to the new ACCP guidelines, the DOACs are suggested, over the VKAs, in patients with lower limb DVT or PE and without cancer (Kearon et al., 2016). The DOACs are not recommended, so far, in patients with

unusual site VTE (thrombosis of splanchnic or cerebral veins), until more evidence will become available.

AF is the most common cardiac arrhythmia. It has an overall prevalence of 5.5%, and increases with aging up to 17.8% in subjects over 85 years old (Heeringa et al., 2006). AF carries a 5-fold increased risk of stroke (Wolf et al., 1991), with a 30-day mortality rate of 24% without treatment (Hylek et al., 2003). The oral anticoagulant therapy is extremely effective in the prevention of thromboembolic complications: it is estimated that VKAs reduce the risk of stroke by 64% compared to placebo (Hart et al., 2007) and that the DOACs further reduce the risk of stroke or systemic embolism by 19% compared to the VKAs (Ruff et al., 2014).

In AF patients, the latest ESC guidelines recommend to evaluate the risk of stroke and systemic embolism using the CHA_2DS_2-VASc score, which includes the following variables: cardiac failure (1 point), hypertension (1 point), age ≥75 years (2 points), diabetes (1 point), stroke (2 points), vascular disease (1 point), age 65–74 (1 point) and female sex (1 point) (Kirchhof et al., 2016). The authors stated that "Oral anticoagulation therapy to prevent thromboembolism is recommended for all male AF patients with a CHA_2DS_2-VASc score of 2 or more" and "all female AF patients with a CHA_2DS_2-VASc score of 3 or more", and that it "should be considered in male AF patients with a CHA_2DS_2-VASc score of 1" and "in female AF patients with a CHA_2DS_2-VASc score of 2, considering individual characteristics and patient preferences" (Kirchhof et al., 2016, p.2920). The DOACs are preferable to the VKAs, except in patients with valvular AF or severe renal impairment (defined as creatinine clearance < 30 ml/min) (Kirchhof et al., 2016).

Prosthetic heart valves carry a thromboembolic risk, which depends on the site (aortic vs. mitral), the type (mechanical vs. biologic) and the characteristics (caged-ball,

tilting disk, bileaflet) of the valve. Lifelong oral anticoagulation with VKAs is recommended for all patients with a mechanical valve, with different INR target ranges according to the site and the characteristics of the valve. In case of a bioprosthetic valve, a limited period of oral anticoagulation (up to 3-6 months) or antiplatelet only (if aortic valve and sinus rhythm) can be considered (Nishimura et al., 2017; Whitlock et al., 2012). The DOACs are contraindicated in patients with prosthetic heart valves (Czuprynska et al., 2017; Nishimura et al., 2017)

1.5 Reversal and neutralisation of the anticoagulant treatment

1.5.1 Risk of bleeding during anticoagulant treatment

The risk of bleeding during parenteral anticoagulant treatment with UFH or LMWH is relatively low. A systematic review and meta-analysis reported major bleeding events in 26 out of 1147 (2.3%) patients treated with UFH and 36 out of 1153 (3.1%) patients treated with LMWH (odd ratio 0.72, 95% CI 0.43-1.20) (Robertson & Strachan, 2017). Even lower incidence rates were reported in a recent cohort study enrolling 12,934 patients with acute VTE, where only 32 patients experienced a major bleeding event (cumulative incidence 2.5 per 1,000 patients) (van Rein et al., 2017).

The reported incidence of major bleeding during oral anticoagulant therapy with VKA is approximately 2% per year in RCTs, but that can reach more than 7% per year in real life data (Wiedermann & Stockner, 2008). Furthermore, VKA related bleeding events carry a high case-fatality rate of 13.4% (95% CI, 9.4-17.4%) (Linkins et al., 2003). The DOACs have a 20-40% lower risk of major bleeding complications compared to VKA, and in particular the risk of intracranial and fatal bleeding is more than halved (Dentali, Riva, et al., 2012; van der Hulle et al., 2014).

Several risk factors are known to increase the risk of bleeding in anticoagulated patients (Palareti & Cosmi, 2009):

- The timing from starting of anticoagulation, since the first three months carry an especially high risk of bleeding events;
- The advanced age. The elderly are particularly vulnerable, since they have reduced metabolic clearance, therefore requiring lower anticoagulant dose, but they also have higher prevalence of co-morbid conditions or interacting medications;
- Co-morbid conditions, such as gastro-intestinal diseases, hepatic or renal diseases, malignancies;
- Concomitant medications, such as antiplatelet or anti-inflammatory drugs;
- The intensity of anticoagulation and, in VKA patients, also the quality of anticoagulation control, expressed by the TTR.

An accurate estimate of the bleeding risk in anticoagulated patients is crucial, in order to guide the intensity and duration of the treatment. Several clinical prediction rules were developed in the past two decades, such as mOBRI (Beyth et al., 1998); HEMORR$_2$HAGES (Gage et al., 2006); HAS-BLED (Pisters et al., 2010); ACCP score (Kearon et al., 2016); VTE-BLEED (Klok et al., 2017). However, these scores are hampered by several drawbacks. Some of these risk factors are not available at the beginning of the anticoagulant treatment or can change during long-term treatment (e.g. compliance, quality of anticoagulation control or concomitant medications). Other variables are not easily available (e.g. platelet function or genetic mutations) or different cut-offs have been used for the same variable in different scores (e.g. age or anaemia). Furthermore, there are contrasting reports on their accuracy. In particular, they appear to have a low predictive value during long-term anticoagulant treatment for VTE (Olesen et al., 2011; Poli et al., 2013; Riva et al., 2014).

1.5.2 Reversal strategies for anticoagulant-related bleeding complications

Several options are available to reverse the effect of the different anticoagulant drugs (J. P. Hanley, 2004). In some cases, withholding the drug could suffice, especially if the anticoagulant has a short half-life. In other situations, specific antidotes (protamine sulfate, vitamin K, idarucizumab, andexanet alfa) or generic products (FFP, PCC, rVIIa) can be useful.

Protamine sulfate is the specific antidote for UFH and LMWH. It binds to heparin, forming an inactive complex. While protamine sulfate can totally reverse the effect of UFH, it has only partial effect on LMWH, probably due to its limited action on small LMWH chains with anti-Xa activity. It is administered by slow intravenous injection and 1 mg of protamine sulfate can neutralise approximately 100 units of heparin. However, the anti-Xa activity of the LMWH is only partially reversed by protamine sulfate (M. Levi, 2015).

Vitamin K1 (phytomenadione) is the specific antidote for VKA, since it can restore the intrinsic production of vitamin K dependent coagulation factors. It requires long time (between 4-6 hours) to reverse the anticoagulant status, but is fundamental to guarantee a prolonged reversal. It can be administered orally or intravenously; the intramuscular route is not recommended because of the risk of haematoma, while the subcutaneous route is not effective. Slow intravenous administration is recommended in order to lessen the risk of anaphylactoid reactions. Vitamin K can be given in dosages between 1 and 10 mg, according to the severity of bleeding (Frumkin, 2013; Holbrook et al., 2012).

Idarucizumab is the specific antidote for dabigatran. It is a monoclonal antibody which can bind and inactivate dabigatran. It usually administered intravenously as two bolus

infusion of 2.5 g each (total dose 5 g). In a large trial, the RE-VERSE AD, idarucizumab was administered to 503 patients who had an uncontrolled bleeding or who needed immediate reversal to undergo an urgent surgical procedure (Pollack et al., 2017). After idarucizumab administration, dabigatran plasma concentrations decreased to ≤ 20 ng/ml in 99.4% of the patients. In patient with uncontrolled bleeding, the median time to effective haemostasis was 2.5 hours, while in patients undergoing surgery the median time to the procedure was 1.6 hours. However, idarucizumab carried also a thromboembolic risk, with thrombotic events occurring in 4.8% of patients at 30 days and 6.8% of patients at 90 days (Pollack et al., 2017).

Andexanet alfa is the specific antidote for the direct factor Xa inhibitors (apixaban, edoxaban, rivaroxaban), but has also the potential to reverse enoxaparin. It is a recombinant form of human factor Xa, which has been modified and is actually inactive. Therefore, it can bind and segregate the factor Xa inhibitors. In a large trial, the ANNEXA-4, andexanet was administered to 352 patients with major bleeding events (Connolly et al., 2019). It was administered intravenously using two different dose regimens: a bolus of 400 mg in 15 minutes followed by an infusion of 480 mg in two hours (if patients on apixaban, or if last dose of rivaroxaban > 7 hours before), or a bolus of 800 mg in 30 minutes followed by an infusion of 960 mg in two hours (if patients on enoxaparin or edoxaban, or if last dose of rivaroxaban ≤ 7 hours before). At the end of the bolus, the median anti-Xa activity was 11.1 ng/ml in patients receiving apixaban and 14.2 ng/ml in patients receiving rivaroxaban. Effective haemostasis was reported in 82% of patients; however, thrombotic events occurred in 10% of patients at 30 days (Connolly et al., 2019).

FFP is commonly used for VKA reversal. It has a long preparation process which results in delayed administration: after testing for ABO blood group compatibility, it

requires approximately 15 minutes to be thawed before administration. In order to effectively replace the coagulation factors, it is usually administered at a dose 15 ml/kg, considering that 1 millilitre contains approximately 1 unit of clotting factors (Makris et al., 2010). Some authors recommend higher doses of FFP up to 30 ml/kg, particularly in case of intracerebral haemorrhage (Goodnough & Shander, 2011; Tomaselli et al., 2017). However, this large volume of FFP can cause volume overload, especially in frail patients. FFP can also transmit infectious agents or cause adverse reactions, such as the transfusion-related acute lung injury (TRALI) (Makris et al., 2010).

PCC are concentrates of vitamin-K dependent coagulation factors derived from normal pooled plasma using different processing techniques, including viral inactivation. They contain a concentration of coagulation factors which is around 25 times higher than normal human plasma, meaning that only 40 ml of PCC might be enough to provide 1000 units of clotting factors (Makris et al., 2010). In 3-factor PCC there are factors II, IX and X, while in 4-factor PCC factor VII is included too. They can contain also the natural anticoagulants protein C and protein S and a small amount of heparin, in order to prevent the activation of clotting factors. They are stored in lyophilised powder form, at room temperature, and can be administered as a small volume (approximately 1-2 ml/Kg) in a short period of time (approximately 10 minutes). They are not blood-group specific. For patients on VKA treatment, the recommended dosages are between 25 and 50 U/kg, according to the INR and the severity of bleeding (Franchini & Lippi, 2010). For patients on DOAC treatment dosages between 25 and 50 U/kg have been reported, or a fixed bolus of 2000 U (Schulman et al., 2018); however, preference is usually given to high doses (50 U/kg) (Siegal et al., 2014; Tomaselli et al., 2017).

There is also activated PCC, containing factors II, VII, IX and X, which are activated during the fabrication process. They are not used for warfarin reversal, but for patients with haemophilia and inhibitors against factor VIII or IX. In patients with haemophilia, they are administered at dosages between 50 and 100 U/kg based on the severity of bleeding. There are small studies evaluating dosages of 50-80 U/kg in patients taking dabigatran or rivaroxaban, but the evidence is low and they should be used with extreme caution in patients at already increased thrombotic risk (Siegal et al., 2014).

rFVIIa can bypass the amplification loop of the coagulation cascade. It is approved for use in patients with haemophilia with inhibitors, although it is frequently used off label for other indications. When used for warfarin reversal, rFVIIa can rapidly reverse anticoagulation, but there are several reports in which it can increase by 1.68 fold the risk of arterial thrombotic events compared to placebo (M. Levi et al., 2010; Mayer et al., 2008). Therefore, its routine use in warfarin reversal is not recommended. Furthermore, since the INR is very sensitive to factor VII levels, rFVIIa can have a larger effect on the INR than on the actual haemostasis. It has also to be considered its short half-life of 1-2 hours, which is not reflected by the INR (Frumkin, 2013). rFVIIa is not recommended for DOAC reversal, because of the lack of clinical data supporting its use (Garcia & Crowther, 2019; Siegal et al., 2014).

1.5.3 Differences between fresh frozen plasma and prothrombin complex concentrates

The main advantages of PCC, compared to FFP, are:

- The smaller volume of PCC required to reverse anticoagulation, which reduces the risk of fluid overload and decrease the time needed for infusion;

- The quicker preparation of PCC, which do not require blood group matching and thawing;
- The better safety profile of PCC, due to viral inactivation.

However, there are still some adverse events reported with the use of PCC, such as allergic reactions, HIT (for PCCs containing heparin) and thromboembolic complications (Franchini & Lippi, 2010). In a meta-analysis of 27 observation studies of warfarin reversal, the rate of thromboembolic complications was 1.8% (95% CI, 1.0-3.0) in patients receiving 4-factor PCC and 0.7% (95% CI, 0.0-2.4) in patients receiving 3-factor PCC (Dentali et al., 2011). The rate of thromboembolic complications in patients receiving 4-factor PCC for major bleeding events during treatment with one of the oral factor Xa inhibitors has been reported to be 4% (Piran et al., 2019).

While FFP is not recommended for DOAC reversal, it is still debated whether there is different efficacy between FPP and PCC, and between 3-factor and 4-factor PCC, when used for VKA reversal. Several studies showed that PCC are more effective than FFP in correcting the INR. For instance, a study showed that the mean INR post-infusion was 2.3 in patients receiving 4 units of FFP and 1.3 in patients receiving PCC 25-50 U/Kg (Makris et al., 1997). Similarly, in another study PCC achieved a more rapid and exhaustive INR reversal than FFP, with mean post-treatment INRs being 1.32 and 2.30, respectively (Cartmill et al., 2000). However, several cohort studies have tried to assess whether the more rapid reduction of INR is associated with better clinical outcomes. For instance, a study showed that PCC were correlated with reduced progression of intracranial haemorrhage compared to FFP, which occurred in 19% of patients receiving PCC vs. 33% of those receiving FFP (Huttner et al., 2006). This effect was probably due to a more rapid INR reversal; in fact, the difference was not

statistically significant when considering only patients with complete INR reversal within two hours (19.2% vs. 28.5%) (Huttner et al., 2006). Another study enrolled patients on warfarin treated with FFP or PCC for emergency warfarin reversal, because of a bleeding, interventional procedure or trauma (Hickey et al., 2013). The authors compared 149 patients receiving FFP (from September 2006 to August 2008) to 165 patients receiving 4-factor PCC (from September 2008 to August 2010). PCC were associated with a more rapid INR reversal and less red blood cell transfusion needed. Furthermore, serious adverse events within seven days (such as mortality, arterial and venous thrombosis, heart failure) were lower with PCC (9.7% vs. 19.5%, p=0.014) (Hickey et al., 2013). Majeed et al. (2014) evaluated the 30-day mortality in 35 patients receiving FFP and 100 patients receiving 4-factor PCC because of a VKA-associated intracerebral haemorrhage. However, after adjustment for age, haematoma volume and localization, mortality rates were similar in the two groups (Majeed et al., 2014).

There are only few RCTs in the context of emergency warfarin reversal. In the study by Boulis et al. (1999) patients with intracranial haemorrhage were randomized to FFP (8 patients) or FFP supplemented with Factor IX complex concentrate (5 patients). The mean time to INR correction was lower in patients receiving FFP and Factor IX complex concentrate than in patients receiving FFP alone (2.95 vs. 8.9 hours) and also the mean volume of FFP required was lower (399 ml vs. 2712 ml). Neurological outcomes were similar in the two groups, although more fluid overload was reported in patients receiving the standard treatment (Boulis et al., 1999). More recently, Sarode et al. (2013) randomized 202 patients with acute major bleeding events during VKA treatment to 4-factor PCC (98 patients) or FFP (104 patients). PCCs were superior in achieving rapid INR reversal (62.2% vs. 9.6% of patients), but they were non-inferior

in achieving haemostatic efficacy (72.4% vs. 65.4% of patients) and mortality rates were also similar in the two groups (Sarode et al., 2013).

There are some reports that 3-factor PCCs have only a suboptimal effect in lowering supra-therapeutic INRs, probably due to the low amount of factor VII (Holland et al., 2009). Therefore, a small amount of FFP, as source of factor VII has been suggested when using 3-factor PCC (Baker et al., 2004), especially if the INR is particularly elevated (Makris & Van Veen, 2011).

Three recently published cohort studies tried to evaluate whether 3-factor and 4-factor PCCs are associated with different efficacy and safety profile in patients with severe bleeding, but they all have the limitation of retrospective design. A group of researchers included patients treated with 3-factor PCC (109 patients) or 4-factor PCC (56 patients) because of emergency warfarin reversal, at a single institution (Voils et al., 2015). Despite the lack of randomization and the lack of explanation about treatment allocation, baseline characteristics were similar in the two groups, except that more patients with gastrointestinal bleeding were present in the 4-factor PCC group, and that more patients in the 3-factor PCC group received also FFP. The level of INR decrease was comparable in the two groups; in fact, the INR 30 minutes after PCC infusion was ≤ 1.5 in 80% of patients receiving 3-factor PCC and 84% of patients receiving 4-factor PCC ($p=0.52$). Mortality was lower in patients treated with 4-factor PCC (9% vs. 31%, $p=0.001$) (Voils et al., 2015). A pooled individual data analysis from 16 stroke registries in nine countries was recently published (Parry-Jones et al., 2015). The authors analysed only patients with intracranial haemorrhage on VKA treatment and baseline INR ≥ 1.3. The following reversal strategies were used: FFP (377 patients), PCC (144 patients 3-factor PCC, 441 patients 4-factor PCC), FFP and PCC (131 patients) or no reversal strategy (454 patients). In contrast with the previous

study, 30-day mortality rate was worse in patients receiving 4-factor PCC alone than in patients receiving 3-factor PCC alone, although this analysis did not keep into consideration patients receiving the combination of FFP and PCC, a group that had a significantly lower case-fatality rate in this study (Parry-Jones et al., 2015). Finally, a small study included patients with severe bleeding receiving 3- or 4-factor PCC for warfarin reversal (84 and 64 patients, respectively) at four different institutions in the United States (Jones et al., 2016). A propensity score matching was used to adjust for differences in treatment, resulting in 36 matched patients. The percentage of INR ≤ 1.4 was comparable in the two groups (84.2% vs. 92.1%, p=0.48). However, both in the unmatched and matched cohorts, the percentage of patients receiving adjunctive FFP was higher among those treated with 4-factor PCC. In patients with baseline INR > 4.0, 4-factor PCC showed better efficacy in INR reversal (Jones et al., 2016).

1.5.4 Neutralisation of the anticoagulant effect in vitro with DOAC Stop®

DOAC Stop® is a hydrophobic binding agent, containing activated charcoal, which can absorb the DOAC from plasma samples *in vitro*. It has been shown to neutralise the effect of apixaban, edoxaban, rivaroxaban and dabigatran up to concentrations of 500 ng/mL in plasma spiked *in vitro* (Exner et al., 2018).

DOAC Stop® has been recently marketed by Haematex Research (Australia) and its first publication in the medical literature dates to 2018. So far, DOAC Stop® has been studied in samples spiked *in vitro* with the DOAC, as well as in samples *ex vivo* from patients treated with the DOAC. It has been consistently shown to normalise routine coagulation assays, such as the PT/INR and the APTT (Exner, Ahuja, et al., 2019; Exner et al., 2018; Platton & Hunt, 2019) and to reduce the rate of false positive lupus anticoagulant assays (Favaloro, Gilmore, et al., 2019; Favresse et al., 2018; Jacquemin

et al., 2018; Ząbczyk et al., 2019). Furthermore, some authors reported that other low molecular weight anticoagulants might be extracted by DOAC Stop®, such as argatroban and lepirudin (Exner, Ahuja, et al., 2019).

Based on the results of recent studies, the following potential uses of DOAC Stop® have been proposed:

1) to identify samples containing the DOAC, among test plasma with prolonged clotting time, especially prolonged APTT (Exner et al., 2018);
2) to eliminate the effect of the DOAC on special coagulation tests, such as the lupus anticoagulant (Favaloro, Gilmore, et al., 2019; Favresse et al., 2018; Jacquemin et al., 2018; Ząbczyk et al., 2019), therefore allowing to perform this assay without stopping the anticoagulant treatment;
3) to eliminate the interference of dabigatran with the calibrators of the thrombin generation assay (Kopatz et al., 2018);
4) to estimate the concentration of the DOAC in a test plasma in a quick and less expensive way, by calculating a "correction ratio" of APTT or the dilute Russell's Viper Venom time (dRVVT) before/after DOAC Stop® treatment (Exner, Favresse, et al., 2019).

However, it has been suggested that there might be a limit of DOAC concentrations (around 360 ng/mL) that can be removed by the DOAC Stop® in *ex vivo* samples from patients receiving apixaban or rivaroxaban (Platton & Hunt, 2019). In another study (Kopatz et al., 2018) the normal plasma treated with DOAC Stop® showed hypercoagulability on the thrombin generation assay, compared to the same untreated plasma, which was particularly evident when low volumes of plasma were treated, although in the range of volumes recommended by the manufacturer (1 mini-tab for 0.5-1.5 mL of plasma). Furthermore, a decrease in the levels of TFPI were observed,

which can explain this increase in thrombin generation (Kopatz et al., 2018). Therefore, although the DOAC Stop® appears to be a promising agent, these results will need confirmation in further studies before it can become part of routine clinical practice.

1.6 Patients psycho-social perspectives on the oral anticoagulant treatment

1.6.1 Compliance and adherence with chronic treatments

Chronic therapies are particularly challenging to follow by both patients and healthcare professionals (Martin et al., 2005; World Health Organization, 2003). While high rates of adherence have been reported in patients with acute conditions, a drop of approximately 50% has been described for patients with chronic diseases after the first six months of treatment (Abdou et al., 2016; Osterberg & Blaschke, 2005).

Compliance and adherence are two important concepts which describe the way patients follow the prescribed treatment regimens (Cramer et al., 2008; Horne et al., 2005). Compliance is defined as "the extent to which the patient's behaviour matches the prescriber's recommendations" (Horne et al., 2005, p.12). However, the term compliance has been criticized as it denotes an imbalance in decision making during the consultation. Thus, in a compliant decision-making style, the clinician decides about the treatment and the patient just follows an instruction. An alternative term is adherence, defined as "the extent to which the patient's behaviour matches agreed recommendations from the prescriber" (Horne et al., 2005, p.12). The term adherence underlines the need for an agreement between the patient and the doctor with regards to treatment prescription. Persistence is defined as "the duration of time from initiation to discontinuation of therapy" (Cramer et al., 2008, p.46). Therefore, while adherence

refers to the way in which a patient takes the medication as prescribed, persistence refers to how long the patient is continuously taking that medication (Abdou et al., 2016).

Anticoagulant treatment is not immune to the adherence challenge. Although monitoring can improve medication adherence in the few days before an appointment, also known as “white-coat adherence”, non-adherence to VKA has been reported to range between 22% and 58%, and was significantly correlated with poor anticoagulation control and worse clinical outcomes (Di Minno et al., 2014; Suryanarayan & Schulman, 2014). Factors associated with poor adherence were high educational level, current employment, low mental health and cognitive functioning (Platt et al., 2008), or a poor patient-physician relationship (Borg Xuereb et al., 2012, 2016). Furthermore, concerns related to lifestyle changes (e.g. the need for periodic laboratory monitoring, the time spent for the test, or restrictions on certain food and drugs) might induce the patients to refuse warfarin treatment (Borg Xuereb et al., 2012). Conversely, a good knowledge of VKA treatment was correlated with better anticoagulation control and adherence (Tang et al., 2003). In order to evaluate the impact of education and patient understanding of their disease, a recently published RCT compared intensive educational interventions (e.g. videos, educational booklets, self-monitoring diary and worksheet) with the standard care in warfarin-naïve AF patients. The intervention group had a higher TTR at 6-month follow-up (76.2% vs. 71.3%, p=0.035), but the difference was not statistically significant at 12-month follow-up (76.0% vs. 70.0%, p=0.44) (Clarkesmith et al., 2013). These results suggested that patients receiving the educational intervention had greater adherence to the treatment, although it probably needed to be repeated after six months.

The DOACs are a favourable alternative to VKA treatment; however, suboptimal adherence can arise from the lack of routine laboratory monitoring and the short half-life of these new drugs means that one missed dose can results in a very low trough concentration and increased thrombotic risk (Heidbuchel et al., 2013). The DOACs have different dose regimens: rivaroxaban and edoxaban should be taken OD, while dabigatran and apixaban BID. Therefore, physicians are allowed to choose the best regimen for each patients, taking into consideration that a OD dosing regimen can reduce the complexity of treatment and improve adherence (Laufs et al., 2011), but also considering that omitting a dose for 24 hours might be less favourable than omitting a dose for 12 hours (Heidbuchel et al., 2013; Vrijens & Heidbuchel, 2015). Factors associated with poor adherence to DOAC have been reported to be male sex, young age, depression, alcohol abuse and other comorbidities, such as heart failure, chronic obstructive pulmonary disease, diabetes (Abdou et al., 2016). Accurate patients' selection, continuous education and counselling and frequent follow-ups, are other strategies that can increase patients' understanding of the anticoagulant treatment and adherence to medication (Ageno et al., 2013; Borg Xuereb et al., 2016). So far, real-life data suggest that adherence in the first few months after initiation of DOAC treatment was acceptable also outside the context of RCTs, but it declined over time. An analysis of patients with non-valvular AF in the United States showed that adequate adherence to the anticoagulant treatment was 83.5% and 78.3% at 3-month follow-up, 73.5% and 65.0% at 6-month follow-up, 62.4% and 52.0% at 12-month follow-up, but only 49.0% and 38.0% at 24-month follow-up, for rivaroxaban and dabigatran users respectively (Coleman et al., 2016). Another analysis, conducted in a real-world German healthcare setting, showed that adequate adherence was 61.4% and 49.5%, for rivaroxaban and dabigatran users respectively ($p<0.001$) during the

first 180 days of follow-up (Beyer-Westendorf et al., 2016). These studies suggested that adherence to rivaroxaban treatment was higher, compared to dabigatran, a finding that might be explained by the difference in dosing regimen, but also by the better side-effect profile of rivaroxaban.

1.6.2 Quality of life and patient satisfaction associated with the anticoagulant treatment

The quality of life (QoL), according to the WHO definition, is "an individual's perception of their position in life in the context of the culture and value systems in which they live and in relation to their goals, expectations, standards and concerns" (WHOQOL Group, 1993, p.153). It is a wide concept that involves several areas: physical health, social condition, economic status, psychological state, satisfaction and welfare (WHOQOL Group, 1993). The perception of physical and mental health, the corresponding diseases and the consequences on physical and functional aspects, are therefore important components of QoL, also known as "health-related QoL".

The health-related QoL should be always considered during the clinical decision-making process, especially when dealing with chronic treatments. Anticoagulation is a long-term treatment for most clinical indications and it can therefore affect patient perception of QoL: it can be associated with positive aspects (such as the reassurance provided by the treatment itself or the contact with supportive healthcare professionals) (Borg Xuereb et al., 2016), but also negative aspects (such as the need for lifestyle changes or regular blood tests, or concerns about possible bleeding complications) (Borg Xuereb et al., 2016; Prins, Marrel, et al., 2009; Samsa et al., 2004). Since patient dissatisfaction can lead to decreased adherence (A. T. Hirsh et al., 2005; Ware & Davies, 1983), poor INR control and worse clinical outcomes (Ho et

al., 2009; Samsa et al., 2004), it is important to evaluate the level of satisfaction associated with the anticoagulant treatment and to identify those patients with low QoL or with dissatisfaction, in order to promote specific interventions. Treatment satisfaction is a patient-reported outcome (PRO) that was recently evaluated also in clinical trials assessing the efficacy and safety of different anticoagulant treatments (Bamber et al., 2013; Prins et al., 2015).

Health-related QoL can be measured using generic scales or condition-specific scales. Generic scales evaluate the QoL in general and can be applied to a wide group of patients. For instance, the 36-item Short Form (SF-36) evaluates eight elements: physical function, physical role, bodily pain, general health, vitality, social function, emotional role, and mental health (Ware & Sherbourne, 1992). The 12-item Short Form (SF-12) was derived from the SF-36, with the aim to evaluate only the physical and mental health status (Ware et al., 1996). Patients satisfaction has also been evaluated using generic scales, such as the 18 multi-item subscales Patient Satisfaction Questionnaire (PSQ-18) which evaluated the satisfaction with medical care (Ware et al., 1983) and the Treatment Satisfaction Questionnaire for Medication (TSQM) which evaluated the satisfaction with medications (Atkinson et al., 2004).

Specific scales assess aspects of the QoL that are linked to particular conditions, for example the health-related QoL of anticoagulated patients. The Duke Anticoagulation Satisfaction Scale (DASS) questionnaire was developed by Samsa et al. (2004). It is a psychometric questionnaire, which measures the QoL and the satisfaction associated with the anticoagulant treatment. The questions explore three possible dimensions of anticoagulation: the positive psychological impacts (such as reassurance from the anticoagulant treatment itself) and the negative psychological impacts, which are further divided into limitations (such as food restriction or reduced physical activities)

and hassles and burdens (such as remembering every day to correctly take the medication, or the need for blood tests). The DASS questionnaire has 25 items, each with seven possible responses (not at all, a little, somewhat, moderately, quite a bit, a lot, and very much) and the score can range from 25 to 175. Six of the 25 items require reverse-coding during the analysis, therefore at the end lower scores represent greater satisfaction and less hassles and burdens. The DASS has high clinical relevance and can help to identify which aspects of anticoagulation are associated with negative impact for each individual patient.

After the initial publication, dealing with the development and preliminary validation, the DASS has been evaluated in several studies (Almeida et al., 2011; Carvalho et al., 2013; Hasan et al., 2015; Matchar et al., 2010; Meyer et al., 2013; Pelegrino et al., 2012; Silva de Assis et al., 2012). From the results of these studies, the following factors were associated with a positive perception of QoL: longer treatment duration (Almeida et al., 2011); higher level of education (Silva de Assis et al., 2012); mechanical heart valve prosthesis as indication to the anticoagulant treatment (Carvalho et al., 2013). Conversely, the following factors were associated with a negative perception of QoL: younger age (Almeida et al., 2011; Hasan et al., 2015; Silva de Assis et al., 2012); higher dose of VKA (Silva de Assis et al., 2012); previous haemorrhagic complications (Almeida et al., 2011).

The Perception of Anticoagulation Treatment Questionnaire (PACT-Q) was developed by Prins, Marrel, et al. (2009), with the aim to assess patient perception associated with the anticoagulant treatment. It consists of 27 items that cover four domains: treatment expectations (7 items, A1-A7), convenience of use of the treatment (11 items, B1-B11), burden of disease and treatment (2 items, C1-C2) and anticoagulant treatment satisfaction (7 items, D1-D7). All questions can be answered

according to a 5-point Likert scale. The first part of the questionnaire (PACT-Q1) evaluates the expectations associated with anticoagulation and should be administered before starting the anticoagulant treatment, while the second part (PACT-Q2) measures the other three domains and is administered after having received the treatment for a while (Prins, Marrel, et al., 2009). In the first validation study, items B and C were summed together in order to evaluate the overall dimension of "convenience" associated with the anticoagulant treatment (Prins, Guillemin, et al., 2009). During the analysis, the items of "treatment expectation" are scored individually; the items of "convenience" are reversed, summed and rescaled on a 0-100 scale; the items of "treatment satisfaction" are summed and rescaled on a 0-100 scale. Therefore, at the end, higher scores correspond to higher convenience/satisfaction.

In the original study for the domain "treatment expectation" highest scores were obtained by patients under 60 years. Patients without any previous experience with medications had higher expectation of symptom relief, while patients with prior experience had higher expectation of side effects. For the domain "convenience" highest scores were obtained by patients aged 65-75 years and patients without any previous experience with medications. Finally, for the domain "treatment satisfaction" highest scores were obtained by patients aged 60-65 years and patients with no clinical events in the first three months of follow-up (Prins, Guillemin, et al., 2009). The PACT-Q has been rigorously translated, following recommended linguistic validation procedures, and is currently available in more than 10 languages (Mohamed et al., 2015; Prins, Marrel, et al., 2009). The PACT-Q is currently under investigation in several clinical studies, such as the PREFER in AF and VTE registries (Agnelli et al., 2015; De Caterina et al., 2014).

There are other questionnaires that evaluated patients' satisfaction associated with the anticoagulant treatment, but they were derived from the previously mentioned questionnaires or they are less validated. The Anti-Clot Treatment Scale (ACTS) is a questionnaire derived from the DASS with some modifications in order to make it applicable to a wider range of patients, irrespective of their underlying conditions or country location (Cano et al., 2012). So far, the ACTS has been evaluated in patients with acute VTE (Bamber et al., 2013; Prins et al., 2015) or AF (Coleman et al., 2013; Fumagalli et al., 2015). There are also other questionnaires which can be applied only to patients with VTE, such as the Deep Venous Thrombosis Quality of Life questionnaire (DVTQOL) (Hedner et al., 2004), the Venous Insufficiency Epidemiological and Economic Study scores for symptoms (VEINES-Sym) and quality of life (VEINES-QOL) (Kahn et al., 2006), and the Pulmonary Embolism Quality of Life Questionnaire (PEmb-QoL) (Cohn et al., 2009).

This data suggests that, in recent years, there has been a great interest in QoL and patients' satisfaction associated with the anticoagulant treatment. However, the studies conducted so far had some drawbacks: the specific QoL questionnaires have never been compared one against the other, in order to establish the most accurate; only the DASS has been applied to patients monitored with the POC coagulometers but in the context of a RCT; and the DASS and the PACT-Q have never been used in Malta, where there is no validated QoL instrument specifically assessing anticoagulated patients available in the Maltese language.

1.7 Rationale and aims

This thesis aimed to provide more evidence on different aspects of anticoagulation and VTE. Taking into consideration the current gaps identified in the literature review and

the actual management of the anticoagulant therapy in the Maltese context, this project was divided into two related parts, a laboratory (Chapters 3-6) and a clinical (Chapters 7-9) part.

The laboratory part, whose methodology is reported in Chapter 2, analysed different areas in which the laboratory can contribute to the management of anticoagulation and VTE. Specifically, the gaps identified in the literature review and the aims to address them were the following:

- The POC coagulometers represent a simpler and quicker alternative to the standard laboratory monitoring of the INR in VKA patients; however, their accuracy was recently questioned by several studies. The study reported in Chapter 3 aimed to assess the accuracy of the POC INR compared to the other INR assays and to a global coagulation assay, the thrombin generation measured with the CAT.
- Despite the importance of a timely diagnosis of VTE, the current diagnostic algorithms still include clinical pre-test probability, laboratory D-dimer and imaging test. The study reported in Chapter 4 aimed to assess the accuracy and the relative importance of several potential biomarkers of acute VTE, such as two different D-dimer assays, the thrombin generation assay, the procoagulant phospholipid-dependent clotting time, and the human soluble P-selectin.
- The thrombin generation and the TEG are global coagulation assays. Through the measurement of the amount of thrombin generated, all the three phases of coagulation (initiation, propagation, termination) can be evaluated, while the TEG can provide information on the kinetics of clot formation/dissolution and its strength. However, variable sensitivity of these assays has been reported to ongoing anticoagulant treatment. The study reported in Chapter 5 aimed to

evaluate the effect of several oral and parenteral anticoagulants on these two assays.

- Different strategies have been proposed to reverse the effect of the anticoagulant drugs in patients with treatment-related bleeding complications. However, no clear superiority of one agent over the others has been demonstrated. Furthermore, the choice of the reversal agent can also be influenced by the available products in the different countries. The studies reported in Chapter 6 aimed to assess the effect of FFP for warfarin reversal *ex vivo*, the effect of different reversal agents for DOAC reversal *in vitro*, and to better evaluate the neutralising effect of DOAC Stop® *in vitro*.

The clinical part focused on the use of the POC coagulometers for VKA monitoring, specifically:

- There is a known correlation between patients' satisfaction and adherence to chronic treatment; however, no validated questionnaire specifically assessing the QoL of anticoagulated patients was available in the Maltese language. The study reported in Chapter 7 aimed to translate and validate two psychometric questionnaires, the DASS and the PACT-Q.
- The use of POC coagulometers, compared to the standard laboratory monitoring of the INR, can simplify the management of VKA patients. While an improvement in the QoL of patients undergoing self-management has been reported, it has not been investigated yet whether the use POC coagulometers by healthcare professionals in anticoagulation clinics is associated with a similar beneficial effect. Malta was the ideal context to perform this study because the two ways of monitoring coexist, being VKA patients monitored with the standard laboratory INR in some anticoagulation clinics and with the POC INR in others. Therefore,

the study reported in Chapter 8 aimed to compare patients' satisfaction associated with the POC INR monitoring compared to the standard INR monitoring.

- While the use of POC coagulometers for PSM can potentially improve the TTR and reduce the risk of thromboembolic events, contradictory findings have been reported when the POC devices are used by healthcare professionals in the anticoagulation clinics. The study reported in Chapter 9 aimed to better assess the efficacy of the use of the POC coagulometers for VKA monitoring, in terms of TTR and number of INR tests.

Finally, the conclusions of these studies, the practical implications and the recommendations for future research and clinical practice are discussed in Chapter 10.

Chapter 2 :

Materials and Methods

2.1 Introduction

This chapter describes the methodology used in the laboratory studies (Chapters 3-6), from blood collection and plasma preparation, to the specific assays that were performed. Detailed methods for the clinical studies (Chapters 7-9) are provided in the respective chapters.

2.2 Blood collection and plasma preparation

Venous blood samples were collected into vacuum coagulation tubes containing whole blood 2 ml and sodium citrate 0.109M/3.2% (Vacuette, Greiner Bio-One, Austria). The coagulation tubes were gently mixed by inversion.

In order to separate the plasma, samples were centrifuged at controlled room temperature for 10 minutes at 2500g using the Eppendorf Centrifuge 5810 (Eppendorf AG, Germany). For assays that needed the PPP, after plasma separation, samples were centrifuged again for 10 minutes at 2500g. After preparation, aliquots of PPP were immediately frozen at -80°C until testing. Further details of blood collection and plasma preparation are reported in each chapter.

2.3 Anticoagulated pool plasma

2.3.1 Plasma spiked with several oral and parenteral anticoagulants

A pool of normal plasma was obtained from anonymised citrated samples with normal PT and APTT analysed in the Coagulation Laboratory, at Mater Dei Hospital (Msida, Malta), between August and November 2018. Samples underwent double centrifugation, in order to obtain the PPP, and they were frozen at -80°C. When an adequate number of samples was collected, the aliquots were thawed in the water bath

at 37°C and the plasma was pooled. Afterwards, the pool of normal PPP was frozen again at -80°C in variable amounts, ranging from 35 to 65 ml, based on the number of planned anticoagulant concentrations in the spiking experiment. On the day of spiking, the normal pooled plasma was thawed in the water bath at 37°C, spiked with different concentrations of one of the anticoagulants, divided into 500 µl aliquots and frozen at -80°C until the analysis. Therefore, all the plasma spiked with the anticoagulants underwent three cycles of freeze-thaw.

Since a progressive decrease of factor VIII levels was reported with every freeze-thaw cycle (Dzik et al., 1989; Gosselin & Dwyre, 2015), the activity of factor VIII was assayed once after every freeze-thaw cycle, over a two weeks period, but no significant decreased was observed (Table 2.1). Similarly, a recently published paper suggested that three is the maximum acceptable number of freeze-thaw cycles to preserve factor VIII stability, as long as the storage is at -80°C (Feng et al., 2018). The activity of factor VII was also evaluated once after every freeze-thaw cycle, but no significant change was observed (Table 2.1).

N. of freeze-thaw cycles	Factor VIII (normal ranges 50-150%)	Factor VII (normal ranges 50-129%)
1	189.9%	111.9%
2	171.9%	115.2%
3	171.9%	107.3%

Table 2.1 Effect of several freeze-thaw cycles on factors VIII and VII levels

There were two batches of normal PPP, whose coagulation parameters and platelet counts are reported in Table 2.2. By definition, PPP should have a platelet count <10 x 10^9/l. All the pooled plasma respected this criterion, considering that the most accurate method for platelet detection is by fluorescence (Wada et al., 2015). The first

batch of normal PPP was used to spike apixaban, edoxaban, rivaroxaban, danaparoid, enoxaparin, and bivalirudin. The second batch of normal PPP was used to spike argatroban, dabigatran, and fondaparinux. However, all the results were compared to the same unspiked normal PPP.

	Normal PPP (batch I)	Normal PPP (batch II)	Warfarinised PPP with INR 2	Warfarinised PPP with INR 3	Warfarinised PPP with INR 4
Platelet (*10^9/l), optical method	12	6	5	6	7
Platelet (*10^9/l), impedance method	8	6	5	6	8
Platelet (*10^9/l), fluorescence method	3	1	1	2	2
Red blood cells (*10^{12}/l)	0	0	0	0	0
Fragmented red blood cells (*10^{12}/l)	0	0	0	0	0
Hb (g/dl)	0	0	0	0	0
PT (sec)	10.5	10.2	23.3	34.1	43.4
INR	1.01	0.98	2.22	3.24	4.11
APTT (sec)	26.8	27.0	36.8	43.5	44.3
APTT (ratio)	0.90	0.91	1.23	1.46	1.49
Fibrinogen (g/l)	4.64	3.99	3.80	3.41	4.31
D-dimer (ng/ml)	395	322	267	64	176

Table 2.2 Coagulation and haematological parameters of the anticoagulated pooled plasma

2.3.1.1 Direct factor Xa inhibitors

Apixaban was purchased from MedChemExpress (USA; lot number 09493) as 10 mg powder. It was initially reconstituted in dimethyl sulfoxide (DMSO) 10 ml (solubility in DMSO 14.25 mg/ml, insoluble in water), to obtain concentration 1 mg/ml. A subsequent dilution 1:9 was performed in deionised water to obtain concentration 0.1 mg/ml. Further dilutions were performed directly in the normal PPP, in order to obtain a broad range of concentrations, ranging from prophylactic to slightly supratherapeutic. In clinical practice, apixaban is administered orally at the following

dosages (European Medicines Agency, 2018c), whose respective plasma concentrations are reported in Table 2.3:

- Primary VTE prevention in orthopaedic surgery: 2.5 mg BID;
- Prevention of stroke and systemic embolism in patients with AF: 5 mg BID (reduced to 2.5 mg BID if ≥ 2 of the following characteristics, including age ≥ 80 years, weight ≤ 60 kg, or creatinine ≥1.5 mg/dl);
- Treatment of VTE: 10 mg BID for the first week, followed by 5 mg BID. The dose 2.5 mg BID can be used after six months of treatment if secondary VTE prevention is required.

The plasma spiked with apixaban was subsequently checked with the specifically calibrated anti-Xa assay (reported in paragraph 2.10) and the following concentrations were confirmed: apixaban 4 ng/ml, 42 ng/ml, 89 ng/ml, 128 ng/ml, 179 ng/ml, 266 ng/ml.

	S. Kitchen et al. (2014), mean	**Testa et al. (2016), mean (min-max)**	**European Medicines Agency (2018c), median (5^{th}-95^{th} percentile)**
Apixaban 2.5 mg BID			
• Peak	62	192 (55-300)	77 (41-146) in orthopaedic surgery 123 (69-221) in AF 67 (30-153) in VTE
• Trough	21	79 (26-248)	51 (23-109) in orthopaedic surgery 79 (34-162) in AF 32 (11-90) in VTE
Apixaban 5 mg BID			
• Peak	128	201 (102-416)	171 (91-321) in AF 132 (59-302) in VTE
• Trough	50	113 (42-283)	103 (41-230) in AF 63 (22-177) in VTE
Apixaban 10 mg BID			
• Peak	NR	NR	251 (111-572)
• Trough	NR	NR	120 (41-335)

Table 2.3 Reported plasma concentrations (ng/ml) of apixaban

Edoxaban (tosylate monohydrate) was purchased from MedChemExpress (USA; lot number 15962) as 10 mg powder. It was initially reconstituted in DMSO 10 ml (solubility in DMSO 50 mg/ml, insoluble in water), to obtain concentration 1 mg/ml. A subsequent dilution 1:9 was performed in deionised water to obtain concentration 0.1 mg/ml. Further dilutions were performed directly in the normal PPP. In clinical practice, edoxaban is administered orally at the following dosages (European Medicines Agency, 2018d), whose respective plasma concentrations (Douxfils et al., 2018; Ruff et al., 2015; Verhamme et al., 2016) are reported in Table 2.4:

- Prevention of stroke and systemic embolism in patients with AF: 60 mg OD;
- Treatment of VTE: 60 mg OD;
- Dose reduction to 30 mg OD is recommended if any of the following characteristics, including creatinine clearance ≤ 50 ml/min, body weight ≤ 60 kg, concomitant treatment with P-glycoprotein inhibitors (ciclosporin, dronedarone, erythromycin, or ketoconazole).

The plasma spiked with edoxaban was subsequently checked with the specifically calibrated anti-Xa assay (reported in paragraph 2.10) and the following concentrations were confirmed: edoxaban 0 ng/ml, 15 ng/ml, 51 ng/ml, 85 ng/ml, 113 ng/ml, 188 ng/ml.

	Ruff et al. (2015), mean (SD)	**Verhamme et al. (2016), median (IQR)**	**Douxfils et al. (2018), median (IQR)**
Edoxaban 30 mg OD			
• Peak	NR	164 (99-225)	NR
• Trough	34.6 (30.9)	16 (8-32)	NR
Edoxaban 60 mg OD			
• Peak	NR	234 (149-317)	170 (125-245)
• Trough	NR	19 (10-39)	36 (19-62)

Table 2.4 Reported plasma concentrations (ng/ml) of edoxaban

Rivaroxaban was purchased from MedChemExpress (USA; lot number 10671) as 10 mg powder. It was initially reconstituted in DMSO 10 ml (solubility in DMSO 14.25 mg/ml, insoluble in water), to obtain concentration 1 mg/ml. A subsequent dilution 1:9 was performed in deionised water to obtain concentration 0.1 mg/ml. Further dilutions were performed directly in the normal PPP. In clinical practice, rivaroxaban is administered orally at the following dosages (European Medicines Agency, 2018f), whose respective plasma concentrations are reported in Table 2.5:

- Patients with acute coronary syndromes or with symptomatic coronary artery or peripheral artery disease: 2.5 mg BID;
- Primary VTE prevention in orthopaedic surgery: 10 mg OD;
- Prevention of stroke and systemic embolism in patients with AF: 20 mg OD (reduced to 15 mg OD if creatinine clearance < 50 ml/min);
- Treatment of VTE: 15 mg BID for 3 weeks, followed by 20 mg OD (eventually reduced to 15 mg OD if high bleeding risk). The dose 10 mg OD can be used after six months of treatment if secondary VTE prevention is required.

The plasma spiked with rivaroxaban was subsequently checked with the specifically calibrated anti-Xa assay (reported in paragraph 2.10) and the following concentrations were confirmed: rivaroxaban 22 ng/ml, 55 ng/ml, 118 ng/ml, 174 ng/ml, 231 ng/ml, 339 ng/ml.

	S. Kitchen et al. (2014), mean (range)	Testa et al. (2016), mean (min-max)	European Medicines Agency (2018f), mean (90% prediction interval)	Douxfils et al. (2018), mean (5^{th}-95^{th} percentile)
Rivaroxaban 10 mg OD				
• Peak	125 (91-195)	NR	101 (7-273)	NR
• Trough	9 (1-38)	NR	14 (4-51)	NR
Rivaroxaban 15 mg OD				
• Peak	NR	208 (77-393)	NR	NR
• Trough	NR	28 (0-88)	NR	NR
Rivaroxaban 20 mg OD				
• Peak	223 (160-360)	236 (61-449)	215 (22-535)	249 (184-343) in AF 270 (189-419) in VTE
• Trough	22 (4-96)	41 (5-119)	32 (6-239)	44 (12-137) in AF 26 (6-87) in VTE

Table 2.5 Reported plasma concentrations (ng/ml) of rivaroxaban

2.3.1.2 Direct thrombin inhibitors

Argatroban (Exembol®, Mitsubishi Tanabe Pharma Europe Ltd, UK; lot number 1780714B) was available as a solution containing 250 mg in 2.5 ml. Three subsequent dilutions 1:9 were performed in DMSO, to obtain the following concentrations: 10000 μg/ml, 1000 μg/ml, and finally 100 μg/ml. Argatroban was subsequently spiked into the normal PPP, in order to obtain the desired concentrations. When used for VTE treatment in patients with HIT, argatroban is administered as a continuous infusion starting at 2 μg/kg/min, which corresponds to a plasma concentration of approximately 0.4 μg/ml. When used for patients with HIT undergoing percutaneous coronary intervention (PCI), argatroban is started at 25 μg/kg/min, which corresponds to a plasma concentration of approximately 5 μg/ml, and a bolus of 350 μg/kg is also administered (Food and Drug Administration, 2017). The plasma spiked with argatroban was subsequently checked with the specifically calibrated DTT assay (reported in paragraph 2.9) and the following concentrations were confirmed: argatroban 0.25 μg/ml, 0.53 μg/ml, 3.10 μg/ml, 5.84 μg/ml.

Bivalirudin (Angiox®, Hälsa Pharma GmbH, Germany; lot number 0007104) was available as 250 mg powder. According to the manufacturer's instructions, it was reconstituted with deionised water 5 ml, to obtain concentration 50 mg/ml. A dilution 1:9 was performed in deionised water to obtain concentration 5 mg/ml. Further dilutions were performed directly in the normal PPP. Although bivalirudin was recently withdrawn from the market, when used for patients with HIT undergoing PCI, bivalirudin was started with a 0.75 mg/kg bolus, followed by a continuous intravenous infusion at 1.75 mg/kg/h. The mean plasma concentration was reported to be 12.4 μg/ml at an infusion rate of 2.5 mg/kg/h (European Medicines Agency, 2018a). The plasma spiked with bivalirudin was subsequently checked with the specifically calibrated DTT assay (reported in paragraph 2.9) and the following concentrations were confirmed: 5.9 μg/ml, 13.8 μg/ml, 31.0 μg/ml.

Dabigatran was purchased from Sigma-Aldrich (USA; lot number FN06041801), as a solution containing dabigatran 100 μg/ml in 1 ml acetonitrile with 10% 0.01 N HCl. A first dilution 1:9 was performed in DMSO to obtain concentration 10 μg/ml. Further dilutions were performed directly in the normal PPP. In clinical practice, dabigatran is administered orally at the following dosages (European Medicines Agency, 2019), whose respective plasma concentrations are reported in Table 2.6:

- Primary VTE prevention in orthopaedic surgery: 220 mg OD (reduced to 150 mg OD if creatinine clearance ≤ 50 ml/min, age ≥ 75 years, concomitant verapamil, amiodarone, quinidine);
- Prevention of stroke and systemic embolism in patients with AF: 150 mg BID;
- Treatment of VTE: 150 mg BID;
- Dose reduction to 110 mg BID is recommended if age ≥ 80 years or concomitant verapamil (dose reduction can also be considered if age 75-80 years, creatinine

clearance 30-50 ml/min, high bleeding risk, or gastritis, esophagitis, gastroesophageal reflux).

The plasma spiked with dabigatran was subsequently checked with the specifically calibrated DTT assay (reported in paragraph 2.9) and the following concentrations were confirmed: dabigatran 0 ng/ml, 44 ng/ml, 92 ng/ml, 148 ng/ml, 176 ng/ml, 276 ng/ml.

	S. Kitchen et al. (2014), mean (95% CI)	**Testa et al. (2016), mean (min-max)**	**European Medicines Agency (2019), mean (25^{th}-75^{th} percentile)**
Dabigatran 220 mg OD			
• Peak	NR	NR	71 (35-162)
• Trough	NR	NR	22 (13-36)
Dabigatran 110 mg BID			
• Peak	NR	191 (31-651)	NR
• Trough	NR	94 (14-386)	NR
Dabigatran 150 mg BID			
• Peak	184 (64-443)	210 (43-538)	175 (117-275)
• Trough	90 (31-225)	91 (16-494)	91 (61-143) in AF 60 (39-95) in VTE

Table 2.6 Reported plasma concentrations (ng/ml) of dabigatran

2.3.1.3 Indirect factor Xa inhibitors

Danaparoid sodium (Orgaran®, Aspen Pharma, Ireland; lot number PL 0065/0125 BN656468) was available as a solution containing 750 anti-Xa units in 0.6 ml. A first dilution 1:9 was performed in normal PPP to obtain concentration 125 U/ml. Danaparoid was subsequently spiked into the normal PPP, in order to obtain the desired concentrations. In clinical practice, danaparoid can be administered subcutaneously or intravenously. The intravenous protocol usually consists in a loading dose (ranging from 1500 to 3750 anti-Xa units based on body weight) followed by a continuous infusion (400 U/h for 4 hours, then 300 U/h for 4 hours, then 150-220 U/h for 5-7 days). The target therapeutic range is 0.5-0.8 anti-Xa U/ml

(Warkentin, 2019). The plasma spiked with danaparoid was subsequently checked with the specifically calibrated anti-Xa assay (reported in paragraph 2.10) and the following concentrations were confirmed: danaparoid 0.33 U/ml, 0.78 U/ml, 1.93 U/ml.

Enoxaparin sodium (Clexane®, Sanofi Aventis, UK; lot number 6L1CMM) was available as a solution containing 20 mg (2000 U) in 0.2 ml. Two dilutions 1:9 were performed in normal PPP to initially obtain concentration 1000 U/ml and subsequently 100 U/ml. Enoxaparin was subsequently spiked into the normal PPP. In clinical practice, enoxaparin is administered subcutaneously at a prophylactic dose (usually 40 mg OD) or therapeutic dose (either 1.0 mg/kg BID or 1.5 mg/kg OD). The target therapeutic range is 0.2-0.5 anti-Xa U/ml for the prophylactic dose, 0.6-1.0 anti-Xa U/ml for the therapeutic BID dose and 1.0-2.0 anti-Xa U/ml for the therapeutic OD dose (Garcia et al., 2012; Lim, 2010). The plasma spiked with enoxaparin was subsequently checked with the specifically calibrated anti-Xa assay (reported in paragraph 2.10) and the following concentrations were confirmed: enoxaparin 0.35 U/ml, 1.06 U/ml, 1.95 U/ml.

Fondaparinux sodium (Arixtra®, Aspen Pharma, Ireland; lot number 0045A) was available as a solution containing 2.5 mg in 0.5 ml. Two dilutions 1:9 were performed in normal PPP to initially obtain concentration 500 μg/ml and subsequently 50 μg/ml. Fondaparinux was subsequently spiked into the normal PPP. In clinical practice, fondaparinux is administered subcutaneously at a prophylactic dose (2.5 mg OD) or therapeutic dose (5 mg OD if body weight < 50 kg, 7.5 mg OD if body weight 50-100 kg, 10 mg OD if body weight > 100 kg) (European Medicines Agency, 2018b). The expected peak plasma concentration for the prophylactic dose is 0.4-0.5 μg/ml, while for the therapeutic doses is 1.2-1.3 μg/ml (Garcia et al., 2012). The plasma spiked with

fondaparinux was subsequently checked with the specifically calibrated anti-Xa assay (reported in paragraph 2.10) and the following concentrations were confirmed: fondaparinux 0.64 μg/ml, 1.64 μg/ml, 2.24 μg/ml.

2.3.2 Warfarinised platelet poor plasma

Warfarinised PPP for the experiments reported in Chapters 5-6 was collected from the remaining plasma of anonymised outpatients' samples receiving warfarin therapy analysed in the Coagulation Laboratory, at Mater Dei Hospital (Msida, Malta), between August and November 2018. Three different pools of warfarinised PPP with different INRs were collected:

1) A pool of samples with INR values between 2.0 and 3.0 (final INR 2.22);
2) A pool of samples with INR values between 3.0 and 4.0 (final INR 3.24);
3) A pool of samples with INR values between 4.0 and 6.0 (final INR 4.11).

The three pools of warfarinised PPP underwent three cycles of thaw-freeze, in order to follow the same preparation procedure of the plasma spiked with the other anticoagulants. Results of the coagulation parameters and platelet counts are reported in Table 2.2

2.4 Prothrombin time and international normalised ratio

The PT assay was performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta). Until December 2015, the PT was performed using the automated coagulation analysers Sysmex CS-2100i or CA-1500 (Siemens Healthcare Diagnostics Products GmbH, Germany) and the Dade® Innovin® reagent (Siemens Healthcare Diagnostics Products GmbH, Germany). From January 2016 onwards, the PT was performed using the automated coagulation analyser ACL TOP 500

(Instrumentation Laboratory, Italy) and the HemosIL® RecombiPlasTin 2G reagent (Instrumentation Laboratory, Italy). Both the Sysmex series and the ACL series are photo-optical coagulometers.

The PT reagent, also called thromboplastin, contains synthetic phospholipids, recombinant human TF and calcium (Instrumentation Laboratory, 2019b; Siemens Healthcare Diagnostics Products GmbH, 2008). In the PT assay, the citrated plasma sample is initially incubated at 37°. Afterwards, the addition of thromboplastin and calcium activates factor VII, which starts the clotting. The PT measures the time to clot formation and evaluates mainly the extrinsic pathway of the coagulation cascade. The PT, expressed in seconds, can be converted into the INR, using the following formula, where ISI is the international sensitivity index specific of each thromboplastin:

$$INR = \left[\frac{\text{patient's PT (sec)}}{\text{mean of the normal range for the PT (sec)}} \right]^{ISI}$$

For the experiments reported in Chapter 3, samples were also analysed with the help of Mr. D. Zammit using an electromechanical coagulometer the Thrombolyzer XRC (Behnk Elektronik, Germany) and the Dade® Innovin® reagent (Siemens Healthcare Diagnostics Products GmbH, Germany; lot number 539289).

In addition, the PT was performed using the manual tilt-tube technique (Estridge & Reynolds, 2012) and the Dade® Innovin® reagent (Siemens Healthcare Diagnostics Products GmbH, Germany; lot number 539289), with the help of Mr. K. Vella. For this technique, 100 µl of thawed PPP were incubated in a glass tube in the water bath at 37° C for 2 minutes. Afterwards, 200 µl of pre-warmed PT reagent were added,

while keeping the tube in the water bath. The timer was started and the tube was gently tilted until clot formation. The fibrin clot usually appears as a thickening. The time to clot formation was recorded in seconds and then converted into INR using the above-mentioned formula, where the mean normal PT obtained from healthy volunteers in our laboratory was 10.6 seconds and the ISI of the Dade® Innovin® reagent, obtained with the calibration reagents and our instruments, was 0.98.

Finally, the INR was also measured by the author using a POC coagulometer (CoaguChek XS Plus, Roche Diagnostics International Ltd, Germany) and specific test strips (lot numbers 233 430-11 and 202 053-11). POC devices can measure the INR directly on whole blood. According to the manufacturer's instructions, the test strips used by the CoaguChek XS system contain a lyophilized reagent that consists of a human recombinant thromboplastin (ISI = 1.0) (Plesch & Wolf, 2006) and a peptide substrate. Following activation of coagulation, when the blood is mixed with the thromboplastin on the test strip, the newly generated thrombin cleaves the peptide substrate. An electrochemical signal is generated and, through a specific algorithm, is subsequently converted into INR (electrochemical clot detection method). The test strips contain also an embedded quality control, which checks each test strip, while performing the INR, in order to detect any possible deterioration due to exposure to heat, light or humidity. An internal QC of the POC device was performed every day before testing. Both capillary and venous blood samples were tested by the author with the POC device. In order to test the capillary blood samples, a finger-prick was performed and the blood was applied on the test strip within 10 seconds. In order to test the non-citrated venous blood samples, blood was collected using a syringe and applied on the test strip, after discharging a few drops. Sixty patients were tested with

the POC coagulometer (Chapter 3) to evaluate its accuracy in comparison to the other INR assays.

2.5 Activated partial thromboplastin time

The APTT assay was performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta), using the automated analyser ACL TOP 500 (Instrumentation Laboratory, Italy) and the HemosIL® SynthASil reagent (Instrumentation Laboratory, Italy). The APTT reagent contains synthetic phospholipids and a negatively charged contact activator (colloidal silica).

The citrated plasma sample is initially incubated at 37°C with the APTT reagent, to activate the contact phase of coagulation, and afterwards recalcified, to start the clotting (Instrumentation Laboratory, 2017i). The APTT measures the time to clot formation and it evaluates mainly the intrinsic pathway of the coagulation cascade. The normal ranges for the APTT, established locally, were 24.8-35.0 sec, ratio 0.89-1.16.

2.6 Lupus anticoagulant

The lupus anticoagulant (LA) assay was performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) with the help of Mr. K. Vella, using the automated analyser ACL TOP 500 (Instrumentation Laboratory, Italy) and the HemosIL® dRVVT Screen and dRVVT Confirm reagents (Instrumentation Laboratory, Italy; lot numbers N0763968 and N0965014). This assay is based on the dilute Russell's Viper Venom time (dRVVT) method and was performed as an integrated test, running screening and confirmatory tests in parallel (Pengo et al., 2009). The Russell's Viper Venom can directly activate factor X present in the plasma sample, which in the

presence of factor V and phospholipids will activate prothrombin and initiate coagulation. The dRVTT Screen reagent has low concentration of phospholipids. If antiphospholipid antibodies are present in the plasma sample, they will bind and sequestrate the phospholipids, therefore the clotting time will be prolonged (Moore, 2016). The dRVVT Confirm reagent has high concentration of phospholipids. It can therefore neutralize the effect of the antiphospholipid antibodies and be normalized (Moore, 2016).

Results of the LA assay are expressed as a ratio between the clotting time of the patient plasma and a normal plasma. In details, results of the dRVVT Screen are expressed as a ratio:

$$\text{dRVVT Screen ratio} = \frac{\text{patient's dRVVT Screen results (sec)}}{\text{mean of the normal range for the dRVVT Screen (sec)}}$$

Results of the dRVVT Confirm are also expressed as a ratio:

$$\text{dRVVT Confirm ratio} = \frac{\text{patient's dRVVT Confirm results (sec)}}{\text{mean of the normal range for the dRVVT Confirm (sec)}}$$

The dRVVT final result is called "normalised dRVVT ratio" (NR) and is a ratio:

$$\text{Normalised dRVVT ratio} = \frac{\text{dRVVT Screen ratio}}{\text{dRVVT Confirm ratio}}$$

The detectable range for the screening test was 16-240 sec, while for the confirm test was 6-121 sec. The manufacturer's cut-off for a positive lupus anticoagulant assays was NR > 1.2 (Instrumentation Laboratory, 2016).

2.7 D-dimer

The D-dimer assay was performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta). Until December 2015, the D-dimer was performed using the automated coagulation analysers Sysmex CS-2100i or CA-1500 (Siemens Healthcare Diagnostics Products GmbH, Germany) and the Innovance® D-dimer (Siemens Healthcare Diagnostics Products GmbH, Germany). From January 2016 onwards, the D-dimer was performed using the automated coagulation analyser ACL TOP 500 (Instrumentation Laboratory, Italy) and the HemosIL® D-dimer HS (Instrumentation Laboratory, Italy). The Innovance® D-dimer and the HemosIL® D-dimer HS are both latex-enhanced turbidimetric immunoassays. They are fully automated assays which provide quantitative results. In these assays, the patient plasma is mixed with a suspension of latex particles coated with a monoclonal antibody directed against specific epitopes of the D-dimer molecule. If the patient plasma contains D-dimer, the particles agglutinate and the aggregates interfere with the transmitted light, detected as increased turbidity (turbidimetric immunoassay). The degree of agglutination is proportional to D-dimer concentration (Tripodi, 2011).

The Innovance® D-Dimer requires a small amount of sample (9 µl) and provide the results within 10 minutes (Siemens Healthcare Diagnostics Products GmbH, 2016). The recommended cut-off for the exclusion of VTE is 500 ng/mL Fibrinogen Equivalent Units (FEU); therefore, values < 500 ng/mL are considered negative and values ≥ 500 ng/mL are considered positive (Coen Herak et al., 2009). The HemosIL® D-Dimer HS requires a small amount of sample (18 µl) and provide the results within 4 minutes (Instrumentation Laboratory, 2017a). Due to the fact that only a fragment of the antibody is used in the HemosIL® D-Dimer HS, it allows a more specific detection of D-Dimer, avoiding the interference with some endogenous factors, like

the rheumatoid factor. A cut-off of 230 ng/mL was used in previous studies (de Moerloose et al., 2005; Scarvelis et al., 2008; van Hylckama Vlieg et al., 2015); therefore, values < 230 ng/mL were considered negative and values ≥ 230 ng/mL were considered positive.

2.8 Factor assays

Factor assays were performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) with the help of Mr. K. Vella, using the automated analyser ACL TOP 500 (Instrumentation Laboratory, Italy) and the following reagents: HemosIL® Q.F.A. Thrombin (Bovine), HemosIL® Factor II deficient plasma, HemosIL® Factor VIII deficient plasma, HemosIL® Factor IX deficient plasma, HemosIL® Factor X deficient plasma, HemosIL® Factor XI deficient plasma, HemosIL® Factor XII deficient plasma (Instrumentation Laboratory, Italy). For the factor VII assay, the HemosIL® Factor VII deficient plasma (Instrumentation Laboratory, Italy) was used for the measurements on the normal PPP reported in paragraph 2.2.1, while the Coagulation Factor VII Deficient Plasma (Siemens Healthcare Diagnostics Products GmbH, Germany) was used for the experiments reported in Chapter 6. In addition, for the PT-based factor assays (II, VII, X), the HemosIL® RecombiPlasTin 2G reagent (Instrumentation Laboratory, Italy) was used; for the APTT-based factor assays (VIII, IX, XI, XII) the HemosIL® SynthASil reagent (Instrumentation Laboratory, Italy) was used, as reported in paragraphs 2.4-2.5.

Fibrinogen was determined using the Clauss method. It is a modified thrombin time, in which the patient plasma is firstly diluted and incubated at 37°C. Then phospholipids, high concentration of thrombin and calcium are added and the clotting time is measured. Since thrombin can catalyse the activation of fibrinogen into fibrin

with formation of the fibrin clot, this clotting time is inversely proportional to the concentration of fibrinogen in the test plasma, which can be estimated from a calibration curve (Winter et al., 2017).

A one-stage modified PT was performed to determine the activity of the factors of the extrinsic pathway (II, VII, X), while a one-stage modified APTT was performed to determine the activity of the factors of the intrinsic pathway (VIII, IX, XI, XII). The patient plasma was diluted and mixed with a plasma specifically deficient in the factor of interest (activity <1%), but with normal levels of the other coagulation factors. Therefore, the patient plasma is the source of the deficient factor. The PT or APTT, as necessary, were performed on this mix. The degree of correction of the clotting time, compared to the deficient plasma, is proportional to the activity of that factor in the test plasma, which can be estimated from a calibration curve. If the patient plasma has a factor deficiency, it will not be able to compensate for the factor deficient plasma and the PT or APTT will still be prolonged. The manufacturer's normal ranges for the factor assays were as follows: fibrinogen 2.0-3.93 g/l; factor II 79-131%; factor VII (HemosIL®) 50-129%; factor VIII 50-150%; factor IX 65-150%; factor X 77-131%; factor XI 65-150%; factor XII 50-150% (Instrumentation Laboratory, 2017b, 2017c, 2017d, 2017e, 2017f, 2017h, 2018, 2019a); factor VII (Siemens®) 70-120% (Siemens Healthcare Diagnostics Products GmbH, 2018).

2.9 Diluted thrombin time assay

The DTT assay was performed in the Coagulation Laboratory at the Royal Hallamshire Hospital (Sheffield, UK) with the help of Mr. K. Hickey, using the automated analyser Sysmex CS-5100 (Siemens Healthcare Diagnostics Products GmbH, Germany) and the Hemoclot Thrombin Inhibitors kit (Hyphen BioMed,

France) with specific calibrators for argatroban, bivalirudin and dabigatran. The Hemoclot Thrombin Inhibitors kit consists of a lyophilised normal pool plasma and a lyophilised preparation of human thrombin (Hyphen BioMed, 2019). The DTT is a modification of the thrombin clotting time, in which the sample is diluted in normal human plasma prior to the analysis (Winter et al., 2017). The DTT is a chronometric test that measures the time to fibrin clot formation after adding exogenous thrombin to the citrated PPP. The addition of exogenous thrombin allows to bypass the phospholipid-dependent pathways of coagulation (extrinsic, intrinsic and common), therefore the thrombin time mainly represents fibrinogen concentration. Thrombin cleaves the fibrinopeptides A and B from the fibrinogen molecule, resulting in its conversion into fibrin.

If direct thrombin inhibitors are present in the test plasma, thrombin activity is inhibited. Therefore, the DTT is directly proportional to the concentration of the direct thrombin inhibitors in the tested plasma. For argatroban, the lower limit of quantification of this assay was 0.03 µg/ml and the upper limit 1.88 µg/ml. For dabigatran, the respective limits were 30 ng/ml and 400 ng/ml.

2.10 Anti-Xa assay

The anti-Xa assay for the indirect factor Xa inhibitors enoxaparin and danaparoid was performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) with the help of Mr. K. Vella, using the automated analyser ACL TOP 500 (Instrumentation Laboratory, Italy) and the HemosIL® Liquid Anti-Xa kit (Instrumentation Laboratory, Italy). The other anti-Xa assays were performed in the Coagulation Laboratory at the Royal Hallamshire Hospital (Sheffield, UK) with the help of Mr. K. Hickey, using the automated analyser Sysmex CS-5100 (Siemens Healthcare Diagnostics Products

GmbH, Germany) and the Biophen DiXal kit (Hyphen BioMed, France) with specific calibrators for apixaban, edoxaban and rivaroxaban, or the Chromogenix Coamatic® Heparin kit (Instrumentation Laboratory, USA) for fondaparinux.

The anti-Xa assay is a one stage chromogenic assay. The anti-Xa kit consists of an exogenous factor Xa reagent and a synthetic chromogenic substrate for factor Xa (Hyphen BioMed, 2018; Instrumentation Laboratory, 2013, 2017g). The exogenous factor Xa cleaves the synthetic chromogenic substrate, with release of a coloured compound (paranitroaniline), which is monitored optically at wavelength 405 nm. If there are indirect factor Xa inhibitors in the sample, they can form a complex with AT and inhibit the exogenous factor Xa (Figure 2.1). If there are direct factor Xa inhibitors in the sample, they can directly bind and inhibit the exogenous factor Xa. Therefore, the measured activity is inversely proportional to the concentration of the anticoagulant drugs in the sample. The anti-Xa assay, after appropriate calibration, can be used to measure UFH, LMWH, danaparoid, fondaparinux, apixaban, edoxaban and rivaroxaban (Winter et al., 2017). For the direct factor Xa inhibitors, the lower limit of quantification of this assay was 30 ng/ml and the upper limit was 400 ng/ml.

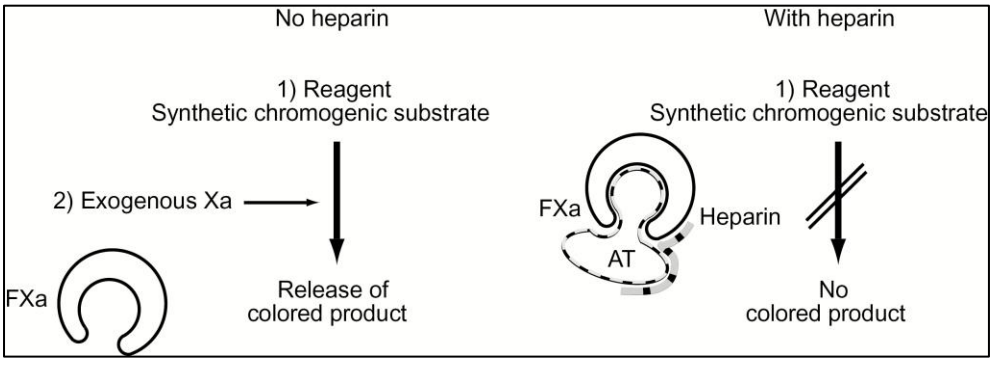


Figure 2.1 Schematic representation of the anti-Xa assay for the indirect factor Xa inhibitors (Winter et al., 2017) (Reproduced with permission, copyright licence no: 4639261176392)

2.11 Procoagulant phospholipid-dependent clotting time

The procoagulant phospholipid-dependent clotting time (PPL) was performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) with the help of Mr. D. Zammit, using the automated analyser ACL TOP 500 (Instrumentation Laboratory, Italy) and the STA Procoag-PPL kit (Diagnostica Stago, France; lot numbers 250412 and 250906). The STA Procoag-PPL kit contains citrated human plasma depleted of procoagulant phospholipids (reagent 1); bovine factor Xa (reagent 2); a normal control and a positive control (Diagnostica Stago, 2015). The PPL was performed as previously described (Exner et al., 2003). As summarised in Figure 2.2, 25 µL of patient plasma were incubated with 25 µL of procoagulant phospholipid depleted plasma (reagent 1) at 37°C. The latter provides all the coagulation factors, while the procoagulant phospholipids are only those of the patient plasma. Afterwards, 100 µL of factor Xa and calcium (reagent 2) was added (B. J. Woodhams, 2014).

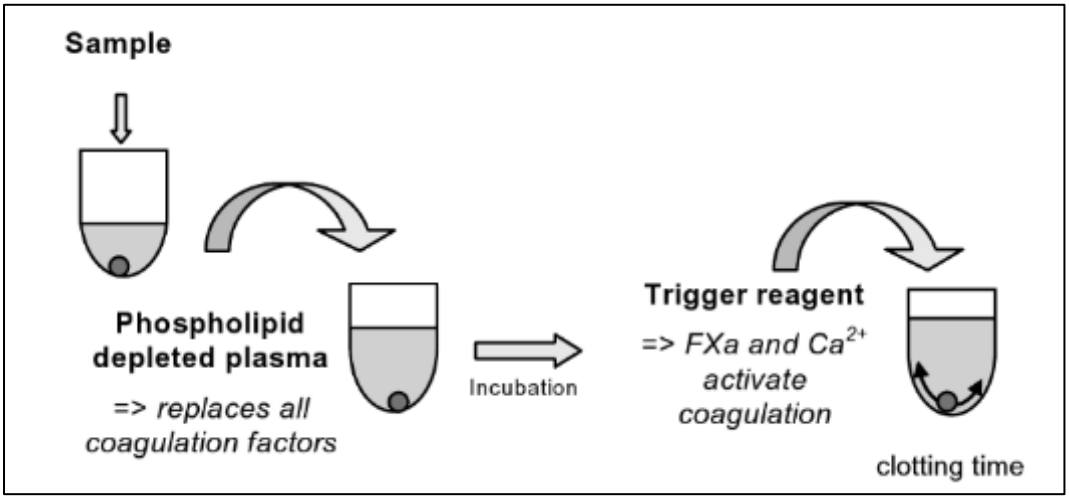


Figure 2.2 Schematic representation of the procoagulant phospholipid-dependent clotting time (B. J. Woodhams, 2014) (Reproduced with permission)

The coagulation cascade was therefore activated at the level of factor Xa, thus eliminating the interference of previous coagulation factors, and the clotting time was recorded. The clotting time is expressed in seconds and is inversely proportional to the level of procoagulant phospholipids in the sample. If there are procoagulant phospholipids in the patient plasma, which were shown to correlate with the functional activity of microparticles (Campello et al., 2014), this factor Xa-based clotting time will be shortened.

Results of the PPL were also expressed as a ratio:

$$\text{PPL ratio} = \frac{\text{patient's clotting time (sec)}}{\text{reference clotting time (sec)}}$$

In order to obtain the reference clotting time, the PPL was tested on plasma samples from 20 healthy controls and the median value was computed. The PPL ratio is < 1 when the PPL is shortened, meaning that there are procoagulant phospholipids in the patient plasma.

2.12 Soluble P-selectin

2.12.1 Instrument

The soluble P-selectin (sP-selectin), a cell adhesion molecule present into the plasma, was measured using an ELISA technique. It was performed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) with the help of Mr. K. Vella, using a microplate processor (DS2® 2-plate ELISA Processing System, Dynex Technologies GmbH, Germany), the software DS-matrix 1.34 (Dynex Technologies GmbH, Germany), and the Human sP-selectin Platinum ELISA kit (Affymetrix, eBioscience, Bender MedSystems GmbH, Austria; lot numbers 113423000 and 133554000).

It is an application of a sandwich ELISA, as summarised in Figure 2.3. The microwells are coated with monoclonal antibody anti-human sP-selectin. If the antigen (human sP-selectin) is present in the test plasma, it binds to these antibodies. A second monoclonal antibody anti-human sP-selectin conjugated with an enzyme is added to the microwells and binds to the sP-selectin captured by the first antibody. A substrate reactive with HRP is added to the microwells and is converted by HRP into a coloured product, whose amount is proportional to the concentration of sP-selectin in the test plasma.

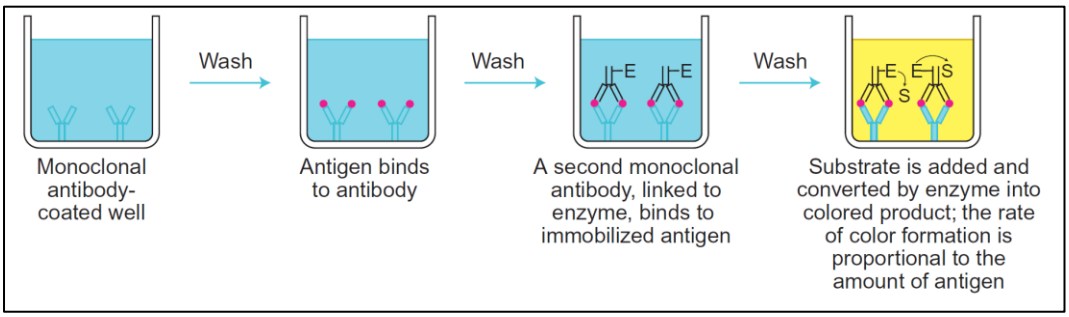


Figure 2.3 Schematic representation of the sandwich ELISA (Foxman, 2012) (Reproduced with permission, copyright licence no: 4641361149015)

2.12.2 Reagents

The Human sP-selectin Platinum ELISA kit (Affymetrix, eBioscience, Bender MedSystems GmbH, Austria) contains a 96-microwell plate coated with monoclonal antibodies anti-human sP-selectin and the following reagents:

- Wash Buffer Concentrate (20x): contains phosphate buffered saline (PBS) with 1% Tween 20. Wash buffer concentrate 50 ml was diluted with deionised water 950 ml;

- Assay Buffer Concentrate (20x): contains PBS with 1% Tween 20 in 10% bovine serum albumin (BSA). Assay buffer concentrate 5 ml was diluted with deionised water 95 ml;
- Sample Diluent;
- Human sP-selectin Standard (lyophilised): it was reconstituted with deionised water according to the volumes stated on the vial, in order to obtain the standard concentration 80 ng/ml. It was subsequently mixed with sample diluent in order to obtain the following seven standard dilutions: 40 ng/ml, 20 ng/ml, 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml;
- HRP-Conjugate (concentrate): contains the enzyme horseradish peroxidase (HRP) conjugated with anti-human sP-selectin monoclonal antibodies. HRP-conjugate concentrate 0.06 ml was diluted with assay buffer 5.94 ml and used within 30 minutes after dilution;
- Substrate Solution: contains tetramethyl-benzidine (TMB), reactive with the HRP;
- Stop Solution: contains 1M phosphoric acid, to inactivate the enzyme;
- Controls (lyophilised): a high and a low control were reconstituted by adding deionised water 100 µl.

2.12.3 Procedures

PPP was prepared and stored at -80°C as described in paragraph 2.1. Before the experiment, the plasma aliquots were thawed in the water bath at 37°C for 5 minutes. On every plate there were also seven standard dilutions, a high and a low control, and a blank. Every sample, standard, control and blank was tested in duplicate. The working protocol consisted of the following steps:

1. The protocol recommended by the manufacturer of the Human sP-selectin ELISA kit was prepared in the DS-matrix 1.34 software;
2. The reagents were prepared as described in paragraph 2.11.2;
3. The 96-microwell plate coated with monoclonal antibodies anti-human sP-selectin was inserted in the microplate processor and all the following steps were automatically performed;
4. Washing step: the plate was washed twice with wash buffer 400 µl per well;
5. The microwell plate was prepared as follows:
    a. Sample diluent 100 µl were pipetted in the Blank wells;
    b. Standard dilutions 100 µl were pipetted in the Standard wells;
    c. In each Sample well (including controls), sample diluent 90 µl were pipetted, followed by test plasma 10 µl. Therefore, samples and controls were all diluted by 10 times. If there is sP-selectin in the test plasma, it binds to the specific antibodies in the microwells;
    d. HRP-Conjugate anti-human sP-selectin 50 µl was pipetted into each well, to bind the sP-selectin captured by the antibodies of the microwells;
6. First incubation: the plate was incubated at ambient temperature (18-25°C) for two hours, with shaker at medium speed (400 rpm);
7. Washing step: the plate was washed three times with wash buffer 400 µl per well, to remove the unbound HRP-Conjugate antibodies;
8. Substrate solution 100 µl was pipetted into each well, to react with the bound HRP-Conjugate;
9. Second incubation: the plate was shaken for 3 seconds at low speed, and afterwards incubated at ambient temperature (18-25°C) for at least 15 minutes. Colour development on the plate, which is proportional to the concentration of sP-selectin,

was monitored and the reaction was stopped when the highest standard developed a dark blue colour;

10. Stop solution 100 µl was pipetted into each well, to stop the reaction;
11. The absorbance of each microwell was read using a spectrophotometer with 450 nm as primary wavelength and 620 nm as reference wavelength;
12. Using the dedicated software, the plate was read as follows: blank wells were used to blank the plate reader, while the standard wells were used to create a standard linear regression curve. The average absorbance from each set of samples, tested in duplicate, was plotted against the standard curve to convert them into the sP-selectin concentrations (expressed as ng/ml). Since samples were diluted 10 times, results were multiplied by 10 times.

2.12.4 Coefficients of variation

According to manufacturer's instructions (Affymetrix eBioscience, 2015), the intra-assay CV for sP-selectin is 7.8% and the inter-assay CV is 5.4%. The lower limit of detection of the assay is 0.20 ng/ml.

2.13 Fluorogenic Calibrated Automated Thrombin Generation Assay

2.13.1 Instrument

The thrombin generation was performed by the author in the Coagulation Laboratory at the Royal Hallamshire Hospital (Sheffield, UK) using a fluorogenic CAT assay. The Fluoroskan Ascent fluorimeter (Thermo Electron Corporation, Helsinki, Finland) was used to measure the fluorescence intensity. Using a dedicated software

(Thrombinoscope BV version 3.4.0.154, Maastricht, The Netherlands), the following CAT parameters were calculated (van Veen et al., 2008):

- Lag time: the time from the start of the run, when the sample is activated with the trigger, to the initiation of the thrombin generation, defined as a deviation of the signal of more than two standard deviation from the baseline (clotting time). It is expressed in minutes;
- ETP: the area under the thrombin generation curve (total amount of thrombin generated). It is expressed in nM*minutes;
- Peak thrombin: the highest concentration of thrombin that can be generated. It is expressed in nM;
- Time to peak: the time from the start of the run to the peak of thrombin. It is expressed in minutes;
- Velocity index: it is derived from the other parameters, using the following formula, and expressed in nM/minutes:

$$\text{Velocity index} = \left[\frac{\text{peak}}{(\text{time to peak} - \text{lag time})} \right]$$

2.13.2 Reagents

2.13.2.1 Thrombin calibrator

Lyophilised thrombin calibrator was purchased from Thrombinoscope BV (The Netherlands). The lot number TC1509/01 with activity 580 nM was used for the experiments performed in 2016 (reported in Chapters 3-4), while the lot number TC1810/01 with activity 640 nM was used for the experiments performed in 2019

(reported in Chapters 5-6). Activity was adjusted accordingly in the measurement program. The thrombin calibrator allows to estimate the amount of thrombin generated over time in the test wells based on the calibrated amount of thrombin activity generated in the calibrator wells. The calibrator wells can correct for inter-subject differences in plasma colour and substrate consumption. According to manufacturer's instructions, calibrator was stored in the fridge (2-8°C) until use. Before the experiments, it was reconstituted with deionised water 1ml and used within four hours of reconstitution.

2.13.2.2 Trigger solution

Lyophilised PPP-Reagent and PPP-Reagent-Low were purchased from Thrombinoscope BV (The Netherlands). They contain a mixture of phospholipids and TF and are used as triggers to initiate the generation of thrombin in PPP. The PPP-Reagent contains TF at 5pM, while the PPP-Reagent-Low contains TF at 1pM. The use of a trigger with low TF increases the sensitivity to factors VIII, IX and XI. The lots number PPP1509/01 (TF 5pM) and PPL1506/01 (TF 1pM) were used for the experiments performed in 2016 (reported in Chapters 3-4), while the lots number PPP1803/01 (TF 5pM) and PPL1805/01 (TF 1pM) were used for the experiments performed in 2019 (reported in Chapters 5-6). According to manufacturer's instructions, the trigger solution was stored in the fridge (2-8°C) until use. Before the experiments, it was reconstituted with deionised water 1ml and used within four hours of reconstitution.

2.13.2.3 Fluorogenic substrate

FluCa-kit, containing Fluo-Buffer and Fluo-Substrate, was purchased from Thrombinoscope BV (The Netherlands). The Fluo-Substrate contains the fluorogenic substrate in DMSO, while the Fluo-Buffer contains calcium chloride in hydroxyethyl-piperazine ethane-sulfonic acid buffer (HEPES) buffer. When the FluCa solution is dispensed by the machine, the citrate PPP is recalcified and the reaction is started. The lot number FC1507/01 was used for the experiments performed in 2016 (reported in Chapters 3-4), while the lot number FC1810/01 was used for the experiments performed in 2019 (reported in Chapters 5-6).

According to manufacturer's instructions, the Fluo-Buffer was stored in the fridge (2-8°C) until use. At its first use, the Fluo-Substrate was thawed in the water bath for two minutes, vortexed and stored at room temperature in an opaque container, in order to protect it from light. In order to prepare the FluCa solution, the Fluo-Buffer was firstly incubated in the water bath at 37°C for 30 minutes. Shortly before starting the experiment, the Fluo-Substrate was added to the warmed Fluo-Buffer in a ratio 1:40 and immediately vortexed.

2.13.3 Procedures

PPP was prepared and stored at -80°C as described in paragraph 2.2. Frozen aliquots were shipped to the Coagulation Laboratory at the Royal Hallamshire Hospital (Sheffield, UK) in dry-ice. Before the experiment, aliquots were thawed in the water bath at 37°C for five minutes. Thrombin generation was performed on the CAT, as previously described (Hemker et al., 2003).

The working protocol consisted of the following steps:

1. Preparation of the reagents: the thrombin calibrator and the trigger solution were reconstituted; the Fluo-Buffer was warmed in the water bath at 37°C;
2. Thrombin calibrator 20 µl were manually pipetted (using reverse pipetting) into the calibrator wells of the 96-well U bottom microtiter plate;
3. Trigger solution 20 µl were manually pipetted into the test wells of the same plate;
4. PPP 80 µl were manually pipetted into each well;
5. The plate was inserted into the fluorimeter and incubated at 37°C for 10 minutes;
6. The FluCa solution was prepared, by mixing Fluo-Substrate with warmed Fluo-Buffer;
7. The reaction was initiated after automated dispensing FluCa solution 20 µL in each well;
8. The fluorescence intensity was measured for at least one hour.

Every experiment was performed in duplicate, using two test wells and 1-2 calibrator wells. Three in-house QC plasma samples were tested in each run: 1) a normal QC (a pool of five FFP donations); 2) a hypo-coagulable QC (a pool of plasma from patients on warfarin with INR >1.8, final INR 2.0); 3) a hyper-coagulable QC (plasma from a patient with thrombotic thrombocytopenic purpura, obtained from therapeutic apheresis). Results were accepted only if all the QC samples were within their established local ranges.

Since the direct thrombin inhibitors can interfere with the activity of the thrombin calibrator and inhibit the conversion of the fluorescent substrate by the calibrator, the manufacturer recommends to avoid the addition of these anticoagulant drugs into the calibrator wells. One trial was done with dabigatran 92 ng/ml and dabigatran 276 ng/ml in the calibrator wells and the estimated ETP was found to be 3.8 times and 25.9

times higher, respectively, than the ETP of the same concentrations obtained with the unspiked normal PPP in the calibrator well. Conversely, when dabigatran 276 ng/ml was tested in the calibrator well after DOAC Stop® treatment, the ETP was correctly estimated. Therefore, the results of these preliminary trials confirmed the concentration-dependent interference with the thrombin calibrator and the potential application of DOAC Stop® in this setting (Kopatz et al., 2018). However, for all the experiments involving direct thrombin inhibitors, the same unspiked normal PPP was used in the calibrator well, as per manufacturer's instructions.

2.13.4 Coefficients of variation

In order to establish the CV of the CAT, several calculations were made, using the formula SD/mean * 100. The CV were firstly calculated in 2016; however, due to the elapsed time and the change in the lot number of the reagents, they were recalculated in 2019. The intra-assay CV were calculated by analysing the normal PPP 10-20 times in the same run. At TF 5pM, the intra-assay CV for all CAT parameters were all < 5% (Table 2.8). The inter-assay CV were calculated by analysing the results of the QCs performed in 10-15 different runs over a two weeks period. At TF 5pM, the inter-assay CV for the ETP ranged from 5.0% to 11.8% for the normal QC, from 5.5% to 8.2% for the hypocoagulable QC, and from 3.7% to 11.8% for the hypercoagulable QC (Table 2.8). At TF 1pM, the inter-assay CV for the ETP calculated in 2016 from 10 different runs were 10.4% for the normal QC, 12.7% for the hypocoagulable QC and 11.2% for the hypercoagulable QC.

	Intra-assay CV		Inter-assay CV					
	Normal PPP		Normal QC		Hypocoagulable QC		Hypercoagulable QC	
	Year 2016 (n=10)	Year 2019 (n=20)	Year 2016 (n=10)	Year 2019 (n=15)	Year 2016 (n=10)	Year 2019 (n=15)	Year 2016 (n=10)	Year 2019 (n=15)
Lag time	1.7%	2.5%	7.9%	3.7%	6.3%	6.8%	9.3%	4.9%
ETP	4.3%	3.1%	11.8%	5.0%	8.2%	5.5%	11.8%	3.7%
Peak thrombin	3.3%	3.4%	12.5%	10.3%	11.4%	6.7%	12.0%	9.8%
Time to peak	0%	2.4%	5.0%	5.2%	4.4%	4.6%	7.2%	5.0%

Table 2.7 Summary of the coefficients of variation of the CAT at TF 5pM

2.14 Thromboelastography

2.14.1 Instrument

The TEG was performed by the author at Mater Dei Hospital (Msida, Malta) using the instrument TEG®5000 (Thromboelastograph Hemostasis Analyser, Haemoscope, Haemonetics Corporation, USA) and a dedicated software (TEG Analytical Software version 4.2.3, Haemoscope, Haemonetics Corporation, USA). The following main TEG parameters were calculated (Haemoscope Corporation, 2007):

- Reaction time or R-time: the time from the start of the run, when the sample is activated with calcium, to amplitude 2 mm (beginning of clot formation, which corresponds to the enzymatic part of the coagulation cascade);
- Coagulation time or K-time: the time from R to amplitude 20 mm (clot kinetics, represented by the achievement of a certain level of clot firmness);
- α-Angle: the angle formed by the slope of the TEG curve between the R-time and the K-time (clot kinetics, represented by the rapidity of fibrinogen conversion to fibrin);

- MA: the greatest amplitude of the TEG curve (maximum clot strength);
- Percent lysis at 30 minutes after MA (LY30): the reduction of the area under the TEG curve from MA until 30 minutes after MA (clot lysis);
- Percent lysis at 60 minutes after MA (LY60): the reduction of the area under the TEG curve from MA until 60 minutes after MA (clot lysis).

In some experiments the following secondary TEG parameters were also considered:

- Time to the split point (SP): the time from the start of the run to the split of the TEG trace;
- Time to MA (TMA): the time from the start of the run to the MA (time needed to form a stable clot, representing a global measurement of clot kinetics);
- Clot firmness as shear elastic modulus strength (G parameter): obtained exponentially from the MA;
- Elasticity constant (E parameter): obtained as normalisation of the G parameter;
- Thrombodynamic potential index (TPI): obtained as ratio E/K (global coagulation status);
- Coagulation index (CIx): derived from R, K, angle, MA (global coagulation status);
- Amplitude at 30 minutes after MA (A30);
- Amplitude at 60 minutes after MA (A60);
- Clot lysis (CL30): the value of A30 relative to MA (fibrinolytic status at 30 minutes after MA);
- Clot lysis (CL60): the value of A60 relative to MA (fibrinolytic status at 60 minutes after MA);
- Lysis time estimate (LTE): the estimate of the time between the MA and amplitude 2 mm.

2.14.2 Reagents

2.14.2.1 Native TEG

Calcium chloride ($CaCl_2$ 0.2M) and plain disposable cups and pins for the TEG Hemostasis System were purchased from Haemonetics Corporation (USA). The following lots of $CaCl_2$ were used: 160087AA, 160374AA 160620AA, 170262AA, 170697BA. The following lots of plain disposable cups and pins were used: HMO3736, HMO3852, HMO4193.

2.14.2.2 TEG with TPA

The addition of TPA was used to show the fibrinolytic part of the TEG curve. A solution of TPA was prepared locally from Actilyse® 50 mg, lot number 502112 (Boehringer Ingelheim, UK; lot number 502112). Actilyse® contains alteplase, a tissue plasminogen activator used as thrombolytic drug in the treatment of acute massive PE, stroke and myocardial infarction. According to the manufacturer's instructions, 50 mg of Actilyse® in powder form were diluted with 50 ml of sterile distilled water, to obtain TPA concentration 1 mg/ml (Boehringer Ingelheim Limited, 2019). Subsequently, 5 ml of this TPA solution were mixed with 45 ml of a solution of HEPES buffer and $CaCl_2$, in order to obtain TPA concentration 0.1 mg/ml. It was divided into small aliquots of 0.2 ml each and frozen at -80°C until use. TPA was initially diluted, aliquoted and frozen in June 2016. It has been previously reported that there is no decline in TPA activity when stored at -80°C for up to seven years (Shaw et al., 2009). After a few trials with TPA 0.1 mg/ml 10 µl, it was noted that this concentration was too high and was not producing any visible TEG curve, therefore two further dilutions

0.05 mg/ml and 0.025 mg/ml were tried (Figure 2.4). TPA concentration 0.025 mg/ml was producing a reasonable effect, however there was high variability among different subjects when TPA was used on the TEG performed on CWB (Figure 2.5).

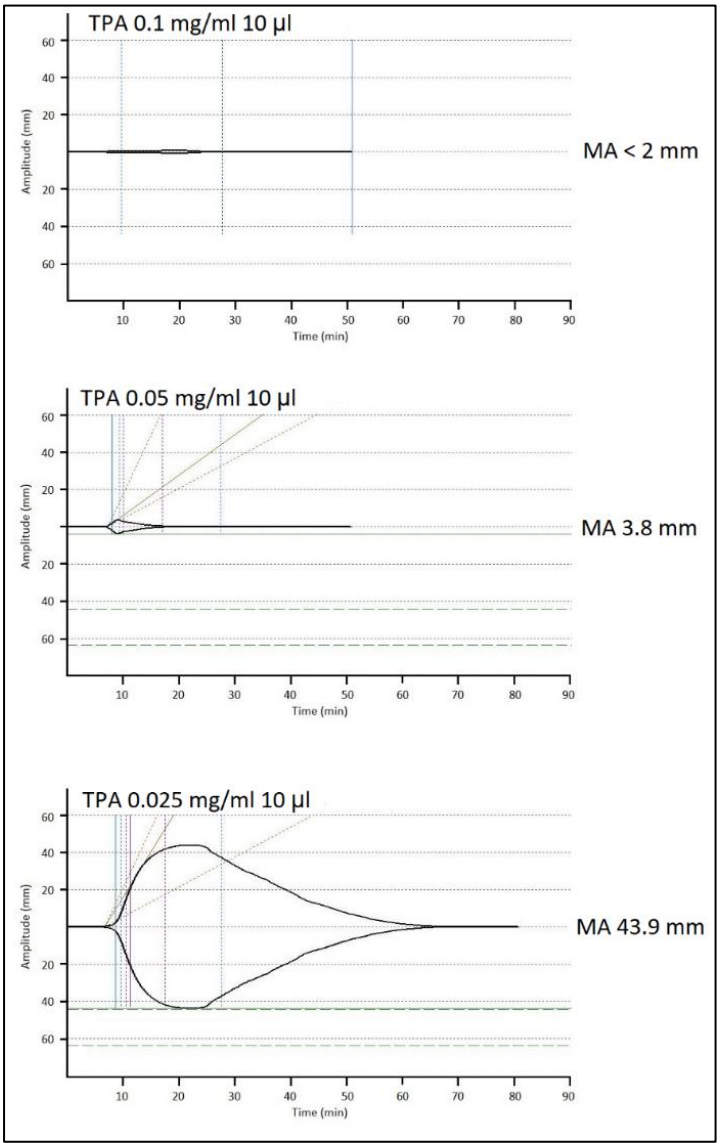


Figure 2.4 The effect of different concentrations of TPA on the TEG on citrated whole blood

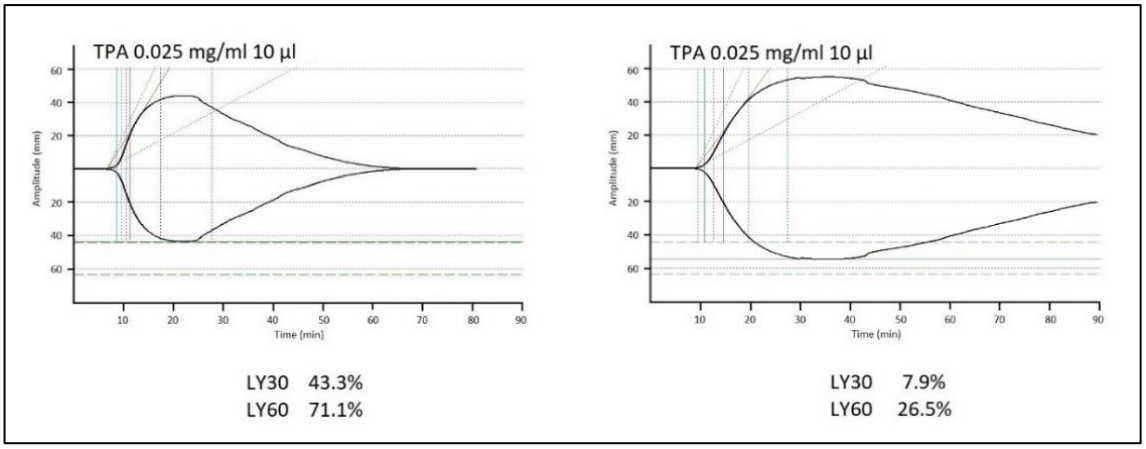


Figure 2.5 Example of inter-subject variability in fibrinolysis when using TPA on the TEG on citrated whole blood

The following modifications of this assay were proposed: performing the TEG on citrated PPP, reducing TPA volume to 5 µl, and testing also an intermediate TPA concentration 0.0375 mg/ml. However, it was noticed that with a further cycle of freeze-thaw, the TPA was losing part of its activity (Figure 2.6). This incidental finding has been previously reported in another study (Calo et al., 2017). In particular, the effect of TPA concentration 0.025 mg/ml was very small, especially on the LY30.

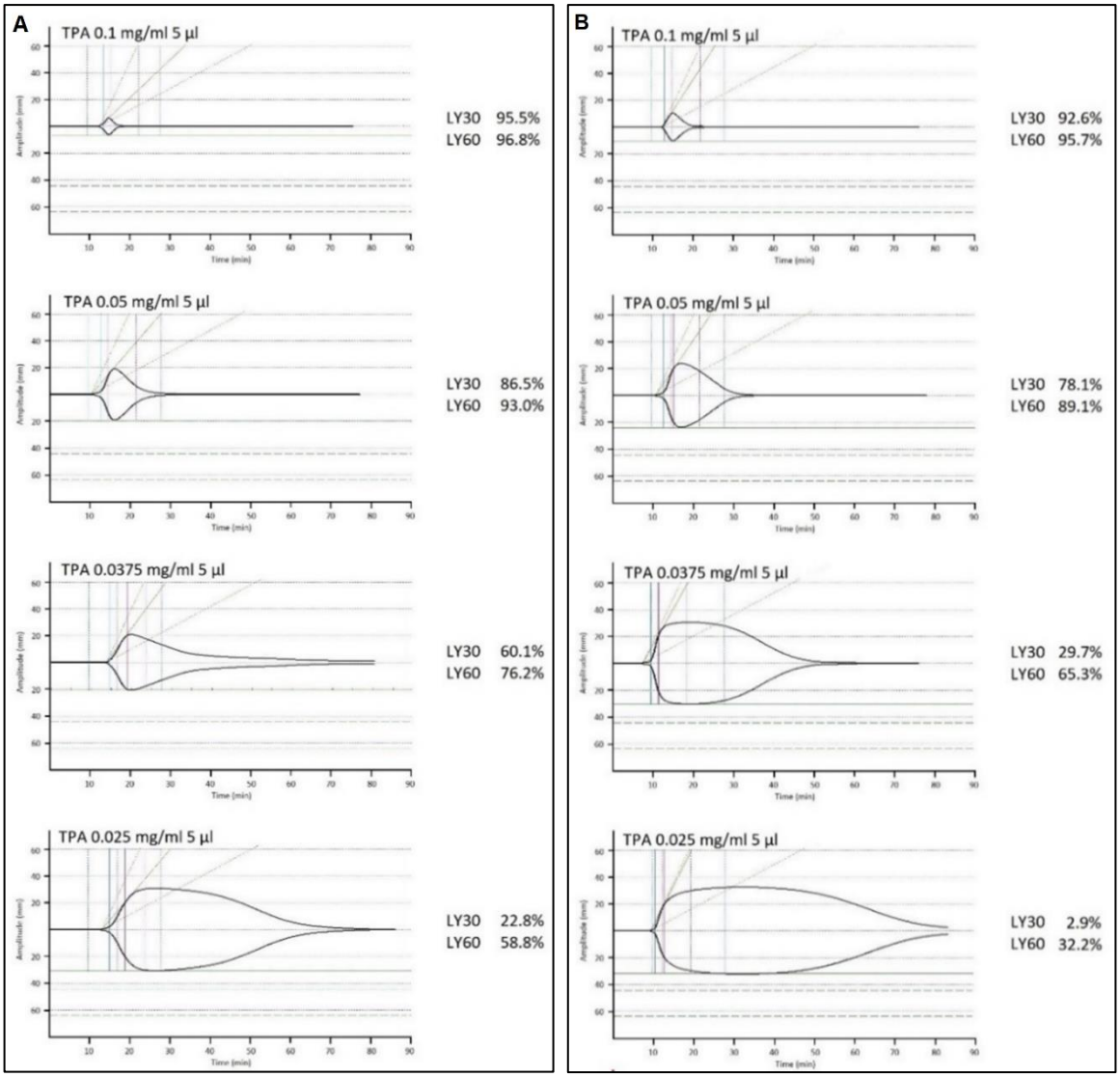


Figure 2.6 Differences between fresh TPA (A) and TPA prepared in advance (B) on the TEG performed on citrated PPP

In order to choose the most appropriate TPA concentration, several trials were performed. The rationale was to find a TPA concentration that was sufficient to show the fibrinolytic part of the TEG curve without too much interference with the other TEG parameters. Four TPA concentrations (0.1 mg/ml, 0.05 mg/ml, 0.0375 mg/ml, and 0.025 mg/ml) 5 µl mixed with citrated normal PPP 325 µl were tested with the APTT, PT and D-dimer assays but there was no progression in the results (Table 2.9). Results of the APTT and the PT were expected, because these assays measure only

the initiation of the coagulation cascade. However, the D-dimer, which measures fibrin degradation products, was expected to be raised. The fact that D-dimer in the samples treated with TPA was comparable to the same untreated normal PPP could be explained by the need for incubation in order to first form the clot and then lyse it. When the D-dimer was tested again after the PPP was run on the TEG with TPA at 37°C, it was unmeasurably high even after several dilutions. Fibrinogen was also tested after the PPP was run on the TEG with TPA and the result was below the lower limit of detection of this assay (< 0.35 g/l).

	TPA 0.1 mg/ml	**TPA 0.05 mg/ml**	**TPA 0.0375 mg/ml**	**TPA 0.025 mg/ml**	**Normal PPP**
APTT (sec)	27.1	27.1	27.0	26.8	27.5
APTT (ratio)	0.91	0.91	0.91	0.90	0.92
PT (sec)	10.5	10.4	10.4	10.5	10.4
INR	1.01	1.00	1.00	1.01	1.00
D-dimer (ng/ml)	392	410	404	393	409

Table 2.8 Results of APTT, PT and D-dimer in samples treated with increasing concentrations of TPA

It was finally decided to use a single batch of TPA with concentration 0.0375 mg/ml for all the experiments. Therefore, the previous TPA aliquots with concentration 0.1 mg/ml were thawed at room temperature and further diluted with HEPES buffer to reach the established concentration 0.0375 mg/ml. This solution was divided into 250 aliquots of 30 μl which were frozen at -80°C until the analysis. Before each experiment, TPA was warmed at room temperature. All the experiments with TPA were performed using this TPA solution (which underwent two cycles of freeze-thaw) on PPP, corresponding to a final TPA plasma concentration 0.52 μg/ml. A previous study conducted with TPA on the ROTEM evaluated TPA plasma concentrations 0.5 μg/ml and 1.0 μg/ml (White et al., 2018).

2.14.3 Procedures

2.14.3.1 Native TEG using citrated whole blood

Venous blood samples were collected into vacuum coagulation tubes using a butterfly needle. The first 2 ml of CWB were discarded. Samples were left standing at room temperature for 30 minutes and they were gently inverted five times before starting the TEG. The TEG was performed following the manufacturer's instructions. Plain disposable cups and pins were loaded onto the TEG analyser. Calcium chloride ($CaCl_2$ 0.2 M) 20 µl was pipetted at the bottom of the cup, followed by CWB 340 µl. The TEG was started immediately.

2.14.3.2 Native TEG using citrated platelet poor plasma

PPP was prepared and stored at -80°C as described in paragraph 2.2. Before the experiment, plasma aliquots were thawed in the water bath at 37°C for five minutes. Plain disposable cups and pins were loaded onto the TEG analyser. Calcium chloride ($CaCl_2$ 0.2M) 30 µl was pipetted at the bottom of the cup, followed by citrated PPP 330 µl. The TEG®5000 user manual recommends to increase the volume of calcium chloride to 30 µl when running citrated PPP instead of CWB, in order to compensate for the increased volume of plasma (Haemoscope Corporation, 2007). Therefore, since the final volume in the TEG cup is constant at 360 µl, the volume of citrated PPP should be 330 µl. The TEG was started immediately.

Since several previous studies analysing citrated plasma used calcium chloride ($CaCl_2$ 0.2M) 20 µl and citrated PPP 340 µl (Lu et al., 2013; Maatman et al., 2018; Rönsholt et al., 2015), a preliminary experiment was performed to compare these two protocols.

A single batch of pooled normal PPP and a single lot of calcium chloride ($CaCl_2$ 0.2M LOT 170697BA) were used for this experiment to reduce variability. The normal PPP was analysed a total of 40 times using both TEG channels during a 2-day period: on the first day 10 times with calcium chloride 20 µl, followed by 10 times with calcium chloride 30 µl; on the second day this sequence was inverted. For this experiment, samples were run until they reached the MA.

CVs were calculated using the formula SD/mean * 100 and compared using the statistical program R (version 3.5.1; Vienna, Austria) and the package cvequality, which uses an asymptotic test to measure the equality of the CVs (Feltz & Miller, 1996; Marwick & Krishnamoorthy, 2019; R Core Team, 2015).

Results are reported in Table 2.10. The mean MA using calcium chloride 30 µl was significantly lower than calcium chloride 20 µl (difference 1.1 mm; 95% CI, 0.3-2.0; p=0.013). Even though the CVs were not statistically significant different, given the improvement in some of them, it was decided to perform all the following experiments using calcium chloride 30 µl and citrated PPP 330 µl.

	$CaCl_2$ 0.2M 20 µl (n=20)			**$CaCl_2$ 0.2M 30 µl (n=20)**			**P values**	
	Mean	**SD**	**CV**	**Mean**	**SD**	**CV**	**Student's t-test (comparison of means)**	**Feltz and Miller asymptotic test (comparison of CVs)**
R time (min)	11.48	1.20	10.4%	12.27	1.30	10.6%	0.053	0.95
K time (min)	2.88	0.78	27.1%	2.68	0.56	20.7%	0.37	0.27
Angle (deg)	51.16	7.27	14.2%	52.45	6.32	12.0%	0.55	0.48
MA (mm)	33.58	1.38	4.1%	32.45	1.37	4.2%	0.013	0.91

Table 2.9 Comparison between two different protocols ($CaCl_2$ 0.2M 20 µl vs. 30 µl) for the TEG on citrated PPP

2.14.3.3 TEG with TPA using citrated platelet poor plasma

The TEG with TPA was performed on citrated PPP, instead of CWB, in order to reduce inter-subject variability (paragraph 2.14.2.2). PPP was prepared and stored at -80°C, as described in paragraph 2.2. Before the experiment, plasma aliquots were thawed in the water bath at 37°C for 5 minutes. TPA solution 0.0375 mg/ml was prepared and stored at -80°C as described in paragraph 2.13.1.2 Before the experiment, one aliquot was thawed at room temperature.

Plain disposable cups and pins were loaded onto the TEG analyser. Calcium chloride ($CaCl_2$ 0.2M) 30 µl was pipetted at the bottom of the cup, followed by TPA solution 0.0375 mg/ml 5 µl, and afterwards citrated PPP 325 µl. The TEG was started immediately. All samples with TPA were run until they reached the LY60.

2.14.4 Normal ranges

At Mater Dei Hospital (Msida, Malta), TEG is usually performed on CWB. Therefore, there were no local normal ranges established for the TEG using citrated PPP. To establish the normal ranges, plasma samples from 20 healthy controls (10 females and 10 males) were analysed. These subjects had no history of cancer, thrombosis or major chronic diseases, regular intake of anticoagulant or oral contraceptive, and they have previously given their consent for the creation of normal ranges. The calculation of normal ranges was performed as follows:

1) The results were divided into two groups by sex. Each TEG parameter in each group was assessed for normality using the Kolgomorov-Smirnov test (with the Lilliefors Significance Correction) and the Shapiro-Wilk test. For these tests a non-significant result ($p>0.05$) indicates normality.

2) For TEG parameters that followed a normal distribution, a parametric test (the independent samples t-test) was used to assess for differences between the two sexes. If there was a significant difference, normal ranges were calculated separately for females and males; otherwise a single normal range was calculated. The range was calculated using the formula mean - 1.96*SD for the lower bound and mean + 1.96*SD for the upper bound. This range is supposed to contain approximately 95% of data of a normally distributed variable.
3) TEG parameters that did not follow a normal distribution were first tested for outliers by creating box-plots in SPSS. In this type of graph, the rectangle contains 50% of the values and the horizontal line inside the rectangle is the median. The lines (whiskers) protruding from the box reach the lowest and the highest values. However, where present, "outliers" are represented as circles far away from the whiskers, and "extremes" as asterisks. SPSS considers "outliers" those values that are more than 1.5 box-lengths from the edge of the rectangle and "extremes" those values that are more than three box-lengths from the edge of the rectangle (Pallant, 2016). After removal of extreme outliers, if the population passed the normality test, parametric tests were used and ranges were calculated with the above-mentioned formulas.
4) If the population was still not normally distributed, data underwent a log-transformation (using the natural logarithm, logn) and were tested again for normality. If the population was then normally distributed, parametric tests were used to compare distributions in males and females.

5) If the population was still not normally distributed, a non-parametric test (the Mann-Whitney U test) was used to assess for differences between the two sexes.
6) In these last two cases, the normal ranges were calculated with a lower limit being the 2.5^{th} percentile and the upper limit being the 97.5^{th} percentile.

The normal ranges for the native TEG and the TEG with TPA obtained following this procedure are reported in Chapter 5 (paragraphs 5.4.3.1 and 5.4.4.1, respectively).

2.14.5 Coefficients of variation

In order to establish the CV of the TEG, several calculations were made, using the formula SD/mean * 100. The intra-assay CV for the native TEG were calculated by analysing the normal PPP 10 times on a single day, using both TEG channels. They were 12.8% for the R time, 23.2% for the K time, 11.6% for the angle, and 8.1% for the MA. The intra-assay CV for the TEG with the addition of TPA were calculated by analysing the normal PPP with TPA 10 times on a single day, using both TEG channels. They were 8.7% for the R time, 23.5% for the K time, 10.5% for the angle, 7.7% for the MA, 40.8% for the LY30, and 22.2% for the LY60.

The inter-assay CV for the native TEG were calculated from the analysis of the normal PPP on 20 different days. They were 25.4% for the R time, 20.2% for the K time, 14.2% for the angle, and 5.6% for the MA. The inter-assay CV for the TEG with the addition of TPA were calculated from the analysis of the normal PPP with TPA on 20 different days. They were 13.0% for the R time, 84.4% for the K time, 26.2% for the angle, 11.6% for the MA, 70.0% for the LY30, and 42.8% for the LY60.

Furthermore, the results of the QC that have been performed from January to December 2018 were also analysed. There are two different levels of QC (level I, LOT

1101-1201; level II, LOT 1022-1202; Haemonetics Corporation, USA). Reference ranges are established by the manufacturer and the main difference between the two levels lies in the MA, being low with level II and normal with level I. Quality control is performed on a daily basis using both channels, while QC Level I and Level II are run on alternate days. The QCs contain a lyophilised preparation of citrated animal plasma. After reconstitution, calcium chloride ($CaCl_2$ 0.2M) 20 µl and 340 µl of the reconstituted control is pipetted into the TEG cup. Only those QCs with all the four parameters available were considered. The inter-assay CVs were 13.7% for the R time, 10.6% for the K time, 3.0% for the angle, and 6.9% for the MA (Level I, n=156) and 12.0% for the R time, 6.3% for the K time, 1.1% for the angle and 7.3% for the MA (Level II, n=160).

Taken together, these results suggest that the MA is the most consistent parameter across the different samples considered, with CVs not exceeding 11.6%. Variability in the fibrinolytic parameters LY30 and LY60 was extremely high, especially with regards to the inter-assay CVs (Table 2.15).

	Intra-assay CV		**Inter-assay CV**			
	PPP (n=10)	**PPP + TPA (n=10)**	**PPP (n=20)**	**PPP + TPA (n=20)**	**QC Level I (n=156)**	**QC Level II (n=160)**
R time	12.8%	8.7%	25.4%	13.0%	13.7%	12.0%
K time	23.2%	23.5%	20.2%	84.4%	10.6%	6.3%
Angle	11.6%	10.5%	14.2%	26.2%	3.0%	1.1%
MA	8.1%	7.7%	5.6%	11.6%	6.9%	7.3%
LY30	NC	40.8%	NC	70.0%	NC	NC
LY60	NC	22.2%	NC	42.8%	NC	NC

Table 2.10 Summary of the coefficients of variation of the TEG on citrated PPP

This data was also used to check if there was any difference between the two channels. When the runs of the PPP performed on the same day were compared, channel 2 gave a prolonged R time (12.84 min channel 2 vs. 11.0 min channel 1; difference 1.84 min,

95% CI, 0.01 to 3.67; p=0.049). However, when the runs of the PPP with TPA performed on the same day were compared, a non-significant opposite trend was observed (12.24 min channel 2 vs. 13.54 min channel 1; difference -1.3 min, 95% CI -2.68 to 0.08; p=0.075). No significant differences emerged from the comparison of the runs of the PPP with and without TPA on different days and QC level 1. The results of QCs level 2 showed that channel 2 gave a slightly shorter K time (0.83 min channel 2 vs. 0.87 min channel 1; difference 0.04 min, 95% CI 0.02 to 0.05; p<0.001), a slightly increased angle (80.57° channel 2 vs. 79.93° channel 1; difference -0.65°, 95% CI -0.91 to -0.38; p<0.001) and a slightly increased MA (34.91 mm channel 2 vs. 33.97 mm channel 1; difference -0.94 mm, 95%CI -1.72 to -0.17; p=0.003). Taken together these results suggested that although there might be small differences between the two channels, these were unlikely to affect the results. It was decided anyway to perform all experiments at least in duplicate, using both channels, and to calculate the mean value, in order to remove the potential of a channel bias.

2.15 Statistics

Data distribution was evaluated using the Shapiro-Wilk normality test, where a p value <0.05 rejects the assumption a normal distribution. Continuous variables were expressed as mean with standard deviation (SD) for normally distributed variables, or as median with interquartile range (IQR), for not-normally distributed variables. Categorical variables were expressed as counts and percentages. Continuous variables were compared using Student’s t-test or the Mann-Whitney U test, as appropriate. Categorical variables were compared using the Chi square or Fisher's exact tests, as appropriate.

Data analysis was performed using SPSS v.21 (SPSS Inc., Chicago, Illinois, USA) and STATA/SE v.12 (StataCorp LP, College Station, Texas, USA). Two-tailed p values less than 0.05 were considered statistically significant. Throughout this thesis, p values ≤ 0.10 were reported with three decimal places, while p values > 0.10 were reported with only two decimal places. Details of specific statistical analyses are provided in the methods sections of each chapter.

2.16 Ethical approval

All the studies described in this thesis were reviewed and approved by the Faculty of Medicine and Surgery Research and Ethics Committee (FREC) and the University of Malta Research and Ethics Committee (UREC), whose approval letters are included in Appendix B. Protocol 21/2015 refers to the studies reported in Chapters 3-6, while protocol 07/2016 refers to the studies reported in Chapters 7-9. Patient information sheets and consent forms are included in Appendices C1-C2.

Chapter 3 :

Point-of-care Coagulometers for VKA Monitoring

3.1 Introduction

VKA are still the main anticoagulant treatment in several countries. However, due to their pharmacological properties (narrow therapeutic window, high inter-patient variability, several food/drug interactions), they need periodical monitoring and dose adjustment (Ageno et al., 2012). VKA are monitored using laboratory assays that evaluate the extrinsic pathway of coagulation, such as the PT, which is usually expressed as INR in order to standardize the results obtained by different laboratories. The manual tilt-tube (Estridge & Reynolds, 2012) was the PT assay recommended by the WHO (World Health Organization, 1999), but nowadays the PT is usually performed using automated coagulation analysers, either photo-optical or electromechanical. In addition, the last two decades saw the advent of portable coagulometers (Triller et al., 2015). The use of POC coagulometers is an attractive alternative to the standard laboratory INR, due to several advantages associated with this way of monitoring, such as the more practical use, the less invasive procedure and the availability of immediate results. Studies showed that, in selected groups of patients, home testing can allow more frequent monitoring, but can also increase patient involvement in their own care and their satisfaction (Bauman et al., 2015; Matchar et al., 2010). A meta-analysis of RCTs reported better outcomes, compared to the usual care, expressed by a significant reduction of major thromboembolism and mortality, without any increased risk of major bleeding complications (Bloomfield et al., 2011). However, while the correlation between different photo-optical or electromechanical automated analysers in previous studies was excellent (Bai et al., 2008; Tekkesin & Kılınc, 2012), it has been reported that the POC and the laboratory ways of measuring the INR may vary in their results (Biedermann et al., 2015; Hur et al., 2013) and this finding can potentially lead to clinical disagreement and differences

in VKA dosing (Biedermann et al., 2015; Lawrie et al., 2012; Vacas et al., 2003). Furthermore, it is still uncertain which assay actually correlates better with the overall coagulation potential, expressed by global coagulation assays, such as the thrombin generation.

3.2 Aims

The aims of this study were:

- To evaluate the level of agreement of four different INR assays which used the same thromboplastin (a photo-optical automated coagulometer, an electromechanical automated coagulometer, a POC coagulometer, and the manual tilt-tube technique), in comparison to the thrombin generation, as global coagulation assay, in order to establish the most sensitive and accurate way to measure the INR;
- To evaluate the accuracy of the POC coagulometer compared to the laboratory INR assays.

3.3 Methods

3.3.1 Study population

Between August and September 2015, adult patients who were attending the Anticoagulation Clinic at Mater Dei Hospital (Msida, Malta) for VKA monitoring were evaluated. Overall, 60 patients were enrolled: 30 patients who were deemed eligible for POC monitoring according to the local protocol at that time (target INR $\leq$ 3.0; $\geq$ 3 consecutive INRs in the therapeutic range; absence of severe comorbidities, such as APS, renal or liver failure, active cancer, triple therapy with two antiplatelet

drugs and warfarin) and 30 random patients (to increase the chance to find a wider range of INR values).

This study was approved by the University of Malta Research and Ethics Committee (protocol 21/2015, Appendix B). After explaining the rationale and the design of this study, eligible patients received an information sheet and, if they agreed to take part in this study, they were asked to sign a consent form. Both English and Maltese versions of the information sheet and consent form were available for patients (Appendix C1).

3.3.2 Blood collection and tests performed

Blood was collected using a syringe and a 21G needle and three vacuum coagulation tubes, containing sodium citrate 0.109M/3.2% (Vacuette, Greiner Bio-One, Austria), were filled. The first tube was processed according to the local standard operating procedure: it was sent to the Coagulation Laboratory using the pneumatic tube system where it was centrifuged (paragraph 2.2). The plasma was immediately analysed with the PT/INR assay using an automated photo-optical coagulometer (Sysmex CS-2100i or CA-1500, Siemens Healthcare Diagnostics Products GmbH, Germany) and human recombinant thromboplastin (Dade® Innovin®, Siemens Healthcare Diagnostics Products GmbH, Germany), as previously detailed (paragraph 2.4). The other two tubes were carried manually to the Coagulation Laboratory and, within two hours from blood collection, they underwent double centrifugation (2500 g for 10 min twice, with plasma separation in between), in order to obtain the PPP. The PPP was divided into 300 µl aliquots and immediately stored at -80°C until further analysis. A previous study showed that the INR is not affected by one cycle of freeze-thaw (Grau et al., 1999).

Prior to the following INR assays, samples were thawed at room temperature, with continuous gentle agitation for 10 minutes on a roller mixer. The INR on thawed PPP was tested using an electromechanical coagulometer (Thrombolyzer XRC, Behnk Elektronik, Germany) and the same lot of human recombinant thromboplastin. Furthermore, 30 samples were randomly chosen from the overall cohort and the PT was performed using the manual tilt-tube technique and the same lot of human recombinant thromboplastin. The INR was calculated from the PT using the formula reported in paragraph 2.4.

Frozen PPP aliquots were shipped to Sheffield (UK) in dry ice. The CAT was performed in duplicate (using one calibrator well and two test wells) at TF 5pM (paragraph 2.13), and samples were run for 60 minutes. All the samples were tested in February 2016.

On the day of enrolment, the POC INR was obtained in all patients using the CoaguChek XS Plus (Roche Diagnostics) coagulometer with capillary and venous blood samples. Non-citrated venous blood was obtained from the syringe used to collect the blood, after filling the three above-mentioned coagulation tubes and after discarding few drops. Capillary blood was obtained by finger-prick and applied on the test strip within 10 seconds (paragraph 2.4).

3.3.3 Statistical analysis

The following information were collected: baseline characteristics of the population (age, gender, body weight); past medical history (hypertension, diabetes mellitus, heart failure, coronary arteries disease, peripheral arteries disease, AF, previous stroke or transient ischemic attack, chronic pulmonary diseases, gastro-intestinal diseases, thyroid diseases, malignancy); details of the warfarin treatment (indication for

anticoagulant treatment, starting date, INR results in the previous three months, current warfarin dose); concomitant medications.

Data distribution was evaluated using the Wilk-Shapiro test. Continuous variables were reported as mean (SD) or as median (IQR), and compared using the Student's t-test or the Mann-Whitney U test, as appropriate. Categorical variables were reported as counts and percentages, and compared using the Chi square or Fisher's exact tests, as appropriate.

The INR values, measured using different methodologies, were correlated using the non-parametric Spearman's rank correlation test, in order to calculate the correlation coefficients (r). The correlation coefficient can range from -1 to 1, where absolute values equal to 1.0 indicate a perfect positive or negative correlation, while absolute values less than 0.50 indicate a poor correlation. A one-way repeated-measures analysis of variance (ANOVA) test, with Bonferroni's post-hoc correction, was used to compared the mean INR values obtained by the different assays.

A Bland-Altman plot (or difference plot) was created to evaluate the statistical agreement among the different INR assays. In this plot, the mean of the two assays results is placed on the x-axis and the difference between the two assays results is placed on the y-axis (Bland & Altman, 1999). The mean difference between the two assay results is the estimated mean bias, while the 95% limits of agreement are calculated as mean bias ± 1.96 SD (Bland & Altman, 1999). Furthermore, the size of the circles represents the frequency of the INR differences.

The clinical agreement among the different INR assays was evaluated by considering each INR result into three categories, i.e. below/within/above the therapeutic range, which was 2.0-3.0 (for patients with AF, VTE or aortic valve replacement) or 2.5-3.5 (for patients with mitral valve replacement). For the analysis reported in this chapter,

the statistical program STATA/SE v.12 (StataCorp LP, College Station, TX, USA) was used.

3.4 Results

3.4.1 Study population

The baseline characteristics of the 60 patients included in this study are reported in Table 3.1. Mean age was 68.5 ± 11.5 years, 43.3% were males. Approximately two thirds of the patients were on VKA treatment for AF (63.3%), one third for VTE (26.7%), and only a minority for mechanical heart valve replacement (8.4%) or cerebrovascular accidents (1.7%). The duration of anticoagulant treatment was more than a year in 73.3% of the study subjects. None of these patients were receiving dual antiplatelet therapy, and none had a known APS.

	N. of patients = 60
Age (years), mean (SD)	68.5 (11.5)
Male sex, n (%)	26 (43.3%)
Indication for anticoagulant treatment:	
• Atrial fibrillation, n (%)	38 (63.3%)
• Venous thromboembolism, n (%)	16 (26.7%)
• Aortic valve replacement, n (%)	4 (6.7%)
• Mitral valve replacement, n (%)	1 (1.7%)
• Cerebrovascular accident, n (%)	1 (1.7%)
Duration of the anticoagulant treatment:	
• ≤ 3 months, n (%)	6 (10.0%)
• 3-6 months, n (%)	6 (10.0%)
• 6-12 months, n (%)	4 (6.7%)
• > 1 year, n (%)	44 (73.3%)
Current warfarin dose (mg), median (IQR)	4 (3-5)
Comorbidities:	
• Hypertension, n (%)	49 (81.7%)
• Diabetes mellitus, n (%)	22 (36.7%)
• Dyslipidaemia, n (%)	32 (53.3%)
• Coronary artery disease, n (%)	18 (30.0%)
• Hypothyroidism, n (%)	8 (13.3%)
• Previous stroke, n (%)	3 (5.0%)
• Chronic obstructive pulmonary disease, n (%)	5 (8.3%)
• Malignancy, n (%)	8 (13.3%)
• Smokers: current, n (%) / previous, n (%)	5 (8.3%) / 13 (21.7%)
• Obesity, n (%)	29 (48.3%)
Concomitant medications:	
• Antiplatelets, n (%)	5 (8.3%)
• Steroids, n (%)	1 (1.7%)
• Statins, n (%)	35 (58.3%)
• Angiotensin-converting enzyme (ACE)-inhibitors or Angiotensin II receptor blockers (ARBs), n (%)	42 (70.0%)
• Diuretics, n (%)	34 (56.7%)
• Beta-blockers, n (%)	22 (36.7%)
• Calcium channel blockers, n (%)	11 (18.3%)
• Digoxin, n (%)	14 (23.3%)
• Levothyroxine, n (%)	8 (13.3%)
• Proton pump inhibitors, n (%)	10 (16.7%)
• Metformin, n (%)	17 (28.3%)

Table 3.1 Baseline characteristics of the study population (Riva et al., 2017) (Reproduced with permission)

3.4.2 Different INR methodologies

INR results for the Sysmex CS-2100i/CA-1500, the CoaguChek XS Plus on capillary blood and venous blood were available for all 60 patients. INR results for the Thrombolyzer XRC were available for 59 patients, since for one patient there was a limited amount of plasma available due to difficult blood sampling. Only 30 randomly chosen samples were tested with the manual tilt-tube technique. Using the standard analyser at the time of this study (the Sysmex CS-2100i/CA-1500), mean INR was 2.46 (± 0.75), median INR was 2.31 (IQR 1.95-2.74), while the INR range was 1.37-4.92. The INR values obtained with the other assays were slightly higher (Table 3.2).

Instrument (n of tests)	*Mean INR (SD)*	*Median INR (IQR)*	*INR range*
Sysmex CS-2100i/CA-1500 (60)	2.46 (0.75)	2.31 (1.95-2.74)	1.37-4.92
CoaguChek XS Plus (capillary blood) (60)	2.74 (0.86)	2.6 (2.2-3.1)	1.4-5.8
CoaguChek XS Plus (venous blood) (60)	2.74 (0.82)	2.6 (2.2-3.0)	1.4-5.7
Thrombolyzer XRC (59)	2.71 (0.85)	2.52 (2.14-2.97)	1.34-5.33
Manual tilt-tube technique (30)	2.65 (0.75)	2.55 (2.23-2.95)	1.41-4.66

Table 3.2 Summary of the INR results using different INR assays (Adapted from Riva et al., 2017. Reproduced with permission)

The INR measurements obtained using the different assays and arranged by increasing INR values are reported in Figure 3.1. Results of the one-way ANOVA showed that there was a statistically significant difference between the mean INR value obtained with the Sysmex CS-2100i/CA-1500 compared to the CoaguChek XS Plus either on capillary or venous blood, the Thrombolyzer XRC and the manual tilt-tube technique ($p<0.001$ for all tests).

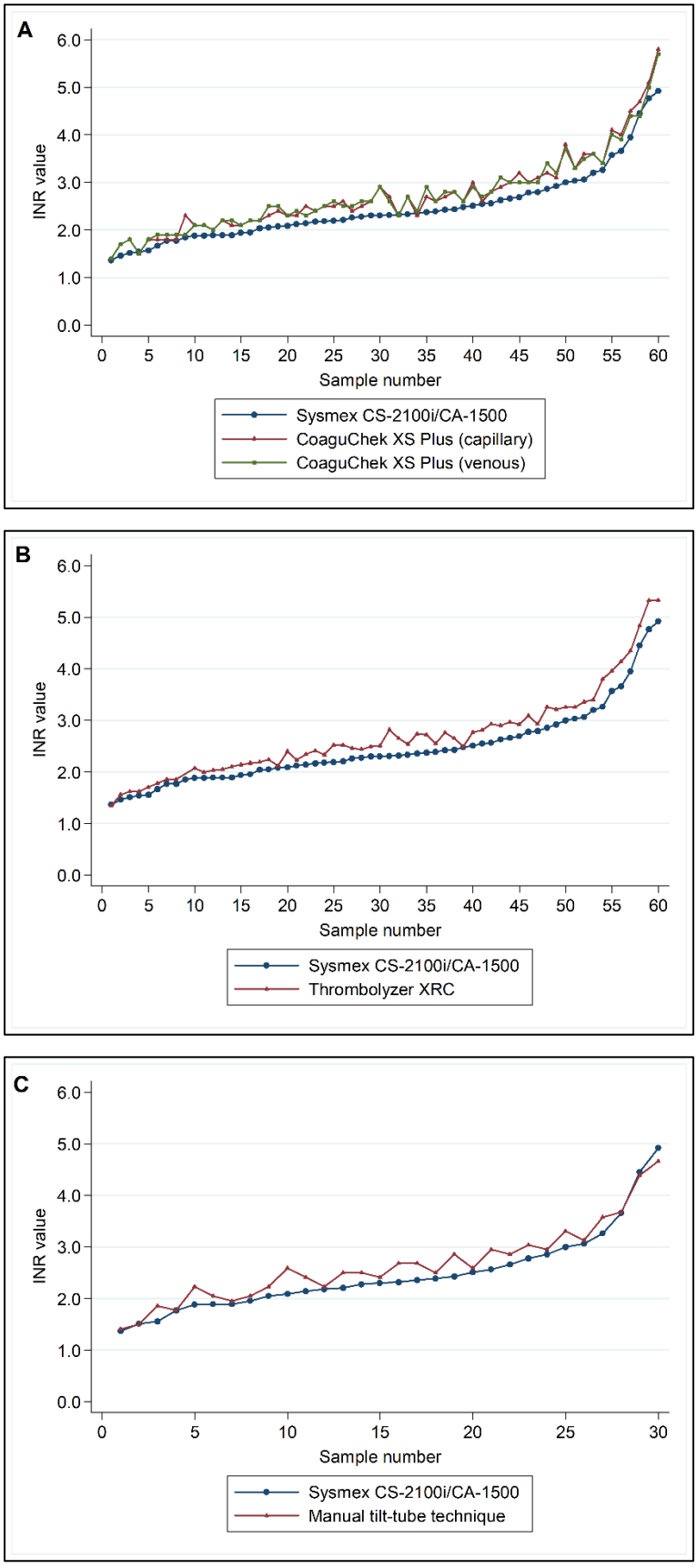


Figure 3.1 Comparison between the INR values obtained from the Sysmex CS-2100i/CA-1500 and the other INR assays, arranged by increasing INR values: CoaguChek XS Plus on capillary and venous blood (A), Thrombolyzer XRC (B), manual tilt-tube technique (C)

3.4.3 Calibrated Automated Thrombin Generation Assay

Results of the CAT were available for 59 patients, since the curve was not computable in one patient with VTE (Table 3.3). The comparison between patients with AF (n=38) and VTE (n=15) showed that patients with AF had a slightly prolonged lag time (median value 6.33 vs. 5.17 min, p=0.08) and time to peak (median value 9.33 vs. 7.83 min, p=0.06) compared to VTE patients.

Other variables, which could explain this finding, were also analysed, but no significant differences were identified:

- median INR: 2.35 in AF patients vs. 2.30 in VTE patients (p=0.99);
- median age: 69.5 years in AF patients vs. 67 years in VTE patients (p=0.54);
- anticoagulant treatment duration greater than one year: 71.7% of AF patients vs. 73.3% of VTE patients (p=1.00);
- median TTR in the last three months: 67.8% in AF patients vs. 67.0% in VTE patients (p=0.77).

Overall population			
Parameter	***Mean (SD)***	***Median (IQR)***	***Range***
Lag time (min)	6.35 (1.99)	6 (5.17-7.17)	3.47-13
Peak thrombin concentration (nM)	101.66 (44.51)	91.49 (72.51-121.2)	29.99-269.37
Time to peak (min)	9.23 (2.15)	8.83 (7.67-10.17)	5.97-16
Endogenous thrombin potential (nM*min)	596.75 (265.26)	547.5 (419-722.5)	186.5-1835
Velocity index (nM/min)	36.53 (17.76)	31.34 (25.22-47.91)	8.59-85.19

Comparison between patients with atrial fibrillation and venous thromboembolism *			
Parameter	***AF patients (n = 38)***	***VTE patients (n = 15)***	***p value***
Lag time (min)	6.33 (5.33-7.67)	5.17 (4.8-6.33)	0.08
Peak thrombin concentration (nM)	83.82 (69.05-121.2)	97.57 (77.37-135.39)	0.43
Time to peak (min)	9.33 (8-10.65)	7.83 (7.67-9)	0.06
Endogenous thrombin potential (nM*min)	508.5 (398-722.5)	547.5 (465-803)	0.40
Velocity index (nM/min)	30.6 (24.02-42.72)	38.45 (25.45-47.98)	0.44

Table 3.3 Results of the CAT in the overall population and in the comparison between patients with atrial fibrillation and venous thromboembolism (Riva et al., 2017) (Reproduced with permission)
* Results are reported as median (IQR) and compared using the Mann-Whitney U test

3.4.4 Correlation between the thrombin generation and the different INR assays

The relationship between the ETP on the CAT and the different INR assays was a negative curvilinear correlation (Figure 3.2). The correlation coefficients were as follows:

- between the ETP and the Sysmex CS-2100i/CA-1500: $r = -0.75$ ($p<0.001$);
- between the ETP and the Thrombolyzer XRC: $r = -0.78$ ($p<0.001$);
- between the ETP and the CoaguChek XS Plus on capillary blood: $r = -0.80$ ($p<0.001$);

- between the ETP and the CoaguChek XS Plus on venous blood: r = -0.78 ($p<0.001$);
- between the ETP and the manual tilt-tube technique: r = -0.80 ($p<0.001$).

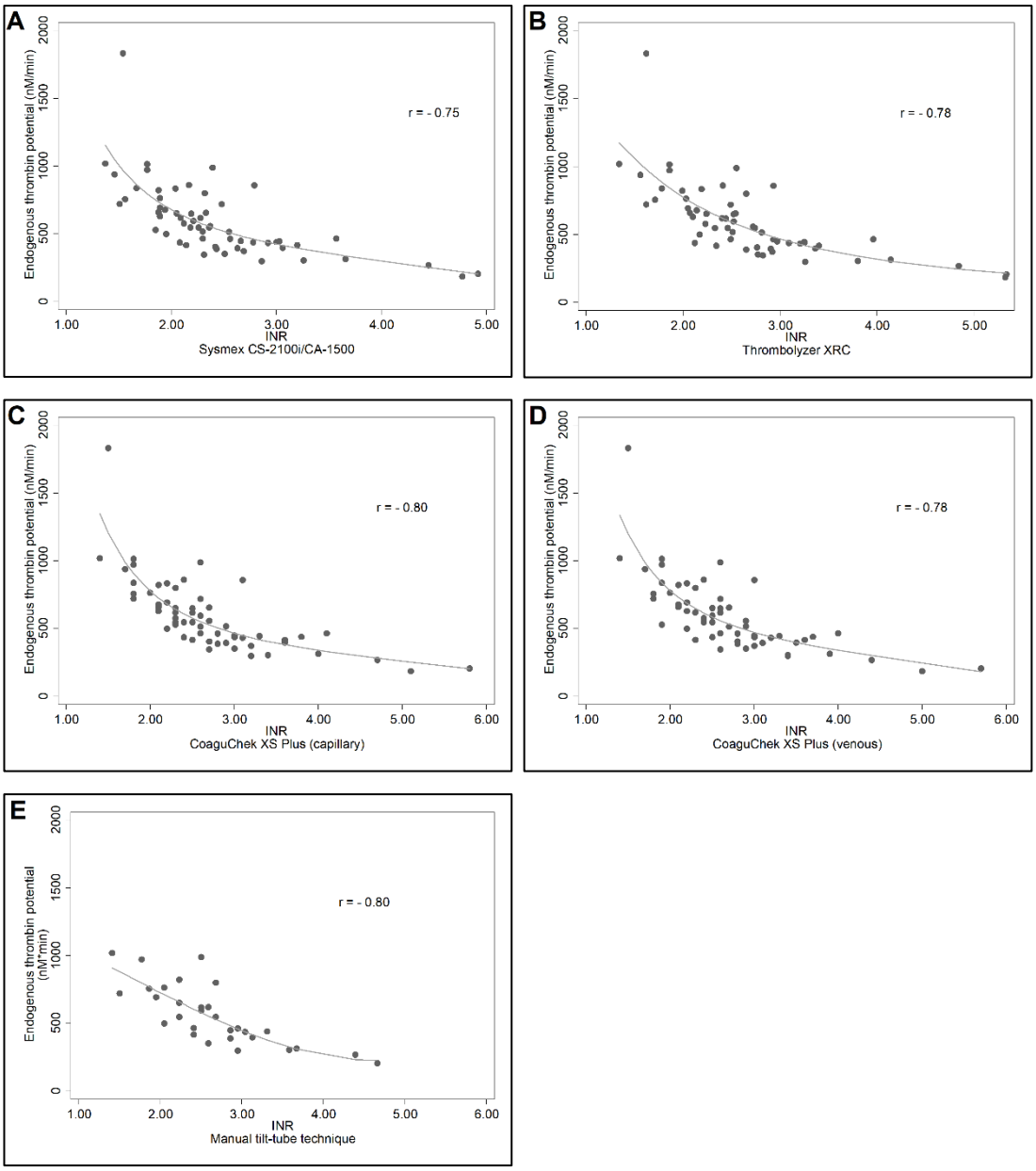


Figure 3.2 Correlation between the endogenous thrombin potential on the CAT and the INR assays: Sysmex CS-2100i/CA-1500 (A), Thrombolyzer XRC (B), CoaguChek XS Plus on capillary blood (C) and on venous blood (D), and the manual tilt-tube technique (E) (Adapted from Riva et al., 2017. Reproduced with permission, copyright licence no: 4643641187274)

3.4.5 Accuracy of the POC coagulometer

The CoaguChek XS Plus performed on capillary blood samples showed a strong positive linear correlation with the other INR assays (Figure 3.3), with the following correlation coefficients:

- between the CoaguChek XS Plus (on capillary samples) and the CoaguChek XS Plus (on venous samples): $r = 0.99$ ($p<0.001$);
- between the CoaguChek XS Plus (on capillary samples) and the Sysmex CS-2100i/CA-1500: $r = 0.97$ ($p<0.001$);
- between the CoaguChek XS Plus (on capillary samples) and the Thrombolyzer XRC: $r = 0.96$ ($p<0.001$);
- between the CoaguChek XS Plus (on capillary samples) and the manual tilt-tube technique: $r = 0.93$ ($p <0.001$).

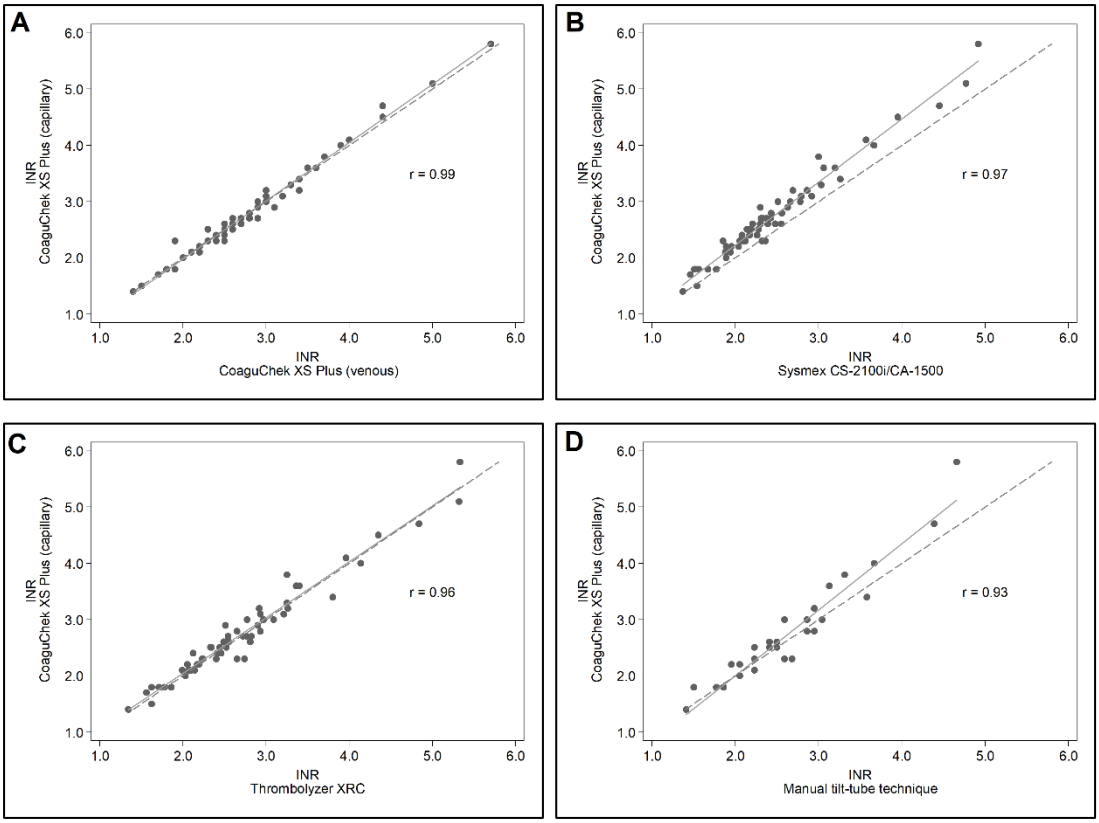


Figure 3.3 Correlation between the INR measured with the CoaguChek XS Plus on capillary blood and the other INR assays: the CoaguChek XS Plus on venous blood (A), the Sysmex CS-2100i/CA-1500 (B), the Thrombolyzer XRC (C) and the manual tilt-tube technique (D) (Adapted from Riva et al., 2017. Reproduced with permission, copyright licence no: 4643641187274)
In each graph, the dashed line represents the perfect correlation, while the continuous line represents the actual correlation between the two INR assays.

The CoaguChek XS Plus tended to overestimate the INR, compared to the other methodologies; however, the mean INR differences were small, as reported in Table 3.4. Specifically:

- The CoaguChek XS Plus performed on capillary blood showed a mean INR difference (or bias) of 0.002 (± 0.11), compared to the CoaguChek XS Plus performed on venous blood, meaning that, with 95% confidence, it might provide INR results up to 0.22 units lower or 0.22 units higher (limits of agreement);

- The CoaguChek XS Plus performed on capillary blood showed a mean bias of 0.28 (± 0.18) INR units, compared to the Sysmex CS-2100i/CA-1500 (95% limits of agreement, -0.07 to 0.63 INR units);
- The CoaguChek XS Plus performed on capillary blood showed a mean bias of 0.04 (± 0.18) INR units, compared to the Thrombolyzer XRC (95% limits of agreement, -0.31 to 0.39 INR units);
- The CoaguChek XS Plus performed on capillary blood showed a mean bias of 0.11 (± 0.30) INR units, compared to the manual tilt-tube technique (95% limits of agreement, -0.48 to 0.70 INR units).

The Bland-Altman plots representing the statistical agreement between the CoaguChek XS Plus and the other INR assays are reported in Figure 3.4.

Comparison	**Spearman's correlation coefficient r (p value)**	**INR difference, mean (± SD)**	**Magnitude of absolute difference, n (%)**		
			< 0.5	**0.5-1.0**	**> 1.0**
CoaguChek XS Plus (capillary) vs. CoaguChek XS Plus (venous)	0.9856 (< 0.001)	0.002 (0.11)	60 (100%)	0	0
CoaguChek XS Plus (capillary) vs. Sysmex CS-2100i/CA-1500	0.9699 (< 0.001)	0.28 (0.18)	53 (88.3%)	7 (11.7%)	0
CoaguChek XS Plus (capillary) vs. Thrombolyzer XRC	0.9646 (< 0.001)	0.04 (0.18)	58 (98.3%)	1 (1.7%)	0
CoaguChek XS Plus (capillary) vs. Manual tilt-tube technique	0.9283 (<0.001)	0.11 (0.30)	29 (96.7%)	0	1 (3.3%)

Table 3.4 Statistical agreement between the CoaguChek XS Plus on capillary blood and the other INR assays (Adapted from Riva et al., 2017. Reproduced with permission)

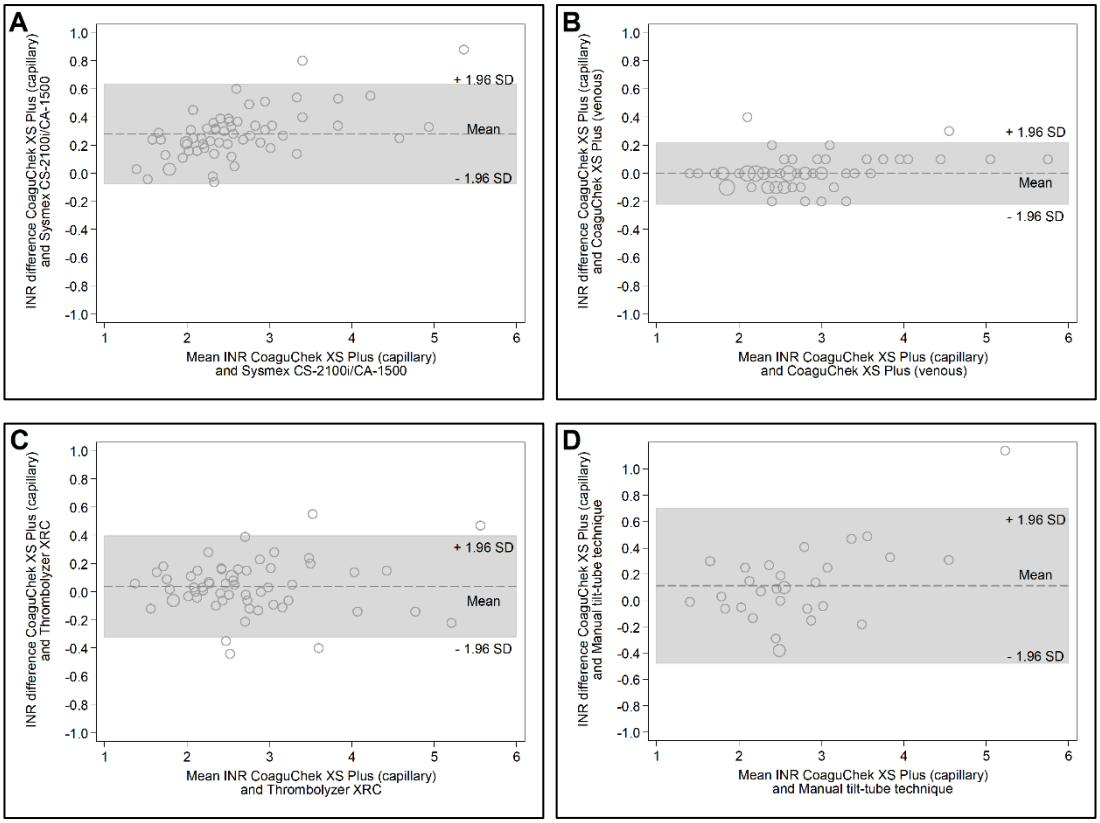


Figure 3.4 Bland-Altman plots representing the difference between the CoaguChek XS Plus on capillary blood and the other INR assays: the Sysmex CS-2100i/CA-1500 (A), the CoaguChek XS Plus on venous blood (B), the Thrombolyzer XRC (C), and the manual tilt-tube technique (D) (Adapted from Riva et al., 2017. Reproduced with permission, copyright licence no: 4643641187274)
The dashed line represents the mean difference, while the grey area represents the 95% limits of agreement

The clinical agreement between the CoaguChek XS Plus on capillary blood and the other INR assays was assessed by categorising the INR results as below/within/above the INR therapeutic range (Table 3.5).

The percentage of INR values that fell within the same clinical category was:

- 93.3% for the comparison between the CoaguChek XS Plus on capillary blood and the CoaguChek XS Plus on venous blood;
- 78.3% for the comparison between the CoaguChek XS Plus on capillary blood and the Sysmex CS-2100i/CA-1500;

- 93.2% for the comparison between the CoaguChek XS Plus on capillary blood and the Thrombolyzer XRC;
- 90.0% for the comparison between the CoaguChek XS Plus on capillary blood and the manual tilt-tube technique.

However, when there was a disagreement between the two INR assays, this would have never led to antagonistic VKA prescription (such as dose reduction vs. dose increase or vice versa).

		CoaguChek XS Plus (venous)		
		Below range	Within range	Above range
CoaguChek XS Plus (capillary)	Below range	9	0	0
	Within range	1	34	1
	Above range	0	2	13

		Sysmex CS-2100i/CA-1500		
		Below range	Within range	Above range
CoaguChek XS Plus (capillary)	Below range	9	0	0
	Within range	8	28	0
	Above range	0	5	10

		Thrombolyzer XRC		
		Below range	Within range	Above range
CoaguChek XS Plus (capillary)	Below range	9	0	0
	Within range	1	33	1
	Above range	0	2	13

		Manual tilt-tube technique		
		Below range	Within range	Above range
CoaguChek XS Plus (capillary)	Below range	4	0	0
	Within range	1	17	1
	Above range	0	1	6

Table 3.5 Clinical agreement between the CoaguChek XS Plus on capillary blood and the other INR assays
The light grey shadow means perfect clinical agreement, while the dark grey shadow means critical clinical disagreement (antagonistic VKA dose adjustment).

3.5 Discussion

This study evaluated the accuracy of four different INR assays compared with the thrombin generation, as global coagulation assay. All the four INR assays (the Sysmex CS-2100i/CA-1500, the CoaguChek XS Plus, the Thrombolyzer XRC, and the manual tilt-tube technique) used human recombinant thromboplastin. It was the first time that these INR assays were compared at the same time with the CAT.

A negative curvilinear correlation was found between the ETP measured on the CAT and the different INR results. The correlation was relatively strong, with correlation coefficients ranging from -0.80 to -0.75. This finding confirmed the negative correlation previously reported between the thrombin generation assay and the Sysmex CA-1500 (Gatt, van Veen, Bowyer, et al., 2008). Nevertheless, in the present study, a better correlation was actually found between the thrombin generation and the CoaguChek XS Plus on capillary blood ($r = -0.80$), than between the thrombin generation and the Sysmex CS-2100i/CA-1500 ($r = -0.75$).

The CoaguChek XS Plus on capillary blood showed a strong linear positive correlation with the other INR assays, with all correlation coefficients above 0.90. In particular, there were optimal correlation and statistical agreement between the CoaguChek XS Plus on capillary blood and the CoaguChek XS Plus on venous blood, with $r = 0.99$ and a mean (SD) INR difference of 0.002 (0.11) INR units. These results suggested that, if the POC INR is correctly performed, there is no difference between a capillary and a non-citrated venous blood sample. Similar results were reported for another POC coagulometer, the CoaguChek XS (Roche Diagnostics, Germany): 162 INR tests were compared and a mean bias of less than ± 0.02 INR units was reported between capillary and venous blood (Plesch & van den Besselaar, 2009).

In the current study, the CoaguChek XS Plus showed a strong correlation with a photo-optical coagulometer (the Sysmex CS-2100i/CA-1500, r = 0.97) and an electromechanical coagulometer (the Thrombolyzer XRC, r = 0.96). Previous studies have shown a good correlation between the CoaguChek XS Plus and either photo-optical coagulometers (Sysmex analysers) or electromechanical coagulometers (STAGO analyser) (Donaldson et al., 2010; Hur et al., 2013; Meneghelo et al., 2015), but they have never been simultaneously compared before.

From a clinical point of view, the disagreement found between the CoaguChek XS Plus on capillary blood and the other INR assays ranged from 6.7% to 21.7% of patients. This clinical disagreement could have resulted in different VKA management, but never in antagonistic behaviours (such as dose reduction vs. dose increase or vice versa). For instance, 13 of the 60 patients (21.7%) would have had clinically contrasting results between the CoaguChek XS Plus and the Sysmex CS-2100i/CA-1500, due to the fact that the CoaguChek XS Plus tended to overestimate the INR by 0.3 INR units on average. In eight of these patients the POC coagulometer gave a result within the therapeutic range, while it was below the therapeutic range using the laboratory INR (INR range using the POC coagulometer 2.00-2.30, INR range using the laboratory INR 1.85-1.95). In the other five patients the POC coagulometer gave a result that was above the therapeutic range, while it was within the therapeutic range using the laboratory INR (INR range using the POC 3.10-3.80, INR range using the laboratory INR 2.69-3.00). In these situations there is a risk of under-dosing the warfarin treatment; however, as demonstrated by a previous study reporting clinical disagreement in 26-29% of patients, the management actually differed only by minor interventions (Lawrie et al., 2012). In addition, since it is usually recommended to avoid frequent switch between POC INR and laboratory INR

measurements, this small difference in the INR values is unlikely to have negative interference with the management of patients on warfarin.

The results of this study have important implications both in the international literature and in the local Maltese context. Despite the recent development of the DOACs, the VKAs are still the ideal anticoagulant treatment in some circumstances, such as in patients with mechanical heart valves or severe renal failure. Due to their ease of use and their confirmed accuracy, portable coagulometers represent a possible alternative to the standard laboratory INR in these patients. In fact, the POC are less invasive and can provide immediate INR results and therefore immediate VKA dose adjustment.

This study is also particularly important in the Maltese context, where approximately 1% of the population is currently anticoagulated (Zammit et al., 2011). While previously the INR testing was centralized at Mater Dei Hospital (Msida, Malta) and was always performed as laboratory INR, in May 2014 there was a decentralisation of this system. Nowadays, the INR can be monitored at the Anticoagulation Clinics in the Health Centres spread around the Maltese islands, where the INR is measured using POC coagulometers and is immediately followed by VKA dose adjustment. Therefore, these results confirmed the accuracy of the CoaguChek XS Plus device compared to the traditional INR laboratory testing.

The main strengths of this study are, first, the fact that samples were tested with both the thrombin generation and four different INR assays. Furthermore, INR assays that use the same human recombinant thromboplastin were chosen, in order to keep constant this analytical variable. Finally, the fact that the POC INRs, the manual INRs and the CAT were each performed by a single person can reduce the variability in these results. However, some limitations of this study need to be acknowledged. First, the number of patients was relatively small, although not dissimilar from previous

studies (Donaldson et al., 2010; Plesch & van den Besselaar, 2009). This resulted in a small number of high INR values (>4.0), therefore preventing the possibility to conduct a sensitivity analysis by categories of INRs. Second, in this study all POC INRs were obtained by a trained doctor, but different accuracy might be obtained when the POC coagulometer is used by patients for self-testing. Third, although the CAT is not considered a validated assay for VKA monitoring, the INR results were compared against the CAT as gold standard, because it can better identify small differences in the accuracy of different assays, being a global coagulation assay.

3.6 Conclusion

The results of this study showed a very good correlation between the different INR assays and the thrombin generation. Although the POC INR is still not considered as a "gold standard" for VKA monitoring, the accuracy of the POC coagulometers was very good, when compared to the other INR methodologies. Therefore, the POC coagulometers can be considered as an accurate and valid alternative to the standard laboratory INR in patients on warfarin treatment, as long as monitoring is performed constantly with the POC devices.

Chapter 4 :

Biomarkers of Venous Thromboembolism

4.1 Introduction

The diagnosis of VTE can be challenging for clinicians. A timely diagnosis is important, since DVT can evolve into PE and PE is a potentially life-threatening disorder. The diagnostic algorithms for DVT and PE involve a composite of clinical pre-test probability, laboratory D-dimer and imaging test. D-dimer has high sensitivity and high negative predictive value, therefore it is used to rule out the suspicion of VTE. However, D-dimer has low specificity, giving false positive results in several conditions, such as infections, heart failure, trauma, cancer (Lippi et al., 2014). Therefore, specific imaging techniques are needed to confirm the diagnosis of VTE. The rationale behind this stepwise approach is to optimize the number of imaging tests and to avoid the unnecessary exposure of patients with low clinical pre-test probability and negative D-dimer to radiations or contrast agents.

Furthermore, different D-dimer assays showed differences in sensitivity and specificity, due to the instruments, calibrators, type of assays and the antibodies against the D-dimer domains (Bates, 2012). There are other coagulation tests which might identify a pro-thrombotic predisposition. For instance, the thrombin generation, an assay currently used only for research purposes, has been evaluated in the diagnostic VTE algorithm in a few studies (Chaireti et al., 2009; F. J. Haas et al., 2011; Wexels et al., 2017), but only small alterations of the thrombin generation parameters have been described in VTE patients. There are some reports that the concentration of soluble P-selectin could be increased in patients with acute DVT compared to normal controls (Ramacciotti et al., 2011; Rectenwald et al., 2005). Finally, the procoagulant phospholipid-dependent coagulation time has never been tested in this setting before. Considering that microparticles are markers of venous thrombosis and the great correlation between the procoagulant phospholipid-dependent coagulation time and

the flow cytometry (Exner et al., 2003), it was hypothesised that this assay could also be useful in patients with suspected VTE.

4.2 Aim

The aim of this study was to assess the accuracy and the relative importance of several laboratory tests as biomarkers to identify patients with acute VTE. Specifically, the accuracy of two different D-dimer assays, the thrombin generation assay (performed with the CAT), the procoagulant phospholipid-dependent clotting time, and the human soluble P-selectin was evaluated.

4.3 Methods

4.3.1 Study population

Between August 2015 and February 2016, a random group of samples analysed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) with a request for D-dimer assay and arriving from the Accident and Emergency Department, were further processed. As per the standard operating procedure, venous blood was collected into vacutainer coagulation tubes, which were sent to the Coagulation Laboratory using the pneumatic tube system. They were centrifuged and the plasma was immediately analysed with the D-dimer assay. For the purpose of this study, the remaining plasma underwent further centrifugation to obtain the PPP, as described in Chapter 2 (paragraph 2.1), and plasma aliquots were frozen at -80°C within two hours from phlebotomy.

All samples were collected before any anticoagulant treatment was administered and before a specific diagnostic imaging was performed. The decision regarding the need

to perform imaging tests was entirely at the discretion of the attending physicians. This collection of samples was performed as a service development initiative. In fact, because of the change of the D-dimer manufacturer in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta), there was the need to validate the new D-dimer assay by testing some samples with both D-dimer assays. Since further tests were performed entirely on residual plasma obtained from anonymised samples, the University of Malta Ethics Committee waived the need for ethical approval.

The samples were divided into three groups:

1) group 1: negative D-dimer without evidence of VTE;
2) group 2: positive D-dimer without evidence of VTE;
3) group 3: patients with VTE confirmed by computed tomography pulmonary angiography, ventilation/perfusion lung scan, or compression ultrasonography.

The planned sample size consisted of at least 25 patients per each group. However, due to some difficulties in the collection of samples with confirmed VTE, the time frame for processing these samples was extended. The first centrifugation step and the D-dimer assay were still performed within two hours. Afterwards the samples were kept at controlled room temperature until further processing, which was performed within 14 hours. There are some data that the mean percentage of change in D-dimer results after samples are stored at room temperature for a maximum of 24 hours is < 10% (Kemkes-Matthes et al., 2011; Zhao & Lv, 2013).

4.3.2 Tests performed

Samples were tested with two D-dimer assays: the Innovance® D-dimer (Siemens Healthcare Diagnostics Products GmbH, Germany) and the HemosIL® D-dimer HS (Instrumentation Laboratory, Italy), as described in paragraph 2.7. The manufacturers'

cut-off for a positive Innovance® D-Dimer was 500 ng/ml, while for the HemosIL® D-Dimer HS was 230 ng/ml. D-dimer was assayed either on fresh plasma or after one cycle of freeze-thaw. It was previously reported that freezing plasma does not alter the D-dimer results (B. Woodhams et al., 2001).

Frozen PPP aliquots were shipped to Sheffield (UK) in dry ice. The CAT was performed in duplicate (using one calibrator well and two test wells) at TF 1pM, and samples were run for 90 minutes (paragraph 2.13). These samples were tested at TF 1pM because it is more sensitive than TF 5pM to the concentrations of procoagulant factors, such as VIII, IX and XI (van Veen et al., 2008). Samples belonging to the three different groups were randomly placed in each plate. In addition, a random group of 14 samples was also tested at TF 5pM. All the samples were tested in February 2016.

The PPL and the sP-selectin were performed at Mater Dei Hospital (Msida, Malta). Both these tests were run into three batches, during the months of January, May and June 2017. The PPL is a factor Xa-based clotting time and was expressed as second and as ratio to a reference clotting time (paragraph 2.11). The concentration of sP-selectin was assayed in duplicate, using the ELISA technique, and expressed as ng/ml (paragraph 2.12). The PPL and the sP-selectin were also tested on plasma from 20 healthy control individuals, with the characteristics described in paragraph 2.13.4.

4.3.3 Statistical analysis

Data distribution was evaluated using the Wilk-Shapiro test. Continuous variables were reported as mean (SD) or as median (IQR), according to data distribution. Categorical variables were reported as counts and percentages, and compared using the Chi square or Fisher's exact tests, as appropriate. The Mann-Whitney U test was

used for the comparison of continuous variables of two groups. The Kruskal-Wallis test was used for the comparison of three groups, while for the post-hoc analysis the Dunn's test with Bonferroni correction of the p values was used. Unadjusted median differences with 95% confidence interval (CI) and median differences adjusted by age and sex were calculated. Results were also graphed as box-and-whisker plots: the line inside the box represents the median value; the edges of the box represent the first and third quartiles; the whiskers represent the lower and the upper adjacent values; the dots represent the outliers (where present).

The correlation between the D-dimers and the other tests was evaluated using the non-parametric Spearman's rank correlation test, in order to calculate the correlation coefficients (r). The correlation coefficient can range from -1 to 1, where absolute values equal to 1.0 indicate a perfect positive or negative correlation, while absolute values less than 0.50 indicate a poor correlation. The clinical agreement between the two D-dimers was calculated by categorising them as positive or negative according to their specific manufacturers' cut-off.

Receiver operating characteristics (ROC) curves were created to assess the accuracy of each test. They assess the c (concordance)-statistics, which is the concordance between predicted events and observed events. A value of c = 1.0 indicates perfect discriminative ability, while c = 0.5 indicates a predictive ability that is no better than chance (J. A. Hanley & McNeil, 1982).

In order to assess the relative importance of each test, as biomarker of acute VTE, a random forest algorithm was applied to the samples with the results of all nine variables available (Innovance® D-Dimer; HemosIL® D-Dimer HS; CAT parameters: lag time, endogenous thrombin potential, peak thrombin, time to peak, velocity index; procoagulant phospholipid-dependent clotting time; sP-selectin). The random forest

consists of 1000 decision trees, each created using three out of the nine possible variables, in random combinations and random order, to split the nodes of the trees. The out-of-bag error, an unbiased estimate of the prediction error of the random forest algorithm, was calculated. The random forest algorithm was applied in two models:

- Model 1: including all potential biomarkers of VTE (considering all the nine variables mentioned above);
- Model 2: excluding the D-dimers (considering only the other seven variables). Since D-dimers are well-known biomarkers of VTE, this analysis aimed to identify other possible biomarkers which might have been covered by the strong predictive value of the D-dimers.

The relative importance of each variable was evaluated through the following parameters:

- Mean minimal depth: the distance from the root of the decisional tree. It is a measure of the predictiveness of each variable, assuming that variables with high predictiveness split the nodes near the root of the trees;
- Accuracy decrease: the mean decrease of the prediction accuracy, after removing that variable from the random forest model. Variables with high importance show high accuracy decrease;
- Gini decrease: the mean decrease in the index of node impurity (Gini index of node impurity), which represents the variance of a node. Variables with high importance show high Gini decrease.

Finally, a decisional classification tree algorithm was applied to the biomarkers identified by the random forest method, in order to find the best cut-off (defined as the cut-off associated with the highest sensitivity and specificity).

For the analysis reported in this chapter, the following statistical programs were used: STATA/SE v.12 (StataCorp LP, College Station, TX, USA); SAS v.9.4 (SAS Institute, Cary, North Carolina, USA); and R (version 3.5.1) with the packages Party and randomForest (Hothorn et al., 2006; Liaw & Wiener, 2002; R Core Team, 2015).

4.4 Results

4.4.1 Study population

A total of 100 samples were collected: 32 negative D-dimer without VTE (group 1), 35 positive D-dimer without VTE (group 2) and 33 with confirmed VTE (group 3). The median age of the study population was 59.0 years (IQR 41.3-70.2); 47% were males. None of the patients without evidence of VTE at the time of D-dimer testing had a diagnosis of VTE in the subsequent three months. Of the 33 patients with confirmed VTE, the events were distributed as follows: PE (n=16), lower limb proximal DVT (n=11), isolated distal DVT (n=3), upper limb proximal DVT (n=1), superficial vein thrombosis of the great saphenous vein (n=2). There was no sex difference between patients with and without VTE (males 45.5% vs. 47.8%, p=0.83), while there was a statistically significant difference in the age, being VTE patients significantly older (69.5 vs. 52.0 years, p<0.001).

Results of each test were available for the following number of samples: Innovance® D-Dimer (n=82); HemosIL® D-Dimer HS (n=98); CAT parameters (n=92); PPL (n=98); sP-selectin (n=94). Some results were unavailable because of insufficient plasma aliquoted or technical issues while performing the tests.

4.4.2 D-dimers

A strong positive linear correlation was found between the Innovance® D-Dimer and the HemosIL® D-Dimer HS, with a correlation coefficient r = 0.97 ($p<0.001$), as reported in Figure 4.1. Values of D-dimer which were above the upper limits of detection of the assays (>4400 ng/ml for the Innovance® D-Dimer, >3610 ng/ml for the HemosIL® D-Dimer HS) were displayed as one unit above the limit.

The clinical agreement between the two D-dimers was evaluated by categorising the results as positive or negative according to their respective manufacturers' cut-off, which were 500 ng/ml for the Innovance® D-Dimer and 230 ng/ml for the HemosIL® D-Dimer HS. The clinical agreement was 93.8%, due to four patients with positive Innovance® D-Dimer but negative HemosIL® D-Dimer HS and one patient with positive HemosIL® D-Dimer HS but negative Innovance® D-Dimer. None of the VTE patients had a negative D-dimer.

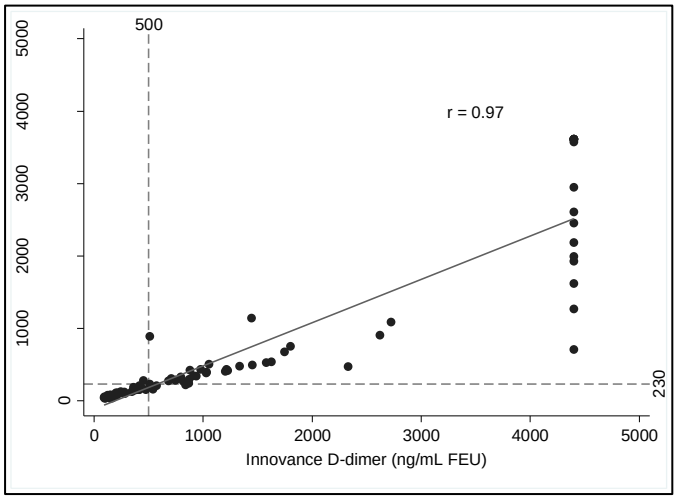


Figure 4.1 Correlation between the two D-dimers (Riva, Vella, et al., 2018) (Reproduced with permission, copyright licence no: 4642400505593)
The vertical dashed line represents the manufacturer's cut-off for the Innovance® D-Dimer. The horizontal dashed line represents the manufacturer's cut-off for the HemosIL® D-Dimer HS.

4.4.3 Calibrated Automated Thrombin Generation Assay

The comparison between patients with and without VTE (Table 4.1 and Figure 4.2) showed that the lag time (median value 5.42 vs. 4.5 min, $p<0.001$) and the time to peak (median value 8.59 vs. 7.33 min, $p=0.004$) were significantly prolonged in patients with VTE. The median differences, adjusted for age and sex, confirmed a significant increase of the lag time (0.84 min, 95% CI 0.30 to 1.38) and the time to peak (1.32 min, 95% CI 0.38 to 2.26), and evidenced a significant decrease of the velocity index (-32.6 nM/min, 95% CI -57.9 to -7.4).

For the comparison of the three groups of patients (negative D-dimer, positive D-dimer, confirmed VTE), classification into negative D-dimer and positive D-dimer was based on the results of the Innovance® D-Dimer (Table 4.2). Patients with positive D-dimer without evidence of VTE compared to patients with negative D-dimer without evidence of VTE showed increased peak (median 318.2 vs. 278.5 nM, $p=0.007$) and increased velocity index (median 121.2 vs. 91.6 nM/min, $p=0.003$).

	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)

Table 4.1 Differences between patients with and without VTE in the CAT results, PPL and sP-selectin (Riva, Vella, et al., 2018) (Reproduced with permission)
Results are reported as median (IQR). Median difference is reported between patients with VTE and patients without VTE, unadjusted and adjusted for age and sex.

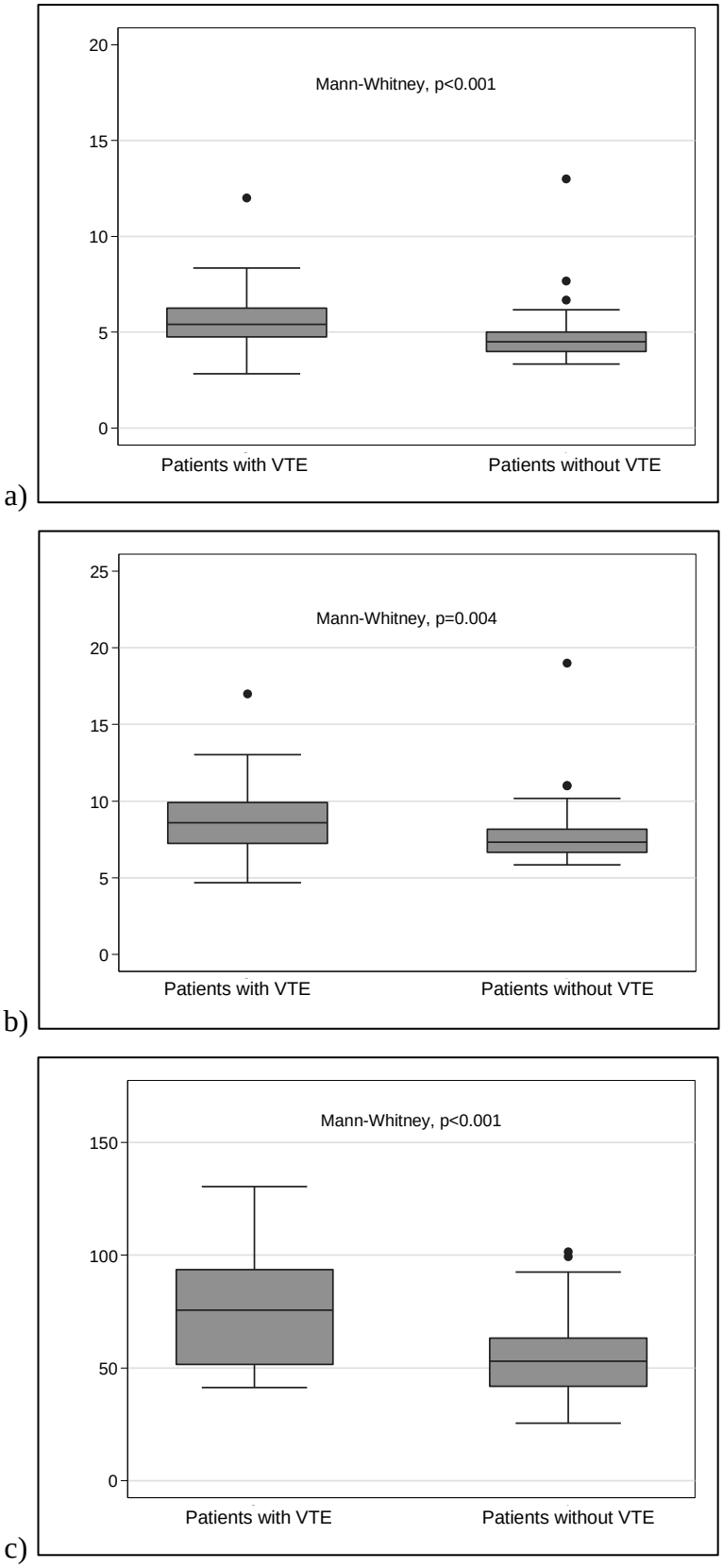


Figure 4.2 Biomarkers significantly raised in VTE patients: lag time (a), time to peak (b) and sP-selectin (c) (Riva, Vella, et al., 2018) (Reproduced with permission, copyright licence no: 4642400505593)

	Group 1: negative DD (Innovance D-dimer < 500 ng/ml) (n=32)	Group 2: positive DD (Innovance D-dimer ≥ 500 ng/ml) (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) #†

Table 4.2 Differences among the three groups of patients (negative D-dimer, positive D-dimer, confirmed VTE) in the CAT results, PPL and sP-selectin (Riva, Vella, et al., 2018) (Reproduced with permission)

Results are reported as median (IQR). After the Kruskal-Wallis test, significant differences were further analysed with the Dunn's test and reported as follows:

- for the comparison group 1 vs. group 2: ° $p \leq 0.01$
- for the comparison group 1 vs. group 3: * $p < 0.05$ # $p < 0.01$
- for the comparison group 2 vs. group 3: § $p < 0.05$ † $p < 0.01$

The comparison of the thrombin generation curves at TF 1pM and TF 5pM (n=14) showed that the 1pM concentration resulted in longer lag time and time to peak, and in reduced peak concentration, ETP and velocity index (Table 4.3). Therefore, it was confirmed that the 1pM concentration is more sensitive than the 5pM and has indeed the potential to amplify the differences between patients with and without VTE.

	TF 1pM	TF 5pM	p value
Lag time (min)	5.33 (4.67-5.5)	3.72 (3.67-4.33)	0.001
Peak thrombin concentration (nM)	307.5 (269.7-339.1)	367.3 (293.2-457.4)	0.001
Time to peak (min)	8.17 (7.67-8.67)	6.73 (6.44-7.11)	0.001
Endogenous thrombin potential (nM*min)	1866.5 (1598-2323.5)	2389 (1827-2932)	0.001
Velocity index (nM/min)	107 (94.8-109.6)	122.9 (100.4-148.4)	0.008

Table 4.3 Comparison between CAT results at TF 1pM and TF 5pM

4.4.4 Procoagulant phospholipid-dependent clotting time

The PPL was not significantly different in patients with VTE compared to patients without VTE (median value 35.7 vs. 35.5 min, p=0.73), as reported in Table 4.1. There was no significant difference also between patients with positive D-dimer compared to patients with negative D-dimer (median value 33.4 vs. 36.8 min, p=0.26), as reported in Table 4.2. The results did not change when the PPL was expressed as ratio. The median PPL clotting time of the 20 healthy controls was 42.8 (IQR 38.1-44.9) and it was significantly longer than patients with VTE (p=0.002) and patients without VTE, either with positive (p<0.001) or negative (p=0.004) D-dimer (Figure 4.3).

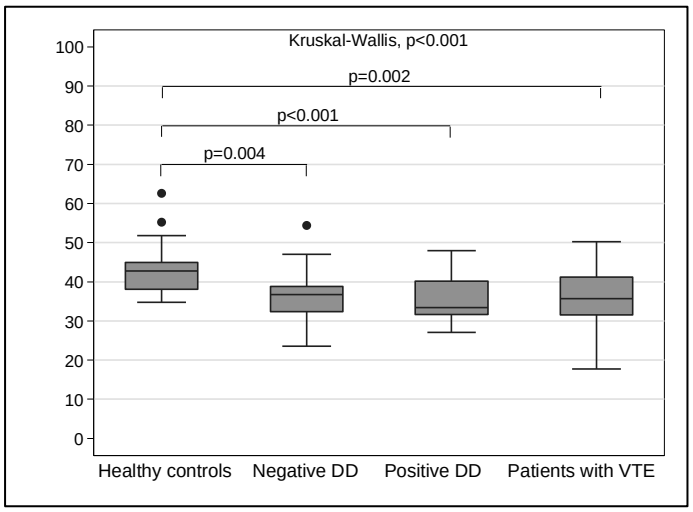


Figure 4.3 Procoagulant phospholipid-dependent clotting time in different subgroups of samples

4.4.5 Soluble P-selectin

VTE patients had significantly higher concentrations of sP-selectin compared to patients without evidence of VTE (median value 75.7 vs. 53.0 ng/ml, p<0.001). The adjusted median difference was 25.1 ng/ml, 95% CI 11.7 to 38.5 (Table 4.1, Figure 4.2). While VTE patients had significantly higher concentrations of sP-selectin compared to both patients with positive D-dimer and negative D-dimer, there was no difference between these last two groups (median value 55.5 vs. 47.9 ng/ml, p=0.33), as reported in Table 4.2.

Results of sP-selectin were available for 13 healthy controls. The median concentration was 40.3 (IQR 37.9-49.4) and it was significantly lower than patients with VTE (p<0.001) and patients without VTE with positive D-dimer (p=0.025) (Figure 4.4).

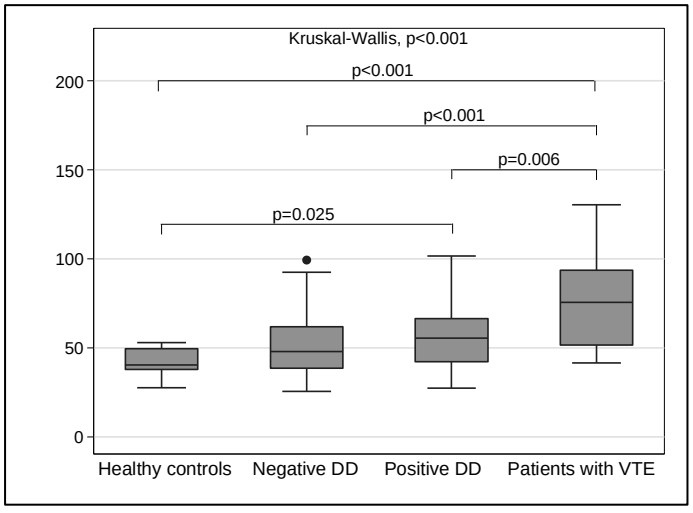


Figure 4.4 Concentration of sP-selectin in different subgroups of samples

4.4.6 Correlation between the two D-dimers and the other assays

The Innovance® D-Dimer showed a poor correlation with the other assays: lag time (r = 0.21, p=0.07), ETP (r = 0.19, p=0.11), time to peak (r = −0.03, p=0.81), peak thrombin concentration (r = 0.38, p=0.0008), velocity index (r = 0.44, p=0.0001), PPL (r = −0.18, p=0.11), sP-selectin (r = 0.38, p=0.0007).

Similarly, the HemosIL® D-Dimer HS showed a poor correlation with the other assays: lag time (r = 0.22, p=0.06), ETP (r = 0.19, p=0.11), time to peak (r = −0.02, p=0.84), peak thrombin concentration (r = 0.38, p=0.0009), velocity index (r = 0.44, p=0.0001), PPL (r = −0.18, p=0.12), sP-selectin (r = 0.36, p=0.002).

4.4.7 Receiver operating characteristic curves

In order to assess the accuracy of each test, their respective ROC curves were created (Figure 4.5). The ROC curves were not created for the D-dimers, because their accuracy might have been influenced by a selection bias, since approximately a third of the population had a confirmed VTE. The best predictive value for a confirmed VTE was reported for the sP-selectin, with an area under the ROC curve (AUC) of 0.77 (95% CI 0.66-0.87). It was followed by the lag time (AUC 0.73; 95% CI 0.61-0.85) and the time to peak (AUC 0.68; 95% CI 0.56-0.81). A poor predictive value was shown by the other tests: PPL (AUC 0.52; 95% CI 0.39-0.65); ETP (AUC 0.48; 95% CI 0.34-0.61); peak thrombin (AUC 0.45; 95% CI 0.31-0.59); velocity index (AUC 0.45; 95% CI 0.31-0.58).

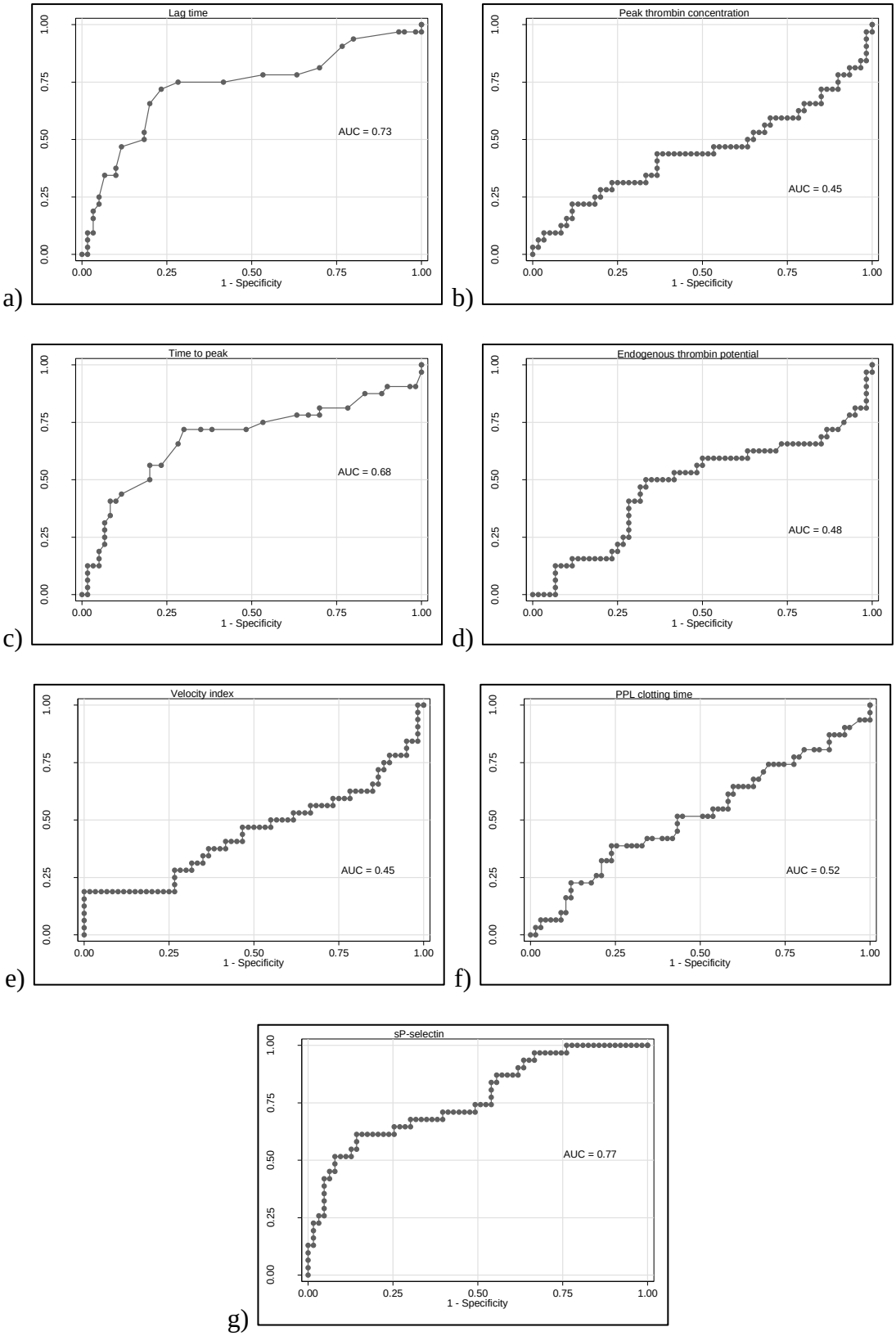


Figure 4.5 ROC curve of each biomarker of VTE: lag time (a), peak thrombin concentration (b), time to peak (c), endogenous thrombin potential (d), velocity index (e), PPL (f), sP-selectin (g) (Riva, Vella, et al., 2018) (Reproduced with permission, copyright licence no: 4642400505593)

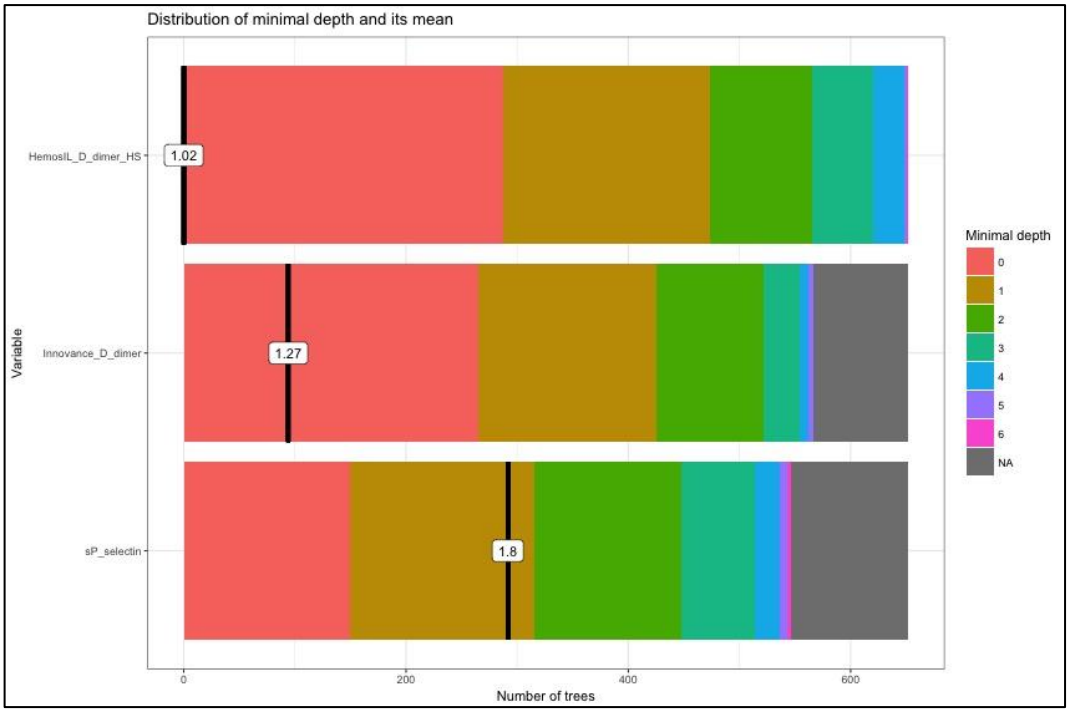
4.4.8 Relative importance of each biomarker in VTE prediction

For the analysis on the relative importance of each biomarker, only 69 samples which had the results of all nine variables available were considered. The random forest plot for the model 1 (including all potential biomarkers of VTE) showed that the two D-dimers were the biomarkers with the highest relative importance, followed by sP-selectin (Figure 4.6). The mean minimal depth was 1.02 for the HemosIL® D-Dimer HS, 1.27 for the Innovance® D-Dimer and 1.80 for the sP-selectin. The out-of-bag error was 10%.

The random forest plot for the model 2 (excluding the D-dimers) showed that sP-selectin was the biomarker with the highest relative importance, with a mean minimal depth 1.51. All the other variables had a mean minimal depth > 2.0 (Figure 4.7). Only variables that were chosen in at least half of the decisional trees of the random forest were represented in Figure 4.7. A classification tree was constructed based on the concentrations of sP-selectin and the best identified cut-off was 74.8 ng/ml, associated with 72.7% sensitivity and 78.2% specificity in this population.

Two logistic models were created to evaluate whether the addition of the sP-selectin concentrations might improve the predictive value of the D-dimers. D-dimers were dichotomised as positive/negative according to their manufacturers' cut-off, while for the sP-selectin the cut-off 74.8 ng/ml was used. In the model evaluating the Innovance® D-Dimer, the AUC increased from 0.534 for the D-dimer alone to 0.737 for the D-dimer in combination with sP-selectin (p=0.0006). In the model evaluating the HemosIL® D-Dimer HS, the AUC increased from 0.730 for the D-dimer alone to 0.825 for the D-dimer in combination with sP-selectin (p=0.0004).

a)



b)

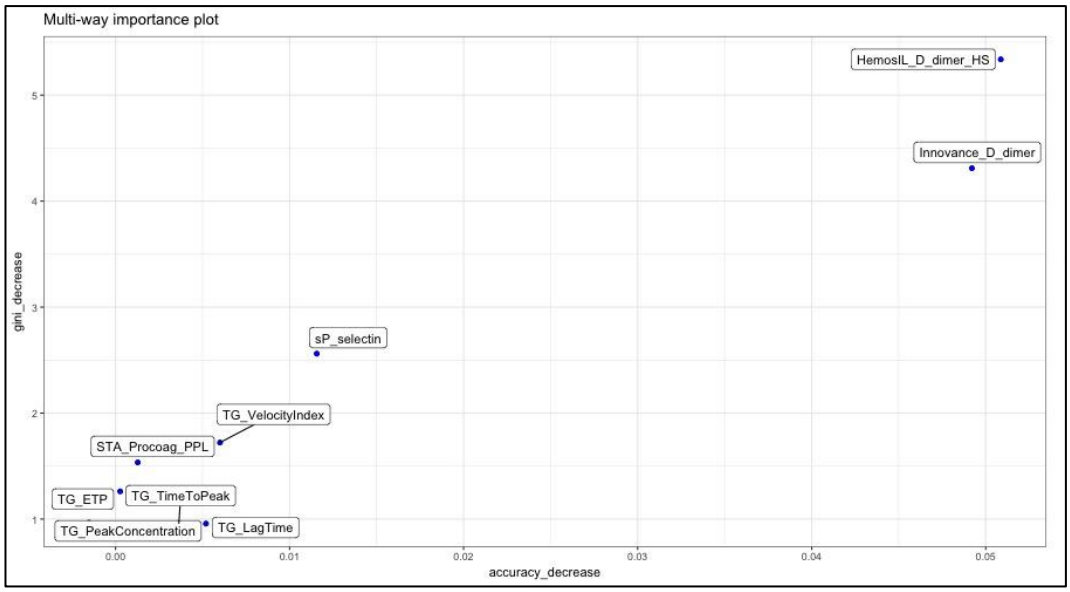
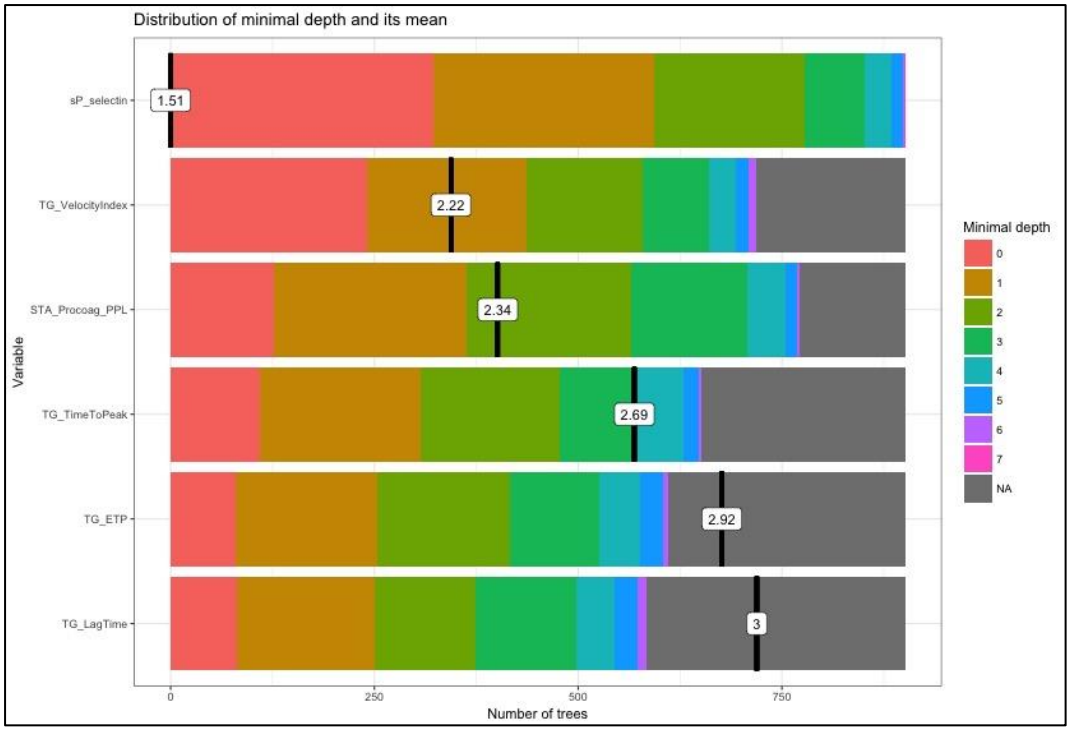


Figure 4.6 Random forest plot (a) and multi-way importance plot (b) for the model including all potential biomarkers of VTE (Riva, Vella, et al., 2018) (Reproduced with permission, copyright licence no: 4642400505593)

a)



b)

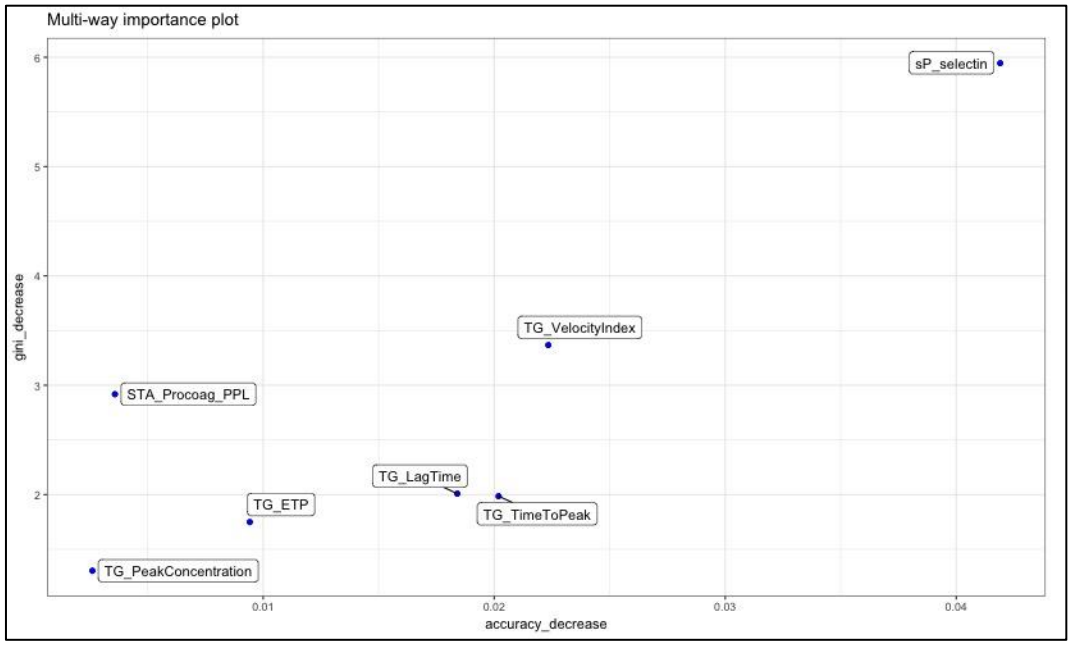


Figure 4.7 Random forest (a) and multi-way importance plot (b) for the model excluding the D-dimers (Riva, Vella, et al., 2018) (Reproduced with permission, copyright licence no: 4642400505593)

4.4.9 Sensitivity analyses in samples with confirmed VTE

No significant differences were observed when the median values of the CAT parameters, PPL and sP-selectin were compared according to the site of thrombosis (16 with PE, 12 with DVT and five with other thrombosis): lag time (p=0.93), peak thrombin (p=0.44), time to peak (p=0.77), ETP (p=0.60), velocity index (p=0.46), PPL (p=0.94), sP-selectin (p=0.35).

When the biomarkers were analysed according to the time to storage (eight samples within 2 hours, nine samples range 2-5 hours, nine samples range 5-10 hours, and seven samples more than 10 hours), some differences were noticed. There was a trend towards longer lag time and time to peak, and reduced peak thrombin concentration and velocity index, while the ETP was not affected by the increasing freezing time (Figure 4.8). No difference was observed for the PPL and the sP-selectin.

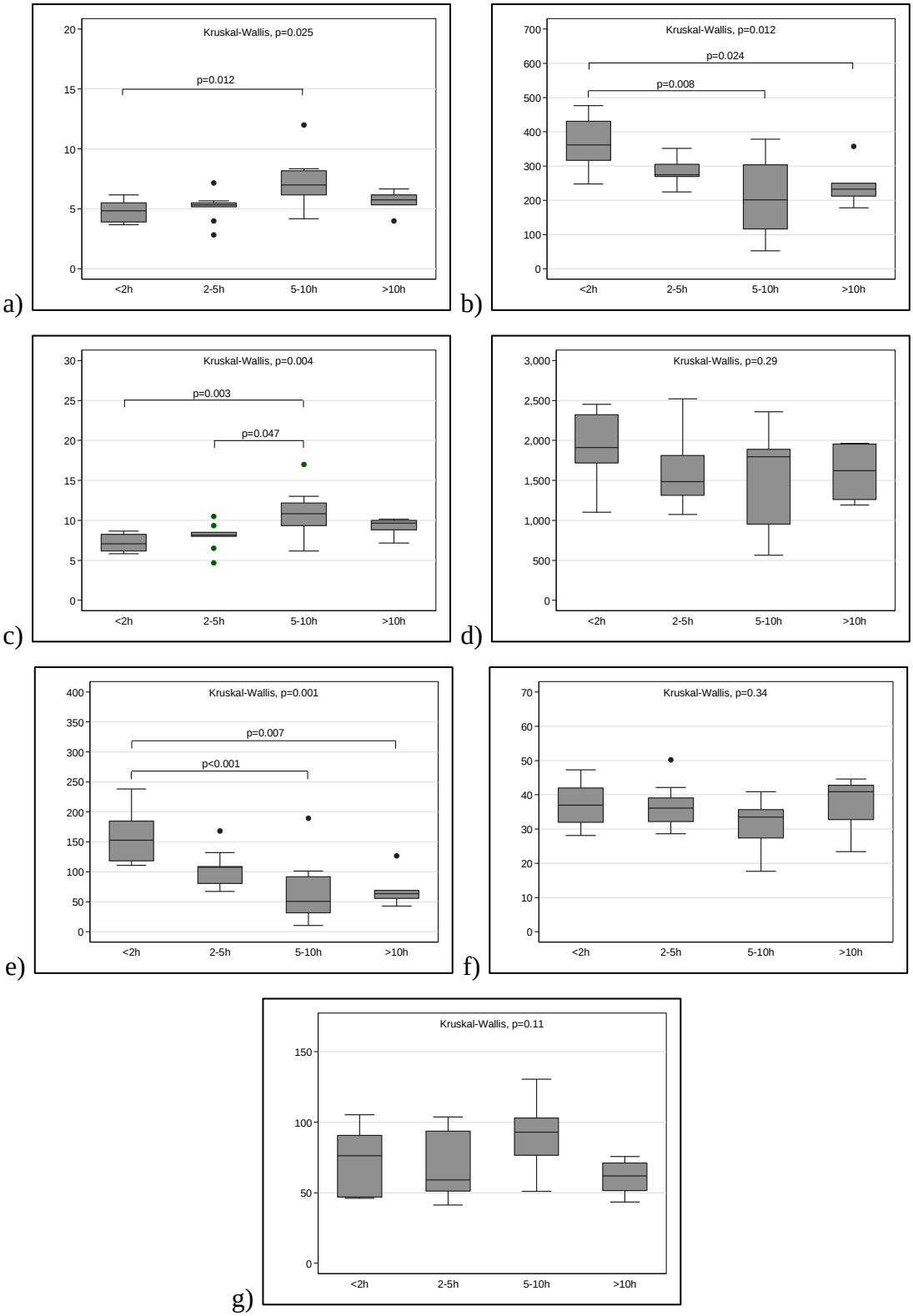


Figure 4.8 Results of the different tests in VTE patients, analysed by the time to storage: lag time (a), peak thrombin (b), time to peak (c), endogenous thrombin potential (d), velocity index (e), PPL (f), sP-selectin (g)

4.5 Discussion

This chapter assessed the accuracy of several laboratory assays (two D-dimers, the CAT, the PPL and the sP-selectin) as potential biomarkers of acute VTE. Among these tests, the two D-dimer assays were confirmed as the principal biomarkers of VTE, while the sP-selectin showed a good predictive value and improved the diagnostic accuracy of the D-dimers when used in combination.

It has been reported in the literature that different D-dimer assays showed some differences in sensitivity and specificity, with the latex-enhanced D-dimers having the highest sensitivity (93-95%), but low specificity (50-53%) for VTE diagnosis (Bates, 2012; Di Nisio et al., 2007). The present study evaluated two latex-enhanced turbidimetric immunoassays, the Innovance® D-Dimer and the HemosIL® D-Dimer HS, and found a strong positive correlation (r=0.97) and good clinical agreement (93.8%) between them.

Among the CAT results, the lag time and the time to peak were significantly prolonged in patients with confirmed VTE. This finding was surprising because in hypercoagulable conditions usually they are shortened, since the lag time represents the time up to the initiation of the generation of thrombin and the time to peak represents the time required to reach the peak concentration of thrombin (Tripodi, 2016). It was initially hypothesised that they might have been a result of the prolonged time to storage of VTE samples, but previous studies involving patients with suspected VTE consistently reported delayed and prolonged thrombin generation in vitro (Chaireti et al., 2009; F. J. Haas et al., 2011; Wexels et al., 2017). It was therefore hypothesised that this reduced thrombin generation potential in plasma *ex vivo* can be a result of the increased thrombin generation *in vivo* with coagulation factors consumption (Chaireti et al., 2009; Wexels et al., 2017). Conversely, discordant results

were available on the ETP, which represents the total amount of thrombin generated. Some studies reported an increased ETP in acute VTE (F. J. Haas et al., 2011; Wexels et al., 2017) while others showed no statistically significant increase (Chaireti et al., 2009; B. J. Hunt et al., 2018), similarly to the current study. However, considering the CAT results overall, it emerged that this assay had a poor diagnostic predictive value for VTE, compared to the other biomarkers.

This study evaluated, for the first time, the accuracy of the PPL in VTE diagnosis. The PPL measures a factor Xa-based clotting time which depends on the presence of procoagulant phospholipids in the patient plasma (Exner et al., 2003; Patil et al., 2016). There are studies showing that procoagulant microparticles are increased in acute VTE, but it is uncertain whether they might be the cause or the consequence of the venous thrombosis (Lacroix, Dubois, et al., 2013). A significant correlation was reported between the PPL and the flow cytometry (Patil et al., 2016), which is the assay usually utilised to detect the presence of microparticles, although it cannot evaluate their functional activity. Conversely, if there are procoagulant microparticles in patient plasma, the PPL is shortened (Exner et al., 2003; Patil et al., 2016). Nevertheless, the results of the present study did not show any significant difference in the PPL between patients with and without VTE.

Finally, the role of sP-selectin was investigated as potential biomarker of acute VTE. P-selectin is an adhesion molecule expressed on the surface of several cells, such as activated platelets and endothelial cells, but can also be released into the blood in soluble form (Pabinger & Ay, 2009). It has been recently reported that P-selectin can be involved in haemostasis, by mediating platelet rolling, generating procoagulant microparticles and enhancing fibrin deposition (Ay et al., 2007; Ay et al., 2008; Cambien & Wagner, 2004). It was found that sP-selectin had a good predictive ability

for VTE and it was the most relevant biomarker, after the D-dimers. A cut-off of 74.8 ng/ml was associated with high specificity (78.2%) and sensitivity (72.7%). There were three previous studies that evaluated the role of sP-selectin in similar contexts. Rectenwald et al. (2005) analysed D-dimer, sP-selectin and microparticles in 30 healthy controls, 22 patients with DVT and 21 subjects with symptoms of DVT but DVT excluded at ultrasound. Mean D-dimer was 7.57 mg/l in patients with DVT, 3.19 mg/l in symptomatic patients without DVT, and 1.53 mg/l in the healthy controls. Soluble P-selectin concentrations were 0.98 (±2.03) ng/mg of total protein in patients with DVT, 0.55 (±0.08) in symptomatic patients without DVT and 0.34 (±0.05) in healthy controls. The authors also identified a cut-off of sP-selectin (0.68 ng/mg of total protein) that was associated with high specificity (81%) and sensitivity (68%) (Rectenwald et al., 2005). Ramacciotti et al. (2011) analysed D-dimer, sP-selectin, microparticles and C-reactive protein in 62 patients with DVT and 116 patients with leg pain but no evidence of DVT at ultrasound. Mean D-dimer was 5.8 mg/l in DVT patients vs. 2.1 mg/l in patients without DVT ($p<0.0001$). Similarly, mean sP-selectin was 87.3 ng/ml in DVT patients vs. 53.4 ng/ml in patients without DVT ($p<0.0001$). Since the D-dimer cut-off ≤ 0.5 mg/l was associated with high sensitivity (98%) but low specificity (29%), they identified a sP-selectin cut-off ≥ 90 ng/ml associated with high specificity (96%) but low sensitivity (28%). However, when these two variables were combined together, specificity was 81% but sensitivity only 43% (Ramacciotti et al., 2011). A recent study by Torres et al. (2017) evaluated different soluble molecules, including sP-selectin, in patients with VTE after the acute phase. Although the sample size was small (15 VTE patients and 20 healthy controls), there were non-significant higher sP-selectin levels in VTE patients, with median concentrations being 90 ng/ml vs. 72 ng/ml ($p=0.099$) (Torres et al., 2017).

The main strengths of this study are the fact that VTE diagnosis was objectively confirmed with the appropriate imaging tests and that different possible biomarkers of VTE were evaluated at the same time. The main limitations of this study were, first, that the sensitivity and specificity of the D-dimers could not be calculated, because they would have required a consecutive enrolment of patients, while the randomly chosen samples included a third of patients with confirmed VTE. Second, the assay results were not available for all samples, due to technical difficulties or insufficient plasma. Third, this sample size was relatively small but not dissimilar to previous studies assessing the role of sP-selectin as biomarker of VTE (Rectenwald et al., 2005; Torres et al., 2017). Fourth, being a laboratory collection of anonymised samples, the only available demographic variables were age and sex and the Wells score (Wells et al., 2003; Wells et al., 2000) or other clinical prediction rules for VTE could not be applied retrospectively. Finally, there were some differences in the time to storage between samples with and without VTE. While there is some data that D-dimer results are stable up to 24 hours (Kemkes-Matthes et al., 2011; Zhao & Lv, 2013), data on the stability for the other assays is scarce.

4.6 Conclusion

The results of this study confirmed that D-dimer is the principal biomarker of VTE and suggested that sP-selectin might be considered an emergent biomarker, although the optimal cut-off still needs to be identified and the assay is hampered by higher costs and more technical difficulties. The CAT parameters showed only a limited relative importance, while it seemed that the PPL had no role in VTE diagnosis. However, these results will need further confirmation in larger management studies.

Chapter 5 :

Anticoagulant Pattern on Global Coagulation Assays

5.1 Introduction

The thrombin generation assay and the TEG are known as global coagulation assays, since they allow the evaluation of the different phases of coagulation (initiation, propagation and termination) and, with some modifications, they can also evaluate fibrinolysis. The thrombin generation measures the amount of thrombin generated over time (van Veen et al., 2008), while the TEG measures the rate of clot formation and its elastic strength (Whiting & DiNardo, 2014). While the TEG has been included in algorithms to guide transfusion after cardiac surgery or liver transplantation (Whiting & DiNardo, 2014), the thrombin generation assay is still mainly used as a research test.

Some studies have evaluated whether the thrombin generation and the TEG can be affected by ongoing anticoagulant treatment (Bowry et al., 2014; Coppell et al., 2006; Dale et al., 2013; Dias et al., 2015; Gatt, van Veen, Bowyer, et al., 2008; Solbeck et al., 2016; Wong et al., 2013), with contrasting results about the sensitivity of these assays. Differences can be due to the wide inter-subject variability, heterogeneity in the tested anticoagulant concentrations, and difficulties in the standardisation of these tests. However, the evaluation of clot strength is particularly important in patients needing surgery or interventional procedures while on anticoagulant therapy, in order to minimise the bleeding and thrombotic risks in the peri-procedural period. Clot strength, in fact, is one of the parameters that could affect bleeding rates during surgery (Thachil et al., 2008).

5.2 Aims

The aims of this study were:

1) To evaluate the effect of several oral and parenteral anticoagulants on two global coagulation assays: the CAT and the TEG;
2) To evaluate the clot strength and the fibrin clot resistance to fibrinolysis using the TEG with TPA in the presence of several oral and parenteral anticoagulants.

5.3 Methods

5.3.1 Samples analysed

Aliquots of warfarinised plasma or citrated PPP spiked with different anticoagulants were prepared as described in Chapter 2 (paragraph 2.3). Aliquots were stored at -80° C until the analysis. The following anticoagulated samples were analysed with the CAT and the native TEG:

- Warfarinised plasma pools:
    - INR 2.22, INR 3.24, INR 4.11;
- Direct factor Xa inhibitors:
    - Apixaban 4 ng/ml, 42 ng/ml, 89 ng/ml, 128 ng/ml, 179 ng/ml, 266 ng/ml;
    - Edoxaban 0 ng/ml, 15 ng/ml, 51 ng/ml, 85 ng/ml, 113 ng/ml, 188 ng/ml;
    - Rivaroxaban 22 ng/ml, 55 ng/ml, 118 ng/ml, 174 ng/ml, 231 ng/ml, 339 ng/ml;
- Direct thrombin inhibitors:
    - Argatroban 0.25 μg/ml, 0.53 μg/ml, 3.10 μg/ml, 5.84 μg/ml;
    - Bivalirudin 5.9 μg/ml, 13.8 μg/ml, 31.0 μg/ml;
    - Dabigatran 0 ng/ml, 44 ng/ml, 92 ng/ml, 148 ng/ml, 176 ng/ml, 276 ng/ml;

- Indirect factor Xa inhibitors:
    - Danaparoid 0.33 U/ml, 0.78 U/ml, 1.93 U/ml;
    - Enoxaparin 0.35 U/ml, 1.06 U/ml, 1.95 U/ml;
    - Fondaparinux 0.64 μg/ml, 1.64 μg/ml, 2.24 μg/ml.

For the TEG with TPA the following concentrations were chosen, based on the results of the native TEG:

- Warfarinised plasma:
    - INR 2.22, INR 3.24, INR 4.11;
- Direct factor Xa inhibitors:
    - Apixaban 89 ng/ml and 128 ng/ml;
    - Edoxaban 51 ng/ml and 85 ng/ml;
    - Rivaroxaban 118 ng/ml and 174 ng/ml;
- Direct thrombin inhibitors:
    - Argatroban 0.53 μg/ml and 3.10 μg/ml;
    - Bivalirudin 5.9 μg/ml and 13.8 μg/ml;
    - Dabigatran 92 ng/ml and 148 ng/ml;
- Indirect factor Xa inhibitors:
    - Danaparoid 0.33 U/ml;
    - Enoxaparin 0.35 U/ml;
    - Fondaparinux 0.64 μg/ml.

5.3.2 Tests performed

All the samples were prepared and analysed between August 2018 and May 2019. The following tests were performed: APTT, PT/INR, CAT and TEG (native and with

TPA). Details of each test are reported in Chapter 2 (paragraphs 2.4-2.5 and 2.13-2.14).

APTT and PT/INR were performed on the day of spiking and they were analysed once. Frozen samples were shipped to Sheffield (UK) in dry ice and the CAT was performed, using two calibrator wells and two test wells. The CAT was performed in duplicate using two wells, at TF 5pM and TF 1pM. Anticoagulated samples of the same pharmacodynamic category (e.g. warfarinised plasma, direct factor Xa inhibitors, direct thrombin inhibitors, indirect factor Xa inhibitors) were run in the same plate. The same unspiked normal PPP was run in every plate as control. Samples were run for 60-180 minutes, as appropriate.

The TEG was performed at Mater Dei Hospital (Msida, Malta) on citrated PPP. The native TEG used citrated PPP 330 µl and $CaCl_2$ 0.2M 30 µl. The native TEG was performed in duplicate, using the two channels. Since only two samples can be run on the TEG at the same time, different concentrations of the same anticoagulant drug were run on the same day, however the other channel was tested on a different day. Samples were run until they reached the MA.

The TEG with TPA was performed to evaluate fibrinolysis (expressed by LY30 and LY60). Since fibrinolysis is immediately stimulated in the presence of TPA, this TEG can measure also the final clot strength under these conditions (expressed by the MA) (Dargaud et al., 2011). For these experiments, citrated PPP 325 µl was mixed with $CaCl_2$ 0.2M 30 µl and TPA 0.0375 mg/ml 5 µl (corresponding to a final TPA plasma concentration of 0.52 µg/ml). Given the high variability of the fibrinolytic parameters, the TEG with TPA was performed 10 times, on different days, for each anticoagulant concentration, alternating the two channels. Every day a first run of native TEG and

TEG with TPA on normal unspiked PPP was performed as baseline. Samples were run until they reached the LY60.

5.3.3 Statistical analysis

Results were expressed as mean (SD) or as median (IQR). Normality was evaluated using the Wilk-Shapiro test. Correlation between the anticoagulant concentrations and the different tests (APTT, PT, CAT parameters, TEG parameters) was evaluated using the non-parametric Spearman's rank correlation test. Results of the TEG performed with and without TPA were compared using the paired samples t-test for normally distributed variables or the non-parametric Wilcoxon matched-pairs signed-ranks test for not-normally distributed variables. The procedure to establish the normal ranges for the native TEG and the TEG with TPA has been described in Chapter 2 (paragraph 2.14.4).

In order to reduce the inter-assay variability, results were also expressed as ratio. CAT results were expressed as ratio to the same unspiked normal PPP that was run in every plate as control. Since a TEG run consists of only two samples and it was unfeasible to always run a normal PPP as control, native TEG results were expressed as ratio to the mean value of the same unspiked normal PPP that was run 10 times on different days. TEG with TPA results were expressed as ratio to the same unspiked normal PPP with TPA that was performed as baseline run on the same day. A MA ratio for each anticoagulant concentration, which consisted of the ratio between the mean MA of the TEG with TPA and the mean MA of the native TEG, was also calculated.

5.4 Results

5.4.1 APTT and PT/INR assays

The direct factor Xa inhibitors had only a modest effect on the APTT, with most of the values within the normal ranges. The highest concentrations of rivaroxaban (231 and 339 ng/ml) showed values outside the upper limit of normal for APTT, while the highest concentration of apixaban (266 ng/ml) was still within the normal ranges. Edoxaban showed a pattern similar to rivaroxaban, although the highest tested concentration was relatively lower (188 ng/ml). Their effect on the PT was more pronounced. Rivaroxaban at concentration 55 ng/ml and edoxaban at concentration 51 ng/ml were already very close to the upper limit of normal for PT, while the effect of apixaban was less pronounced and it was close to the upper limit of normal at concentration 89 ng/ml.

The direct thrombin inhibitors had a more pronounced effect on the APTT, although the effect of dabigatran on the PT was very similar to that of apixaban. The two lowest concentrations of argatroban (0.25 μg/ml and 0.53 μg/ml), corresponding to the ones used for patients with HIT and VTE, had an effect similar to dabigatran. Conversely, bivalirudin and the highest concentrations of argatroban (3.10 μg/ml and 5.84 μg/ml), corresponding to the ones used for patients with HIT undergoing PCI, gave a considerable prolongation of both APTT and PT.

The indirect factor Xa inhibitors showed no prolongation of the PT. Fondaparinux showed also no effect on the APTT. Enoxaparin prolonged the APTT at therapeutic (1.06 U/ml) and supratherapeutic (1.95 U/ml) concentrations. The effect of danaparoid was seen already at prophylactic concentration (0.33 U/ml).

The effect of each anticoagulant on APTT and PT/INR assays is reported in Table 5.1. The linear correlation between different concentrations of the DOAC and the APTT and the PT are represented in Figures 5.1-5.2, respectively. As reported in Table 5.2, there was a very strong correlation between the anticoagulant concentrations and the results of the APTT and the PT, expressed as seconds. Most of the rho coefficients were 1.0, the weakest being the correlation between apixaban and the APTT ($r = 0.83$).

		APTT		PT	
Normal ranges		*24.8-35.0 sec*	*Ratio 0.89-1.16*	*9.2-11.8 sec*	*INR 0.84-1.04*
Warfarinised plasma					
INR 2		36.8	1.23	23.3	2.22
INR 3		43.5	1.46	34.1	3.24
INR 4		44.3	1.49	43.4	4.11
Direct factor Xa inhibitors					
Apixaban	4 ng/ml	28.0	0.94	10.6	1.02
	42 ng/ml	29.2	0.98	11.1	1.07
	89 ng/ml	29.8	1.00	11.7	1.12
	128 ng/ml	30.6	1.03	12.3	1.18
	179 ng/ml	29.9	1.00	12.6	1.21
	266 ng/ml	30.4	1.02	14.4	1.38
Edoxaban	0 ng/ml	28.3	0.95	10.4	1.00
	15 ng/ml	29.4	0.99	11.2	1.08
	51 ng/ml	30.1	1.01	11.8	1.10
	85 ng/ml	31.3	1.05	12.4	1.19
	113 ng/ml	32.8	1.10	13.0	1.25
	188 ng/ml	34.7	1.16	14.3	1.37
Rivaroxaban	22 ng/ml	28.7	0.96	10.6	1.02
	55 ng/ml	30.9	1.04	11.6	1.11
	118 ng/ml	32.0	1.07	13.0	1.25
	174 ng/ml	34.4	1.15	14.9	1.43
	231 ng/ml	35.4	1.19	16.2	1.55
	339 ng/ml	36.9	1.24	19.2	1.83
Direct thrombin inhibitors					
Argatroban	0.25 µg/ml	41.7	1.40	11.9	1.14
	0.53 µg/ml	48.2	1.62	13.0	1.25
	3.10 µg/ml	89.7	3.01	30.2	2.87
	5.84 µg/ml	129.8	4.36	51.3	4.85
Bivalirudin	5.9 µg/ml	107.4	3.60	37.4	3.55
	13.8 µg/ml	150.5	5.05	71.1	6.71
	31.0 µg/ml	202.0	6.78	121.7	11.42
Dabigatran	0 ng/ml	33.4	1.12	10.6	1.02
	44 ng/ml	40.0	1.34	11.4	1.10
	92 ng/ml	46.4	1.56	12.2	1.17
	148 ng/ml	50.7	1.70	12.6	1.21
	176 ng/ml	53.9	1.81	13.2	1.27
	276 ng/ml	62.2	2.09	14.3	1.38
Indirect factor Xa inhibitors					
Danaparoid	0.33 U/ml	36.5	1.22	10.8	1.04
	0.78 U/ml	45.9	1.54	11.1	1.07
	1.93 U/ml	81.1	2.72	11.5	1.10
Enoxaparin	0.35 U/ml	33.2	1.11	10.7	1.03
	1.06 U/ml	46.0	1.54	10.9	1.05
	1.95 U/ml	70.2	2.36	11.2	1.08
Fondaparinux	0.64 µg/ml	31.6	1.06	10.7	1.03
	1.64 µg/ml	32.0	1.07	11.1	1.07
	2.24 µg/ml	33.0	1.11	11.4	1.10

Table 5.1 APTT and PT/INR results with different anticoagulant concentrations
Out of range values have been reported in red colour.

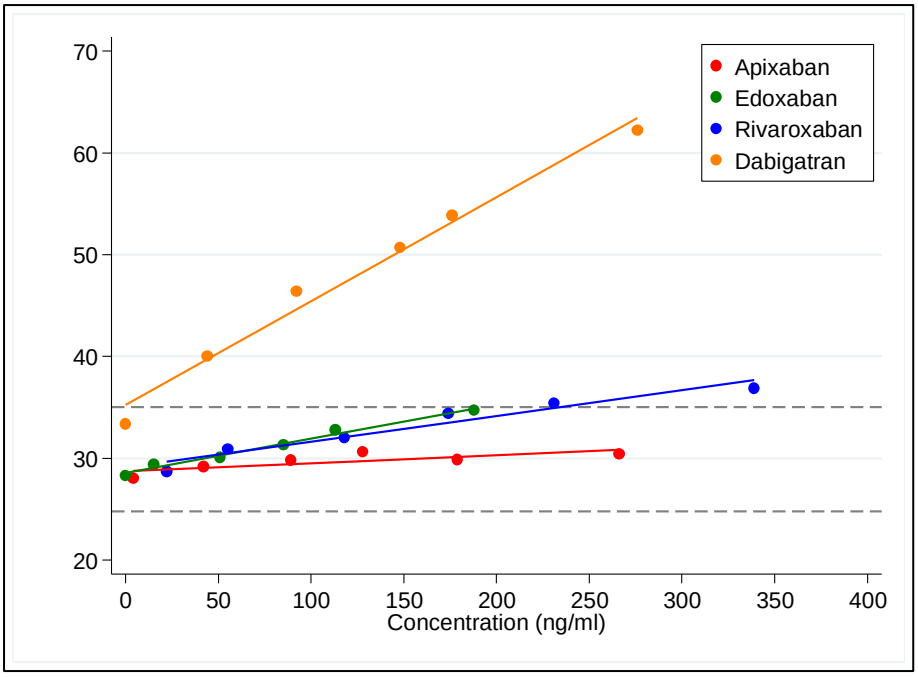


Figure 5.1 Correlation between different concentrations of the DOACs and the APTT
The horizontal dashed grey lines represent the normal ranges of the APTT.

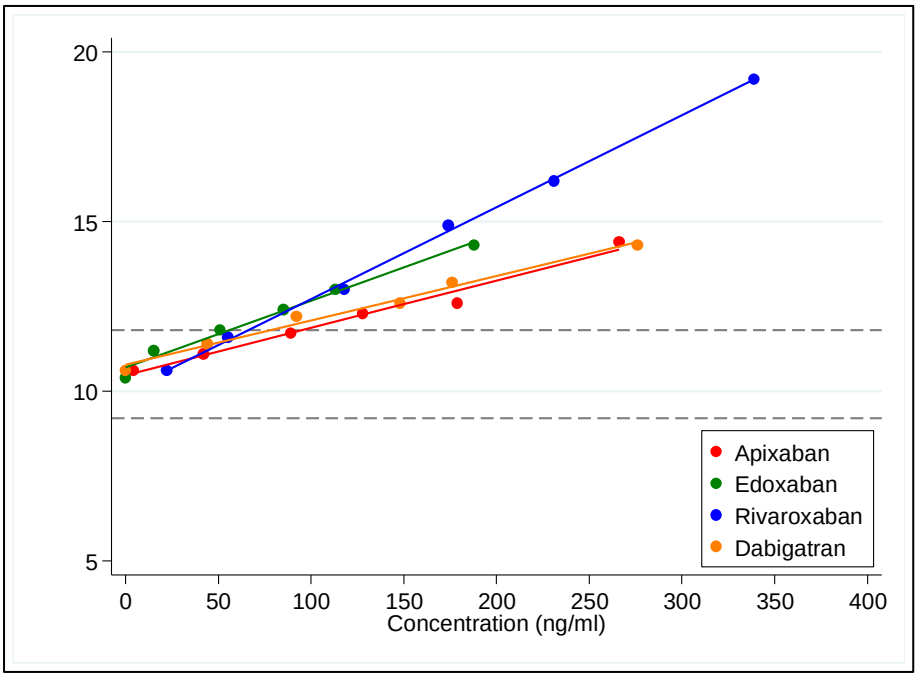


Figure 5.2 Correlation between different concentrations of the DOACs and the PT
The horizontal dashed grey lines represent the normal ranges of the PT.

Anticoagulant	N. of tests	APTT: correlation coefficient (p value)	PT: correlation coefficient (p value)
Warfarin*	3	1.00 (<0.001)	1.00 (<0.001)
Direct factor Xa inhibitors			
Apixaban	6	0.83 (0.042)	1.00 (<0.001)
Edoxaban	6	1.00 (<0.001)	1.00 (<0.001)
Rivaroxaban	6	1.00 (<0.001)	1.00 (<0.001)
Direct thrombin inhibitors			
Argatroban	4	1.00 (<0.001)	1.00 (<0.001)
Bivalirudin	3	1.00 (<0.001)	1.00 (<0.001)
Dabigatran	6	1.00 (<0.001)	1.00 (<0.001)
Indirect factor Xa inhibitors			
Danaparoid	3	1.00 (<0.001)	1.00 (<0.001)
Enoxaparin	3	1.00 (<0.001)	1.00 (<0.001)
Fondaparinux	3	1.00 (<0.001)	1.00 (<0.001)

Table 5.2 Correlation between anticoagulant concentrations and the APTT and PT assays
* For the warfarinised plasma, the INR values have been considered instead of the concentrations.

5.4.2 Calibrated Automated Thrombin Generation Assay

5.4.2.1 Normal ranges of the CAT

The local normal ranges for the CAT on citrated PPP were previously established by testing 37 healthy volunteers at TF 5pM, of whom 28 were also tested at TF 1pM. They are reported in Table 5.3.

	TF 5pM		TF 1pM	
	Lower limit	**Upper limit**	**Lower limit**	**Upper limit**
Lag time (min)	2.2	4.5	5.0	10.3
Endogenous thrombin potential (nM*min)	1540	2978	1159	2317
Peak thrombin (nM)	241	444	71	407
Time to peak (min)	4.7	8.3	7.4	16.2
Velocity index (nM/min)	57.6	163.2	NA	NA

Table 5.3 Normal ranges for the CAT at TF 5pM and TF 1pM

5.4.2.2 Results of the different anticoagulated plasmas on the CAT

Results of the thrombin generation assay at TF 5pM are reported in Table 5.4. Results of the anticoagulated plasmas analysed at TF 1pM are reported in Appendix D (Table D1), as well as CAT results expressed as ratio to normal plasma (Table D2). Results will be discussed according to the different classes of anticoagulant drugs.

		Lag time (min)	*ETP (nM*min)*	*Peak (nM)*	*Time to peak (min)*	*Vel. index (nM/min)*
Normal ranges		***2.2-4.5***	***1540-2978***	***241-444***	***4.7-8.3***	***57.6-163.2***
Warfarinised plasma						
INR 2.22		5.67 (0)	759.0 (8.0)	148.63 (0.58)	8.00 (0)	63.70 (0.25)
INR 3.24		9.00 (0)	408.5 (8.5)	69.75 (1.25)	12.00 (0)	23.25 (0.42)
INR 4.11		11.17 (0.17)	321.5 (5.5)	54.04 (0.71)	14.33 (0)	17.10 (0.68)
Direct factor Xa inhibitors						
Apixaban	4 ng/ml	4.78 (0.17)	2156.5 (66.5)	258.28 (5.17)	8.95 (0)	62.02 (3.72)
	42 ng/ml	6.28 (0)	1813.0 (21.0)	140.30 (1.10)	10.46 (0.17)	33.65 (1.08)
	89 ng/ml	6.61 (0)	1315.5 (53.5)	85.94 (8.09)	10.46 (0.17)	22.51 (3.09)
	128 ng/ml	7.28 (0)	1105.0 (40.0)	64.25 (3.55)	11.12 (0.71)	16.80 (1.66)
	179 ng/ml	8.28 (0)	956.0 (38.0)	48.26 (3.01)	12.29 (0)	12.04 (0.75)
	266 ng/ml	8.95 (0)	828.0 (13.0)	38.47 (0.47)	13.29 (0)	8.86 (0.11)
Edoxaban	0 ng/ml	5.45 (0.17)	1980.0 (97.0)	224.17 (8.78)	10.46 (0.17)	44.75 (1.75)
	15 ng/ml	7.12 (0.17)	1813.0 (50.0)	138.49 (1.89)	15.30 (0.33)	16.93 (0.11)
	51 ng/ml	8.45 (0.17)	1544.5 (30.5)	93.47 (0.62)	18.64 (0.33)	9.18 (0.09)
	85 ng/ml	9.45 (0.17)	1281.5 (7.5)	72.18 (0.39)	21.14 (0.50)	6.18 (0.21)
	113 ng/ml	10.12 (0.17)	1127.5 (20.5)	59.86 (0.80)	22.31 (0.33)	4.91 (0)
	188 ng/ml	11.46 (0.17)	896.5 (49.5)	45.15 (2.16)	24.15 (0.17)	3.56 (0.17)
Rivaroxaban	22 ng/ml	5.28 (0)	1947.5 (40.5)	176.41 (5.50)	10.46 (0.17)	34.15 (2.16)
	55 ng/ml	7.45 (0.17)	1403.0 (12.0)	70.82 (0.68)	18.97 (0)	6.15 (0.15)
	118 ng/ml	8.79 (0.17)	1070.0 (45.0)	47.29 (1.90)	22.31 (0)	3.50 (0.18)
	174 ng/ml	9.62 (0.33)	975.0 (61.0)	41.79 (3.68)	23.48 (0.83)	3.03 (0.37)
	231 ng/ml	10.62 (0)	752.0 (3.0)	29.96 (0.03)	25.65 (0)	1.99 (0)
	339 ng/ml	11.96 (0)	563.0 (14.0)	21.06 (0.09)	27.65 (0)	1.34 (0.01)
Direct thrombin inhibitors						
Argatroban	0.25 µg/ml	7.44 (0.17)	1420.0 (6.0)	221.56 (1.14)	10.45 (0.17)	73.73 (0.38)
	0.53 µg/ml	9.28 (0)	1114.0 (10.0)	147.86 (0.41)	12.11 (0.17)	52.29 (3.22)
	3.10 µg/ml	17.29 (0)	No tail found	5.76 (0.09)	37.16 (3.17)	0.30 (0.04)
	5.84 µg/ml	22.97 (0)	No tail found	3.14 (0.07)	57.69 (3.0)	0.09 (0.01)
Bivalirudin	5.9 µg/ml	20.80 (0.17)	1662.0 (0)	351.89 (6.40)	22.97 (0)	163.34 (15.51)
	13.8 µg/ml	27.14 (1.50)	1726.5 (31.5)	318.04 (4.29)	29.81 (1.50)	119.07 (1.61)
	31.0 µg/ml	41.16 (2.17)	1691.5 (30.5)	268.92 (14.60)	45.00 (2.67)	71.76 (13.16)
Dabigatran	0 ng/ml	4.60 (0.33)	2036.0 (74.0)	292.58 (16.64)	8.27 (0.33)	79.67 (4.53)
	44 ng/ml	7.44 (0.17)	1700.0 (24.0)	269.62 (6.06)	10.78 (0.17)	80.76 (1.81)
	92 ng/ml	10.11 (0.17)	1450.5 (19.5)	255.18 (3.75)	12.78 (0.17)	95.54 (1.40)
	148 ng/ml	12.28 (0.33)	1226.0 (11.0)	226.42 (1.34)	14.95 (0.33)	84.77 (0.50)
	176 ng/ml	13.45 (0.17)	1080.0 (7.0)	205.17 (0.02)	16.12 (0.17)	76.81 (0.01)
	276 ng/ml	16.96 (0.33)	775.0 (10.0)	156.88 (0.09)	19.29 (0.33)	67.13 (0.04)
Indirect factor Xa inhibitors						
Danaparoid	0.33 U/ml	5.00 (0)	654.5 (2.5)	40.27 (0.03)	12.33 (0)	5.49 (0)
	0.78 U/ml	8.33 (0.67)	153.0 (2.0)	4.25 (0.13)	30.33 (1.33)	0.19 (0.01)
	1.93 U/ml	Flat CAT traces				
Enoxaparin	0.35 U/ml	4.33 (0)	1544.5 (11.5)	137.99 (2.89)	10.17 (0.17)	23.69 (1.17)
	1.06 U/ml	6.00 (0.33)	172.0 (3.0)	6.74 (0.19)	18.00 (0.33)	0.56 (0.02)
	1.95 U/ml	Flat CAT traces				
Fondaparinux	0.64 µg/ml	7.33 (0)	1244.5 (52.5)	90.14 (3.66)	14.50 (0.17)	12.60 (0.80)
	1.64 µg/ml	11.83 (0.17)	435.0 (25.0)	24.70 (1.22)	21.17 (0.17)	2.65 (0.13)
	2.24 µg/ml	16.67 (0.33)	218.0 (11.0)	11.01 (0.62)	28.17 (0.50)	0.96 (0.07)

Table 5.4 Results of the different anticoagulant concentrations on the CAT at TF 5pM
Results are reported as mean (SD) of two measurements.
Out of range values have been reported in red colour.

Warfarinised plasma

The thrombin generation curve of the warfarinised plasma with INR 2.22 had a similar shape of the normal PPP (Figure 5.3). At TF 5pM, the time to peak was comparable to the normal PPP, the lag time was increased by 42%, the ETP was only 39% and the peak only 55% of the normal PPP. Similar findings were reported at TF 1pM.

The effect on the lag time was more evident with INR 3.24 and INR 4.11, where it was more than double the normal PPP. Differences between these two INR values were less pronounced, with the ETP at TF 5pM being 21% and 17% of normal PPP with INR 3.24 and INR 4.11, respectively, while at TF 1pM it was 18% and 15% with INR 3.24 and INR 4.11, respectively.

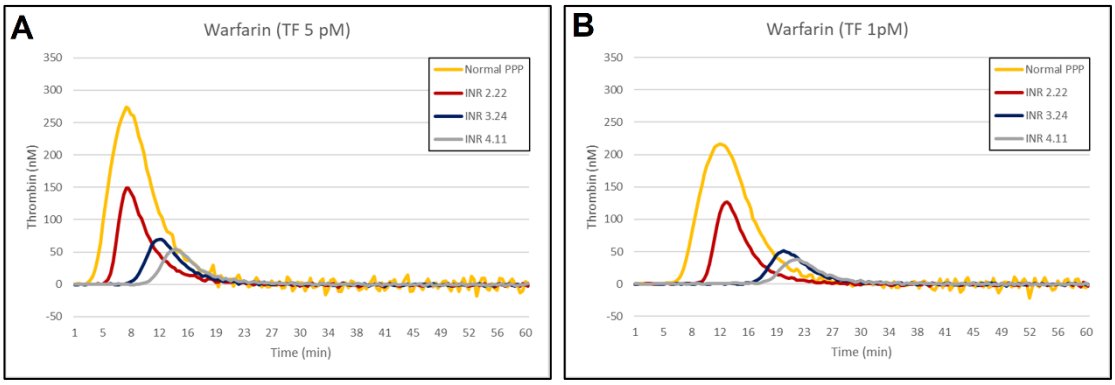


Figure 5.3 Thrombin generation curves of the warfarinised plasma at TF 5pM (A) and TF 1pM (B)

Direct factor Xa inhibitors

The thrombin generation curves of the direct factor Xa inhibitors (apixaban, edoxaban, rivaroxaban) are reported in Figure 5.4. The thrombin generation curves with the lowest concentrations for each anticoagulant had a similar shape of the normal PPP. However, it is noticeable that despite the lower limit of detection of the anti-Xa assay was 30 ng/ml, lower concentrations were already showing some small alterations in

the thrombin generation curves, suggesting that the CAT is a more sensitive assay than the anti-Xa assay.

The shape of the curves showed some differences among the different factor Xa inhibitors. Apixaban had a short time to peak and a plateau before starting the tail. Conversely, edoxaban and rivaroxaban had a smooth ascending curve and a prolonged time to peak. In fact, the tested concentrations of apixaban never doubled the time to peak, while it was doubled with low concentrations of edoxaban (from 15 ng/ml upwards at TF 5pM, from 51 ng/ml upwards at TF 1pM) and rivaroxaban (from 55 ng/ml upwards at TF 5pM, from 118 ng/ml upwards at TF 1pM). Similarly, the lag time, representing the shift of the thrombin generation curve to the right, was doubled with higher concentrations of apixaban (from 179 ng/ml upwards at TF 5pM, from 266 ng/ml upwards at TF 1pM), followed by rivaroxaban (from 118 ng/ml upwards at TF 5pM, from 174 ng/ml upwards at TF 1pM) and edoxaban (from 51 ng/ml upwards at TF 5pM, from 113 ng/ml upwards at TF 1pM).

However, despite differences in the shape of the curve, the ETP, which is the most consistent CAT parameter, showed similar values when similar DOAC concentrations were compared. For instance, edoxaban 113 ng/ml, rivaroxaban 118 ng/ml and apixaban 128 ng/ml had ETP 52%, 50% and 51% of the normal PPP (TF 5pM). Similarly, rivaroxaban 174 ng/ml, apixaban 179 ng/ml and edoxaban 188 ng/ml had ETP 45%, 45% and 42% of the normal PPP (TF 5pM).

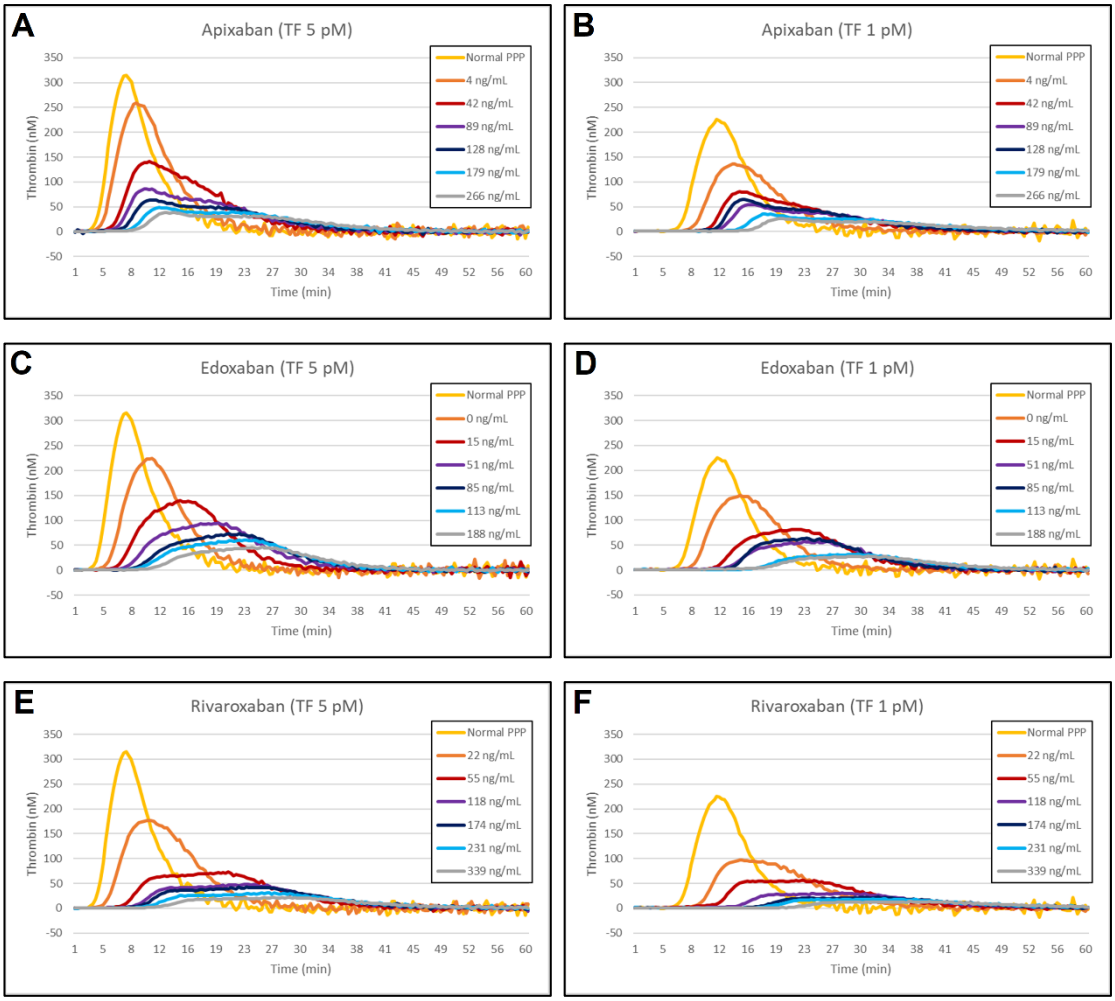


Figure 5.4 Thrombin generation curves of the plasma spiked with the direct factor Xa inhibitors: apixaban at TF 5pM (A) and TF 1pM (B), edoxaban at TF 5pM (C) and TF 1pM (D), rivaroxaban at TF 5pM (E) and 1pM (F)

Direct thrombin inhibitors

The thrombin generation curves of the direct thrombin inhibitors (argatroban, bivalirudin and dabigatran) are reported in Figure 5.5. In general, direct thrombin inhibitors had a more pronounced effect on the lag time, while the peak was only slightly reduced.

The two lower concentrations of argatroban gave a 2-fold prolongation of the lag time, while the two higher concentrations gave a 4- to 6-fold prolongation compared to the normal PPP. Argatroban at concentration 0.25 µg/ml had ETP 73% and at

concentration 0.53 μg/ml 57% of the normal PPP at TF 5pM. Similarly, at TF 1pM, argatroban at concentration 0.25 μg/ml showed ETP 71% and at concentration 0.53 μg/ml 60% of the normal PPP. With higher concentrations of argatroban (3.10 μg/ml and 5.84 μg/ml), the amount of thrombin generated was very low (peak thrombin 5.76 nM and 3.14 nM, which correspond to 2% and 1% of the normal PPP, respectively) and no tail could be seen after 180 min, therefore the ETP could not be calculated.

Bivalirudin showed a different pattern. With increasing concentrations there was an increase of the lag time (from 5- to 10-fold the normal PPP) and the time to peak (from 3- to 6-fold the normal PPP). The ETP was only modestly reduced (between 82% and 86% of normal PPP). Conversely, the peak was very close to the normal PPP or slightly increased with the lowest concentration (120% of the normal PPP at TF 5pM, 140% of the normal PPP at TF 1pM).

The effect of dabigatran was seen mainly on the lag time, which was doubled with concentration 44 ng/ml and it reached more than 4-fold the normal PPP with the highest concentration 276 ng/ml. It is noticeable that despite the lower limit of detection of the DTT assay was 30 ng/ml, the lowest concentration (0 ng/ml according to DTT) was already showing some small alterations in the thrombin generation curves, especially at TF 1pM, suggesting that the CAT is a more sensitive assay than the DTT assay. A paradoxical rise of the peak was observed with low concentrations of dabigatran. Furthermore, the peak thrombin was still >80% of the normal PPP with dabigatran concentration 148 ng/ml at TF 5pM and dabigatran concentration 176 ng/ml at TF 1pM. The ETP was progressively reduced with increasing dabigatran concentration. At dabigatran concentration 176 ng/ml it was 56% of the normal PPP, which was slightly higher than the similar concentrations of the direct factor Xa inhibitors (rivaroxaban 174 ng/ml had 45%, apixaban 179 ng/ml had 45%, edoxaban

188 ng/ml had 42% of the normal PPP).

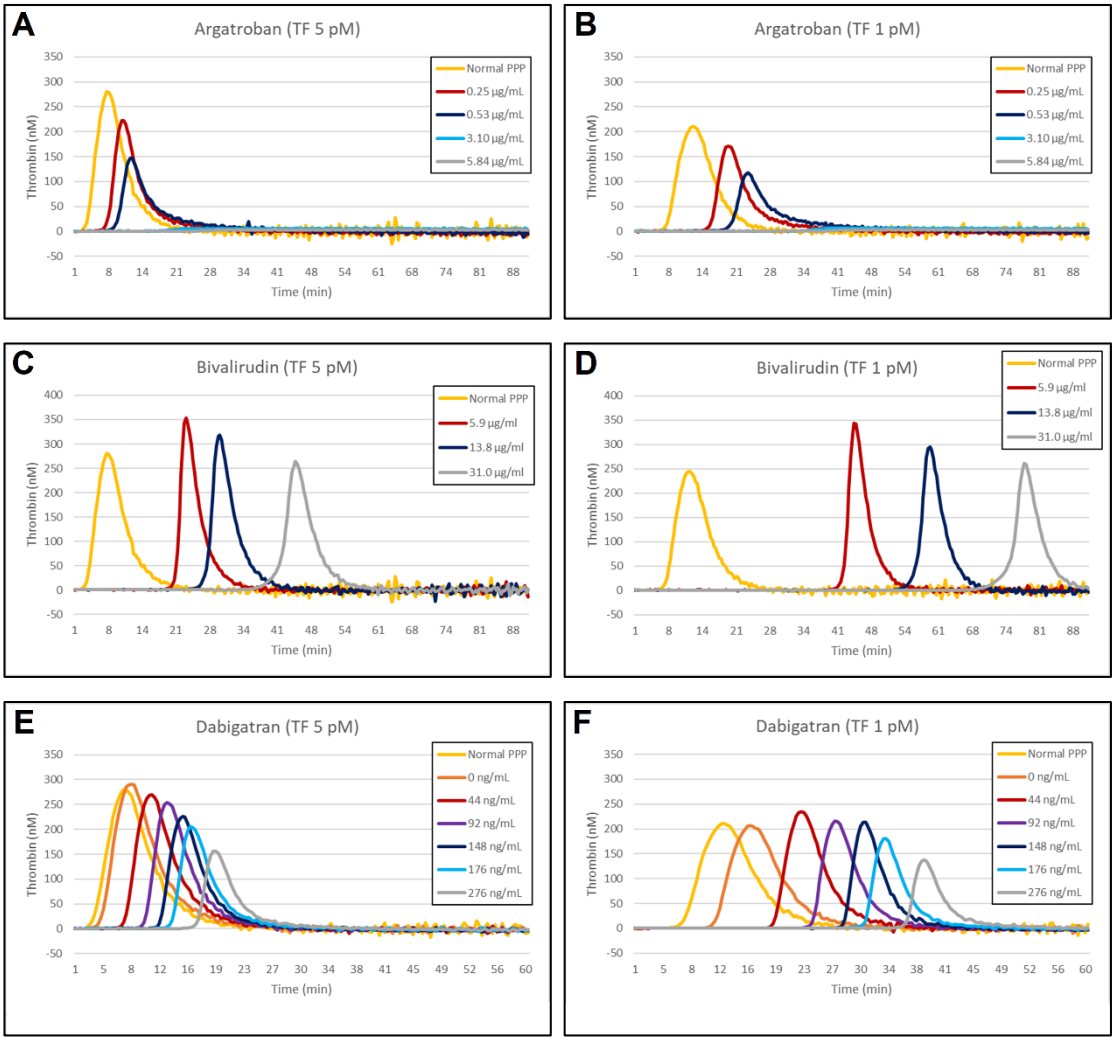


Figure 5.5 Thrombin generation curves of the plasma spiked with the direct thrombin inhibitors: argatroban at TF 5pM (A) and TF 1pM (B), bivalirudin at TF 5pM (C) and TF 1pM (D), dabigatran at TF 5pM (E) and 1pM (F)

Indirect factor Xa inhibitors

The thrombin generation curves of the indirect factor Xa inhibitors (danaparoid, enoxaparin, fondaparinux) are reported in Figure 5.6. The indirect factor Xa inhibitors had a more pronounced effect on the peak, which was significantly reduced to less than 50% of the normal PPP even at prophylactic concentrations. At similar

prophylactic concentrations, danaparoid 0.33 U/ml showed a greater reduction of the ETP compared to enoxaparin 0.35 U/ml (31% vs. 74% of the normal PPP at TF 5pM, 11% vs. 23% at TF 1pM). At TF 5pM, the amount of thrombin generated at therapeutic concentrations, danaparoid 0.78 U/ml and enoxaparin 1.06 U/ml, was very low (peak 4.25 nM and 6.74 nM, respectively). Supratherapeutic concentrations (danaparoid 1.93 U/ml and enoxaparin 1.95 U/ml) gave flat traces. At TF 1pM, enoxaparin 1.06 U/ml gave also a flat trace, while danaparoid 0.78 U/ml prolonged the lag time by 13-fold, generated a very small amount of thrombin (peak 1.05 nM), and no tail could be identified at 120 min, therefore the ETP could not be calculated.

Fondaparinux had also a greater effect on the peak, which was 33% of the normal PP at concentration 0.64 μg/ml, decreasing to 9% and 4% with concentrations 1.64 μg/ml and 2.24 μg/ml (TF 5pM), respectively. The lag time was almost doubled with the lowest concentration (0.64 μg/ml), increasing to 3- and 4-fold with increasing concentrations. The ETP was 65% of the normal PP with concentration 0.64 μg/ml, decreasing to 23% with concentration 1.64 μg/ml and 11% with concentration 2.24 μg/ml.

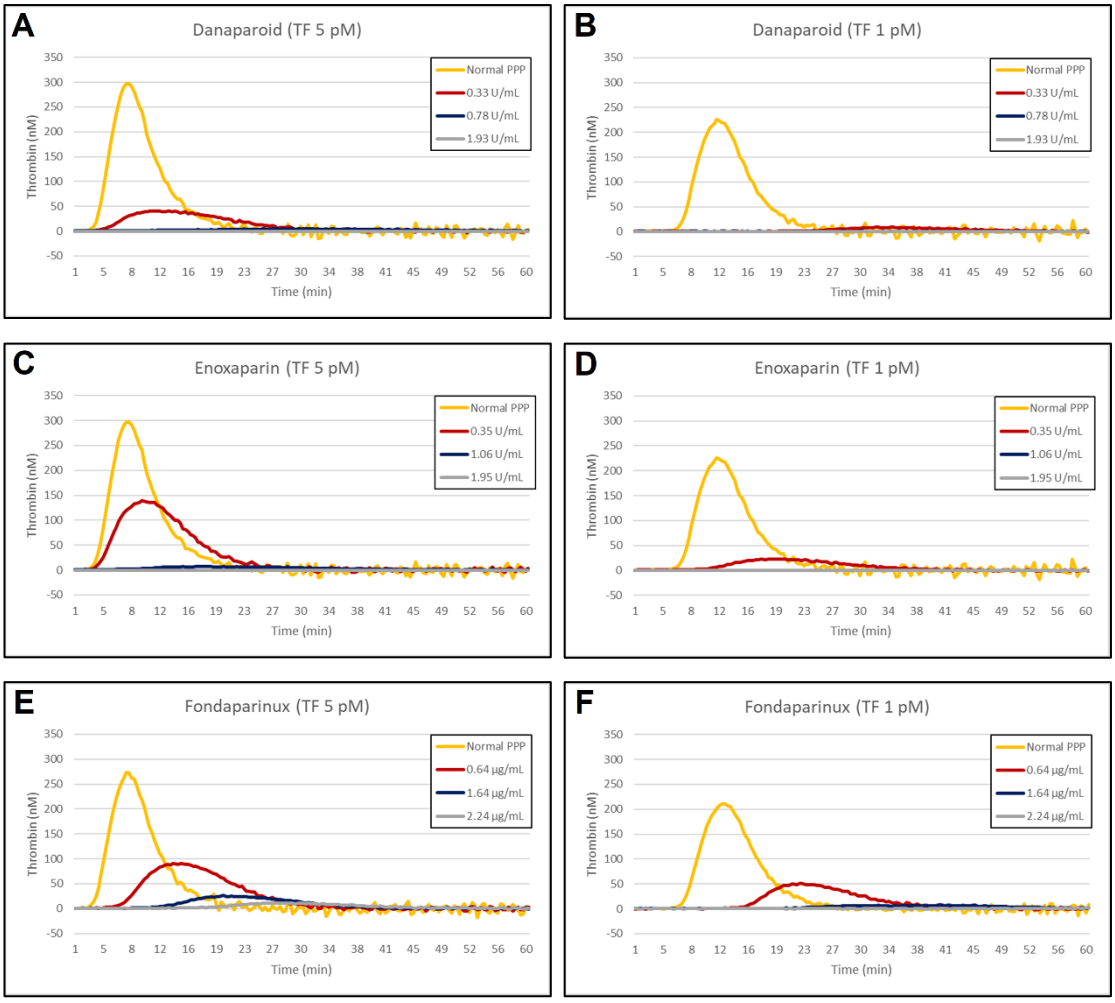


Figure 5.6 Thrombin generation curves of the plasma spiked with the indirect factor Xa inhibitors: danaparoid at TF 5pM (A) and TF 1pM (B), enoxaparin at TF 5pM (C) and TF 1pM (D), fondaparinux at TF 5pM (E) and 1pM (F)

5.4.2.3 Correlation between CAT and anticoagulant concentrations

A negative curvilinear correlation emerged between the ETP and the DOACs concentration (Figure 5.7). With the exception of bivalirudin, there was a strong correlation between all the CAT parameters and the anticoagulant concentrations, with most of the rho coefficients close to 1.0 (Table 5.5). However, correlations for danaparoid, enoxaparin and the ETP with argatroban were not statistically significant, probably due to the low number of tests (n=4). Lag time and time to peak showed a positive correlation, while ETP and peak showed a negative correlation.

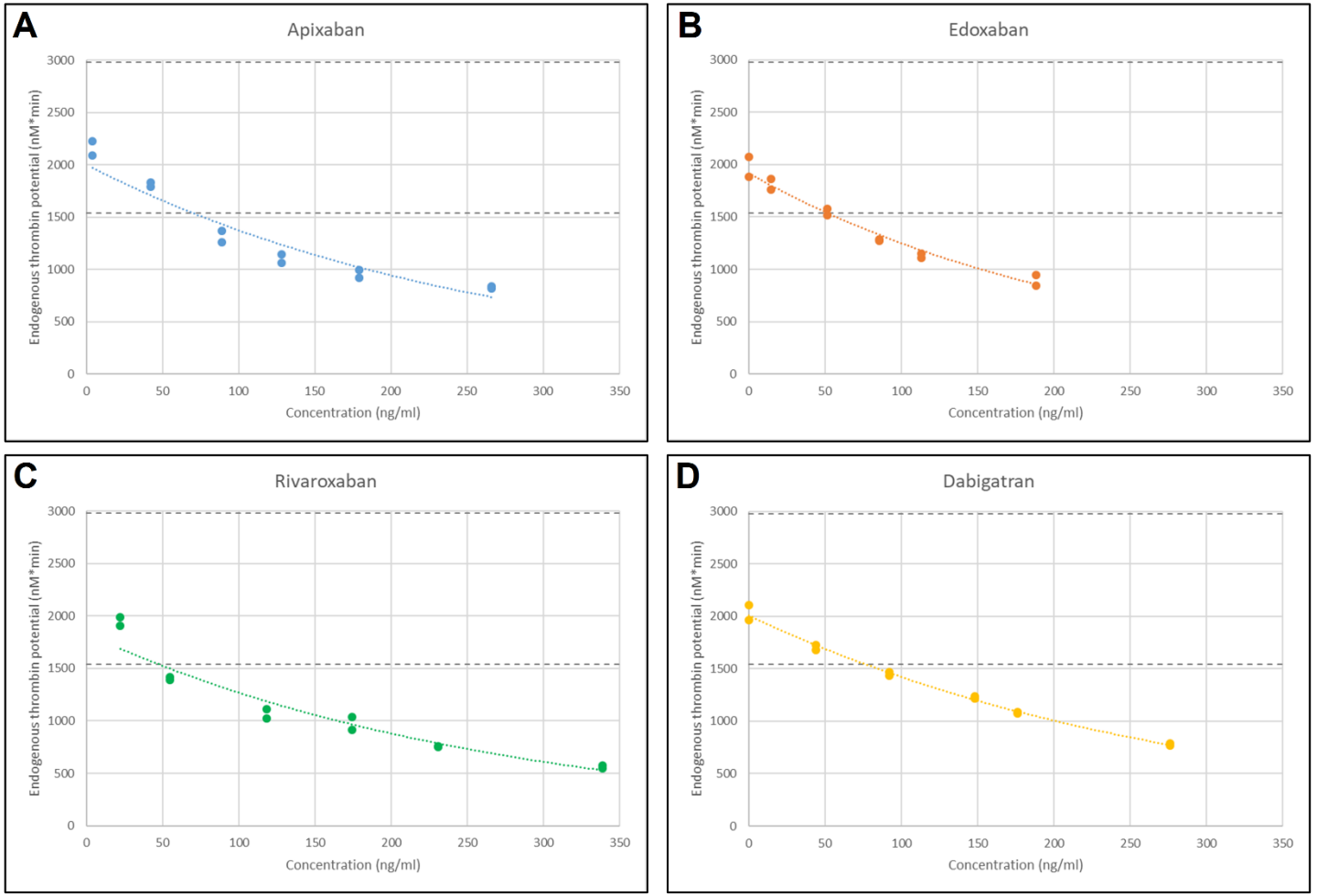


Figure 5.7 Correlation between different concentrations of the direct oral anticoagulants and the endogenous thrombin potential at TF 5pM: apixaban (A), edoxaban (B), rivaroxaban (C), dabigatran (D)

The horizontal dashed grey lines represent the normal ranges of the ETP.

Anticoagulant	N. of tests*	Lag time: correlation coefficient (p value)	ETP: correlation coefficient (p value)	Peak: correlation coefficient (p value)	Time to peak: correlation coefficient (p value)
Warfarin**	6	0.98 (<0.001)	-0.96 (0.003)	-0.96 (0.003)	1.00 (<0.001)
Direct factor Xa inhibitors					
Apixaban	12	1.00 (<0.001)	-0.99 (<0.001)	-0.99 (<0.001)	0.97 (<0.001)
Edoxaban	12	0.99 (<0.001)	-0.99 (<0.001)	-0.99 (<0.001)	0.99 (<0.001)
Rivaroxaban	12	0.99 (<0.001)	-0.98 (<0.001)	-0.98 (<0.001)	1.00 (<0.001)
Direct thrombin inhibitors					
Argatroban	8***	0.99 (<0.001)	-0.89 (0.11)	-0.97 (<0.001)	0.97 (<0.001)
Bivalirudin	6	0.96 (0.003)	0.24 (0.64)	-0.96 (0.003)	0.97 (0.001)
Dabigatran	12	0.99 (<0.001)	-0.99 (<0.001)	-0.99 (<0.001)	0.99 (<0.001)
Indirect factor Xa inhibitors					
Danaparoid	4****	0.94 (0.057)	-0.89 (0.11)	-0.89 (0.11)	0.94 (0.057)
Enoxaparin	4****	0.94 (0.057)	-0.89 (0.11)	-0.89 (0.11)	0.89 (0.11)
Fondaparinux	6	0.97 (0.001)	-0.96 (0.003)	-0.96 (0.003)	0.96 (0.003)

Table 5.5 Correlation between anticoagulant concentrations and the parameters of the CAT at TF 5pM

* Each concentration has been tested in duplicate.

** For the warfarinised plasma, the INR values have been considered, instead of the concentrations.

*** Only 2 concentrations of argatroban (4 tests) could be evaluated for the ETP.

****Only 2 concentrations of danaparoid and enoxaparin (4 tests each) could be evaluated, because of flat CAT traces.

5.4.3 Native thromboelastography

5.4.3.1 Normal ranges of the native TEG

R time, angle and MA were available for all 20 subjects. Conversely, K time was undefined in two females and four males because the MA was < 20 mm in these subjects (Table 5.6).

The four main parameters of the TEG (R time, K time, angle and MA) were normally distributed in the two sexes (all tests for normality $p>0.05$). They were compared using the independent samples t-test assuming equal variances (Levene's test for equality of variances $p>0.05$). There were no statistically significant differences between females

and males in any of these four parameters (p>0.05); therefore, one single normal range was created for both sexes.

	N. of test	Mean	SD	Tests for normality: p values		Independent samples t-test (males vs. females): p values	
				Kolmogorov-Smirnov (Lilliefors Significance Correction)	Shapiro-Wilk	Levene's Test for Equality of Variances	t-test for Equality of Means
R time (min)							
Female	10	12.6	2.6	0.19	0.095	0.59	0.90
Male	10	12.5	1.9	0.20	0.89		
K time (min)							
Female	8	4.4	2.2	0.20	0.60	0.48	0.73
Male	6	4.0	1.6	0.20	0.19		
Angle (deg)							
Female	10	48.6	9.6	0.20	0.72	0.29	0.62
Male	10	46.7	8.3	0.20	0.93		
MA (mm)							
Female	10	25.5	6.9	0.20	0.61	0.66	0.42
Male	10	23.1	6.2	0.20	0.26		

Table 5.6 Preliminary statistical analyses for the calculation of normal ranges for the native TEG on citrated PPP, divided by sex

Before establishing the normal ranges, the four TEG parameters were checked again to confirm their normal distribution in the overall population, including both males and females. All tests for normality gave p>0.05, therefore the normal ranges were calculated using the formula mean ± 1.96 * SD and are reported in Table 5.7. The ranges of the LY30 and the LY60 could not be established because these parameters were 0% in the native TEG using citrated PPP.

	N. of tests	Results, mean (SD)	Normal ranges	
			Lower limit	Upper limit
R time (min)	20	12.6 (2.2)	8.3	16.9
K time (min)	14	4.2 (1.9)	0.5	7.9
Angle (deg)	20	47.6 (8.8)	30.4	64.8
MA (mm)	20	24.3 (6.5)	11.6	37.0

Table 5.7 Normal ranges for the native TEG on citrated PPP

5.4.3.2 Results of the different anticoagulated plasmas on the native TEG

Results of the main parameters of the native TEG are reported in Table 5.8. Results of the secondary parameters of the TEG are reported in Appendix D (Table D3), as well as TEG results expressed as ratio to normal plasma (Table D4). Results will be discussed according to the different classes of anticoagulant drugs.

		R time (min)	K time (min)	Angle (deg)	MA (mm)
Normal ranges		***8.3-16.9***	***0.5-7.9***	***30.4-64.8***	***11.6-37.0***
Warfarinised plasma					
INR 2.22		10.85 (1.34)	2.80 (1.41)	54.10 (15.84)	37.80 (2.83)
INR 3.24		17.60 (2.97)	5.20 (0.42)	34.35 (0.07)	37.05 (0.49)
INR 4.11		16.25 (6.01)	3.75 (1.34)	41.85 (10.82)	38.05 (1.34)
Direct factor Xa inhibitors					
Apixaban	4 ng/ml	16.90 (0.14)	3.65 (2.19)	48.70 (18.10)	36.20 (2.97)
	42 ng/ml	14.15 (0.07)	5.05 (2.05)	38.20 (6.79)	35.40 (4.81)
	89 ng/ml	17.60 (2.26)	3.70 (0.57)	40.45 (7.71)	31.10 (2.55)
	128 ng/ml	15.65 (4.45)	6.00 (3.11)	36.95 (17.89)	33.40 (6.65)
	179 ng/ml	16.90 (0.99)	4.80 (0)	37.05 (2.90)	30.65 (3.04)
	266 ng/ml	16.90 (2.83)	4.90 (2.26)	43.60 (14.28)	28.30 (3.54)
Edoxaban	0 ng/ml	13.75 (0.07)	4.20 (1.41)	39.75 (3.61)	34.80 (6.08)
	15 ng/ml	15.90 (1.56)	3.85 (0.64)	45.60 (6.51)	29.20 (1.56)
	51 ng/ml	15.40 (1.98)	5.10 (1.56)	35.00 (10.61)	30.25 (5.44)
	85 ng/ml	21.55 (8.41)	6.90 (3.11)	32.35 (13.08)	27.85 (3.89)
	113 ng/ml	26.50 (10.04)	8.25 (2.76)	27.10 (5.52)	27.90 (4.81)
	188 ng/ml	28.15 (1.91)	10.85 (0.64)	19.85 (1.34)	29.05 (1.63)
Rivaroxaban	22 ng/ml	13.70 (0.57)	4.15 (0.64)	41.55 (3.89)	31.80 (1.70)
	55 ng/ml	18.00 (2.40)	4.15 (0.92)	42.40 (5.66)	29.90 (0.42)
	118 ng/ml	19.50 (1.56)	5.85 (1.77)	32.75 (7.0)	29.20 (2.12)
	174 ng/ml	19.55 (0.07)	7.50 (2.12)	27.40 (7.92)	28.40 (0.57)
	231 ng/ml	27.30 (1.98)	8.65 (2.05)	24.35 (5.87)	27.10 (0.42)
	339 ng/ml	28.95 (4.60)	12.40 (5.52)	20.70 (7.35)	25.00 (0.85)
Direct thrombin inhibitors					
Argatroban	0.25 µg/ml	19.40 (5.80)	3.45 (1.20)	47.70 (11.31)	31.70 (0.71)
	0.53 µg/ml	24.75 (0.64)	5.80 (0.99)	29.85 (6.86)	29.40 (0.85)
	3.10 µg/ml	40.50 (10.32)	6.80 (2.55)	29.50 (9.33)	32.30 (1.84)
	5.84 µg/ml	65.45 (18.60)	9.05 (2.62)	22.85 (5.30)	31.95 (0.07)
Bivalirudin	5.9 µg/ml	34.75 (11.38)	4.85 (0.07)	29.25 (4.03)	30.95 (0.07)
	13.8 µg/ml	47.05 (4.31)	9.70 (3.68)	22.55 (8.27)	30.55 (0.35)
	31.0 µg/ml	52.85 (17.32)	9.85 (0.49)	24.40 (7.64)	27.75 (1.48)
Dabigatran	0 ng/ml	16.45 (0.35)	4.80 (2.26)	40.90 (11.17)	30.70 (1.70)
	44 ng/ml	20.15 (3.32)	4.40 (1.98)	40.05 (16.48)	31.10 (0.57)
	92 ng/ml	29.15 (1.77)	6.30 (0.14)	33.20 (11.03)	29.00 (1.27)
	148 ng/ml	27.75 (11.38)	3.25 (0.49)	51.30 (11.88)	36.10 (2.97)
	176 ng/ml	29.20 (1.84)	6.15 (4.31)	36.90 (21.21)	35.75 (5.16)
	276 ng/ml	42.25 (13.36)	11.00 (11.60)	35.45 (27.22)	34.25 (3.32)
Indirect factor Xa inhibitors					
Danaparoid	0.33 U/ml	28.90 (15.70)	18.35 (0.78)	12.65 (0.64)	27.10 (5.94)
	0.78 U/ml	Flat TEG traces			
	1.93 U/ml	Flat TEG traces			
Enoxaparin	0.35 U/ml	23.35 (0.35)	11.40 (2.12)	17.40 (0.99)	28.20 (0.99)
	1.06 U/ml	Flat TEG traces			
	1.95 U/ml	Flat TEG traces			
Fondaparinux	0.64 µg/ml	22.55 (3.46)	9.20 (1.98)	22.95 (7.28)	27.85 (0.07)
	1.64 µg/ml	Flat TEG traces			
	2.24 µg/ml	Flat TEG traces			

Table 5.8 Results of the different anticoagulant concentrations on the main parameters of the TEG
Results are reported as mean (SD) of two measurements.
Out of range values have been reported in red colour.

Warfarinised plasma

The thromboelastogram of the warfarinised plasma with INR 2.22 had a similar shape of the normal PPP (Figure 5.8). The effect of increasing INR values was seen mainly as prolongation of the R time and the K time, even though there was only a slight difference between INR 3.24 and INR 4.11. With INR 3.24 the R was 1.38 times and the K was 1.27 times the normal PPP, while with INR 4.11 they were 1.68 times and 1.23 times, respectively. The angle was slightly reduced (70% of normal PPP with INR 3.24 and 86% of normal PPP with INR 4.11). The MA was actually slightly increased, up to 1.2 folds the normal PPP. Of note, given the wide normal ranges, most of the TEG parameters of the warfarinised plasma were within the normal ranges.

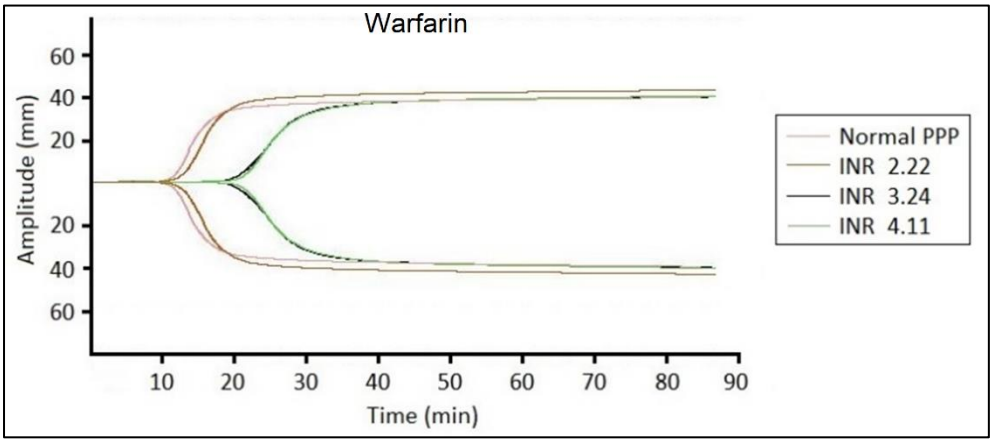


Figure 5.8 Thromboelastograms of the warfarinised plasma

Direct factor Xa inhibitors

The thromboelastograms of the direct factor Xa inhibitors (apixaban, edoxaban, rivaroxaban) are reported in Figure 5.9. As noticed for the CAT, despite the lower limit of detection of the anti-Xa assay was 30 ng/ml, lower concentrations were

already showing some small alterations in the thromboelastograms, mainly in the R and K time.

The TEG showed a different sensitivity to the different factor Xa inhibitors. With increasing concentrations of rivaroxaban and edoxaban there was a progressive increase in the R and K times and a progressive decrease in the angle. The MA was progressively reduced but to a lesser extent than the angle. Conversely, the TEG profile of increasing concentrations of apixaban was erratic. For instance, edoxaban doubled the R time at concentrations from 113 ng/ml upwards and doubled the K time at concentrations from 85 ng/ml upwards. Rivaroxaban doubled the R time at concentrations from 231 ng/ml upwards and the K time at concentrations from 118 ng/ml upwards. Apixaban never doubled the R time and the K time was doubled only at concentration 128 ng/ml, but it was lower at concentrations 179 ng/ml and 266 ng/ml.

When comparing similar concentrations, it appeared that the TEG is more sensitive to edoxaban. For instance, edoxaban 113 ng/ml, rivaroxaban 118 ng/ml and apixaban 128 ng/ml had the R time 2.3-fold, 1.7-fold and 1.36-fold the normal PPP; the K time 3.19-fold, 2.27-fold and 2.31-fold the normal PPP; the angle 50%, 60% and 68% the normal PPP and the MA 84%, 87% and 100% the normal PPP. Similarly, rivaroxaban 174 ng/ml, apixaban 179 ng/ml and edoxaban 188 ng/ml had the R time 1.7-fold, 1.47-fold and 2.45-fold the normal PPP; the K time 2.88-fold, 1.85-fold and 4.19-fold the normal PPP; the angle 50%, 68% and 36% the normal PPP; and the MA 85%, 92% and 87% the normal PPP.

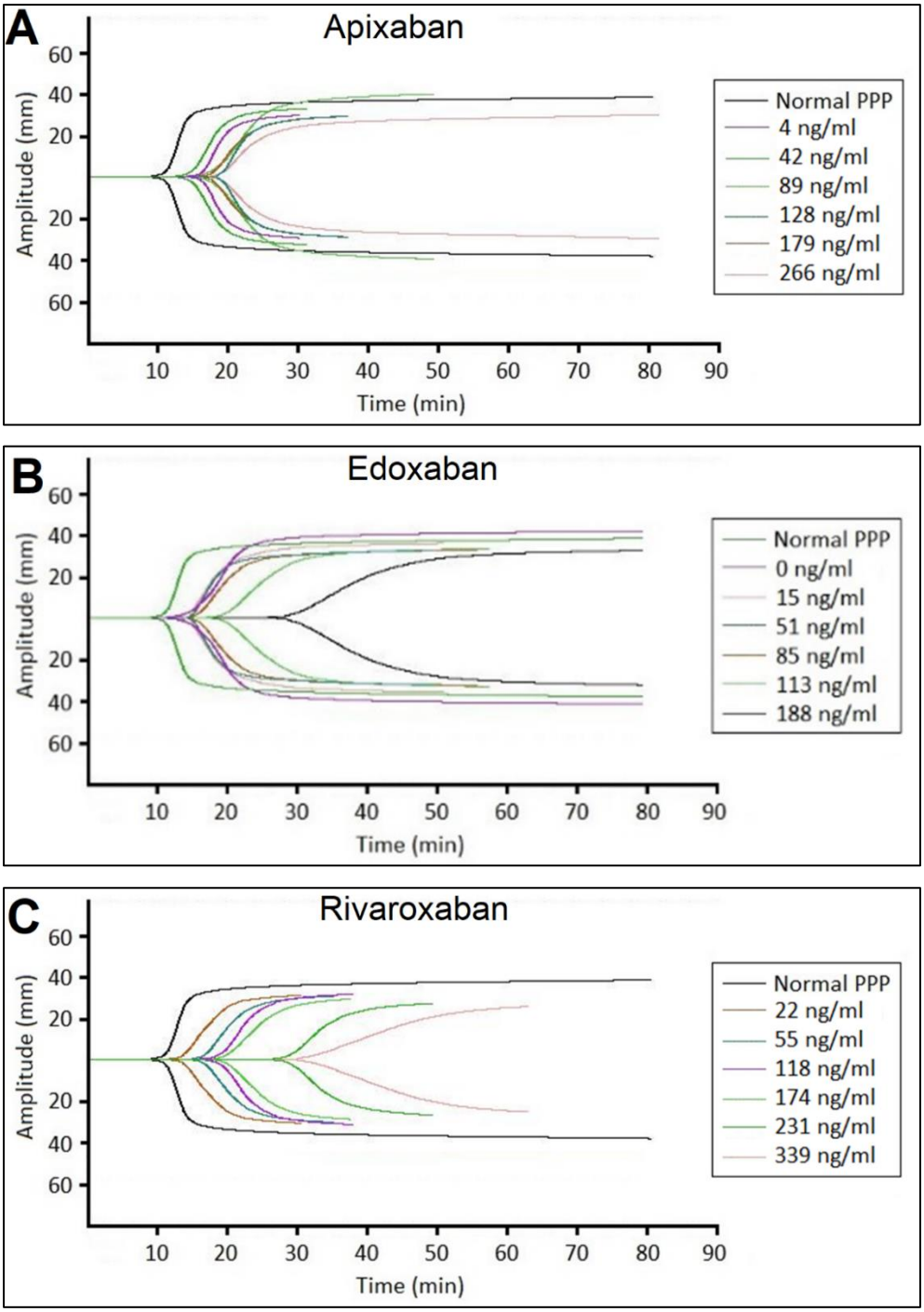


Figure 5.9 Thromboelastograms of the plasma spiked with the direct factor Xa inhibitors: apixaban (A), edoxaban (B), rivaroxaban (C)

Direct thrombin inhibitors

The thromboelastograms of the direct thrombin inhibitors (argatroban, bivalirudin, dabigatran) are reported in Figure 5.10. In general, the direct thrombin inhibitors had a more pronounced effect on the R and K time, while their effect on the MA was erratic.

The two lower concentrations of argatroban gave a 1.52-fold and 1.94-fold prolongation of the R time and a 1.13-fold and 1.87-fold prolongation of the K time. The two higher concentrations gave a more than 3-fold prolongation of R and a more than 2-fold prolongation of K, compared to the normal PPP. With increasing concentrations, the angle decreased from 97% to 47% of normal PPP. The MA was not influenced, and always close to the normal PPP.

Bivalirudin showed a more pronounced effect on both R and K, which were increased by 3.03-fold and 1.88-fold, respectively, with bivalirudin 5.9 μg/ml. With the two higher concentrations, they were more than 4-fold and more than 3-fold, respectively. The angle was reduced from 54% to 41% and the MA from 93% to 83%, with the highest and lowest concentrations, respectively.

With dabigatran the R time was doubled from concentration 92 ng/ml upwards, while the effect on the K time and the angle was more erratic. Similarly to what has been observed with argatroban, the MA was always very close to the normal PPP.

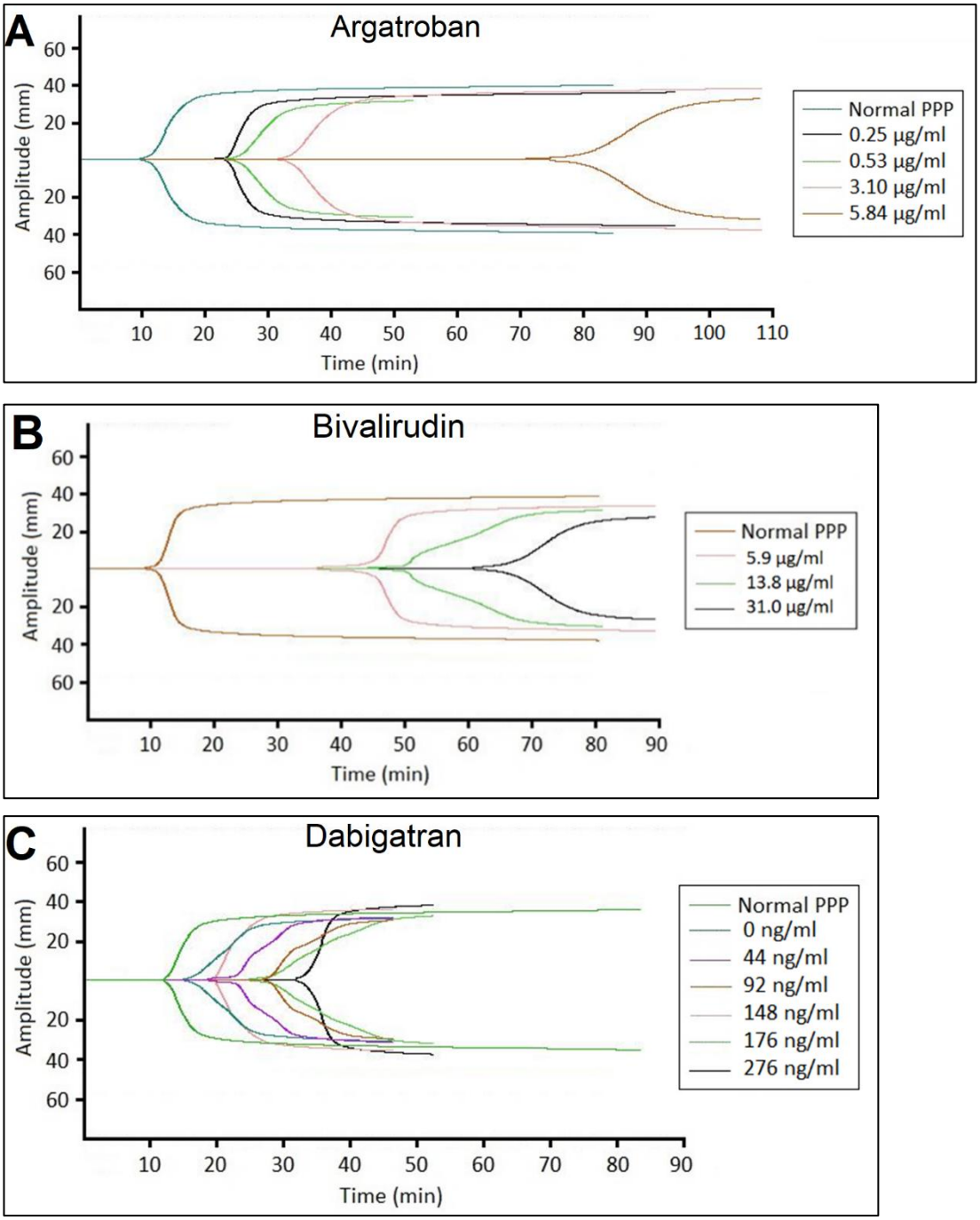


Figure 5.10 Thromboelastograms of the plasma spiked with the direct thrombin inhibitors: argatroban (A), bivalirudin (B), dabigatran (C)

Indirect factor Xa inhibitors

The thromboelastograms of the indirect factor Xa inhibitors (danaparoid, enoxaparin, fondaparinux) are reported in Figure 5.11. In general, the indirect factor Xa inhibitors had a more pronounced effect on the R time (prolonged more than 2-fold the normal PPP) and the K time (prolonged more than 4-fold the normal PPP) with prophylactic concentrations of danaparoid and enoxaparin. The effect of prophylactic fondaparinux was less pronounced, with a 1.77-fold and 2.97-fold prolongation for the R and K, respectively. The angle was reduced to 47% with prophylactic fondaparinux, 32% with prophylactic enoxaparin and 23% with prophylactic danaparoid, while the MA was only slightly reduced, being 87%, 84% and 81% respectively. At therapeutic and supra-therapeutic concentrations, the three indirect factor Xa inhibitors gave flat traces at 120 minutes.

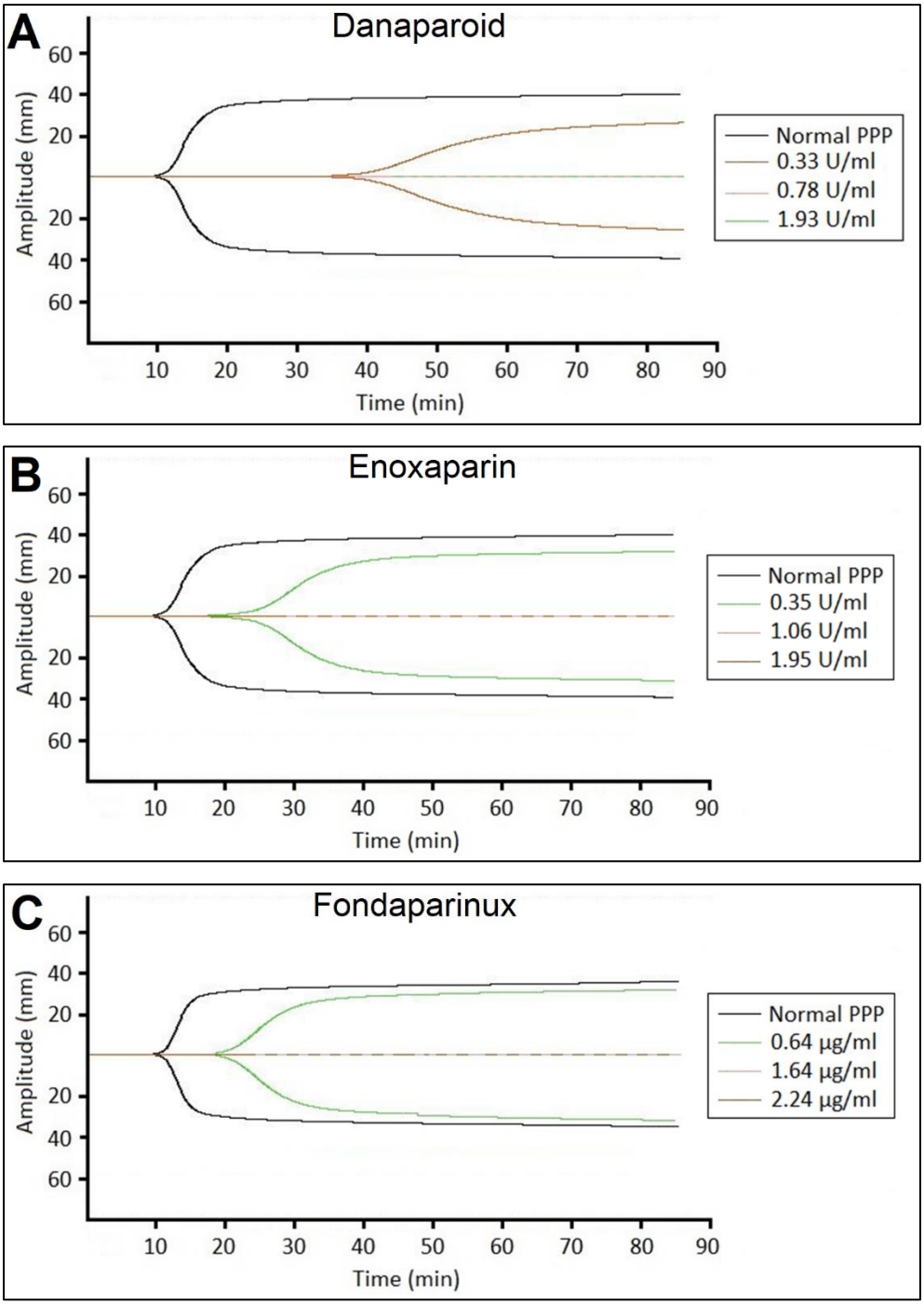


Figure 5.11 Thromboelastograms of the plasma spiked with the indirect factor Xa inhibitors: danaparoid (A), enoxaparin (B), fondaparinux (C)

5.4.3.3 Correlation between native TEG and anticoagulant concentrations

The correlation between the R time and the DOACs concentration is reported in Figure 5.12. The warfarinised plasma showed a strong but not significant positive correlation with the R time, a moderate negative correlation with the angle, and a weak correlation with the K time and the MA (Table 5.9).

Among the direct factor Xa inhibitors, rivaroxaban showed a strong significant correlation for all the TEG parameters. Correlation was positive for the R time and the K time, and negative for the angle and the MA. Edoxaban showed a strong significant positive correlation with the R time and the K time and a strong significant negative correlation with the angle. There was a moderate negative correlation with the MA. Apixaban instead showed a weak positive correlation with the R time and the K time, a weak negative correlation with the angle, and a strong significant negative correlation with the MA.

Among the direct thrombin inhibitors, argatroban showed a strong significant positive correlation with the R time and the K time, a strong negative correlation with the angle, and a weak correlation with the MA. Bivalirudin showed a strong positive correlation with the K time and a strong negative correlation with the MA, and a negative weak correlation with the angle. Dabigatran showed a strong significant positive correlation with the R time and the MA, and a weak correlation with the K time and the angle. Finally, the correlation for the indirect factor Xa inhibitors could not be calculated, because only the lowest concentration for each of these anticoagulants was providing proper TEG curves.

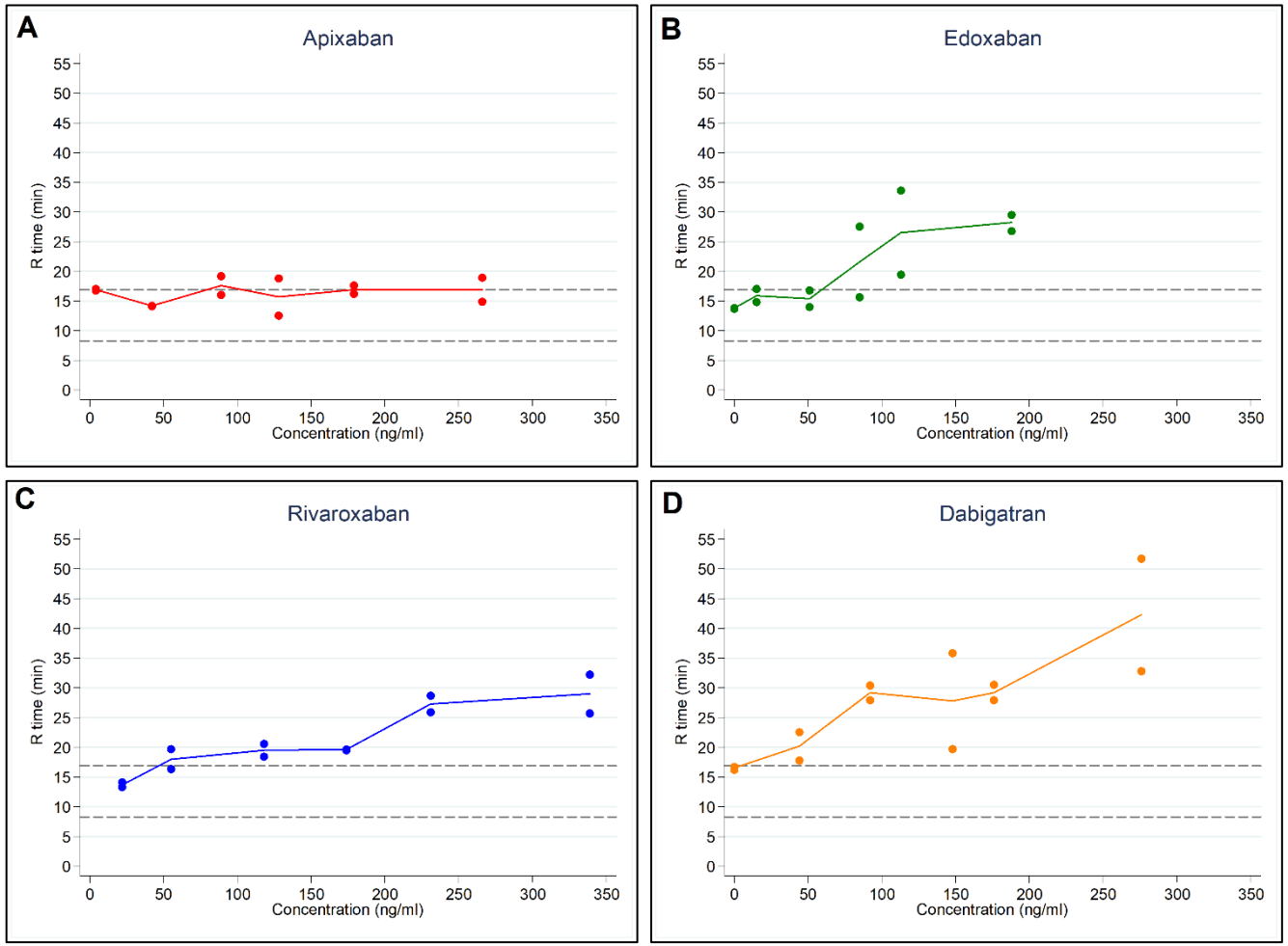


Figure 5.12 Correlation between different concentrations of the direct oral anticoagulants and the R time: apixaban (A), edoxaban (B), rivaroxaban (C), dabigatran (D)
The horizontal dashed grey lines represent the normal ranges of the R time.

Anticoagulant	N. of tests*	R time: correlation coefficient (p value)	K time: correlation coefficient (p value)	Angle: correlation coefficient (p value)	MA: correlation coefficient (p value)
Warfarin**	6	0.72 (0.11)	0.24 (0.65)	-0.48 (0.34)	0.12 (0.82)
Direct factor Xa inhibitors					
Apixaban	12	0.17 (0.60)	0.18 (0.58)	-0.13 (0.69)	-0.68 (0.015)
Edoxaban	12	0.83 (<0.001)	0.85 (<0.001)	-0.74 (0.007)	-0.40 (0.20)
Rivaroxaban	12	0.86 (<0.001)	0.86 (<0.001)	-0.86 (<0.001)	-0.90 (<0.001)
Direct thrombin inhibitors					
Argatroban	8	0.98 (<0.001)	0.83 (0.011)	-0.73 (0.039)	0.20 (0.64)
Bivalirudin	6	0.48 (0.34)	0.72 (0.11)	-0.24 (0.65)	-0.96 (0.003)
Dabigatran	12	0.84 (<0.001)	-0.01 (0.97)	0.01 (0.97)	0.63 (0.028)
Indirect factor Xa inhibitors* **					
Danaparoid	2	NC	NC	NC	NC
Enoxaparin	2	NC	NC	NC	NC
Fondaparinux	2	NC	NC	NC	NC

Table 5.9 Correlation between anticoagulant concentrations and the main parameters of the TEG
* Each concentration has been tested in duplicate
** For the warfarinised plasma, the INR values have been considered instead of the concentrations.
*** The indirect factor Xa inhibitors were not computable because only the thromboelastograms of the lowest concentrations were available

5.4.4 Thromboelastography with the addition of TPA

5.4.4.1 Normal ranges of the TEG with TPA

R time, angle, MA, LY30 and LY60 were available for all 20 subjects. Conversely, K time was undefined in four females and seven males because the MA was < 20 mm in these subjects (Table 5.10).

Four of six main parameters of the TEG (R time, K time, MA and LY60) were normally distributed in the two sexes (all tests for normality $p>0.05$). They were compared using the independent samples t-test assuming equal variances (Levene's test for equality of variances $p>0.05$). There were no statistically significant

differences between females and males in any of these five parameters ($p>0.05$); therefore, one single normal range was created for both sexes.

In the female group, the angle (Kolmogorov-Smirnov test $p=0.13$, Shapiro-Wilk test $p=0.050$) and the LY30 (Kolmogorov-Smirnov test $p=0.039$, Shapiro-Wilk test $p=0.11$) were not normally distributed. Box plots were created to check for outliers, but no outliers or extremes were shown; therefore, data underwent logn transformation. After logn transformation, results of the angle were normally distributed (Kolmogorov-Smirnov test $p=0.20$, Shapiro-Wilk test $p=0.10$ in females, Kolmogorov-Smirnov test $p=0.20$ Shapiro-Wilk test $p=0.32$ in males). They were compared using the independent samples t-test, which showed no significant difference between males and females ($p=0.58$); therefore, one single normal range was created for the angle.

After logn transformation, results of the LY30 were not normally distributed in the male group (Kolmogorov-Smirnov test $p=0.022$, Shapiro-Wilk test $p=0.019$). Therefore, the original data were considered and compared between sexes using the independent samples Mann-Whitney U test. There was no statistically significant difference between females and males ($p=0.63$); therefore, one single normal range was created also for the LY30.

	N. of test	Mean	SD	Tests for normality: p values		Independent samples t-test (males vs. females): p values	
				Kolmogorov-Smirnov (Lilliefors Significance Correction)	Shapiro-Wilk	Levene's Test for Equality of Variances	t-test for Equality of Means
R time (min)							
Female	10	12.7	2.9	0.20	0.91	0.72	0.67
Male	10	13.3	2.3	0.20	0.91		
K time (min)							
Female	6	5.1	3.4	0.20	0.26	0.056	0.28
Male	3	2.7	0.4	NC	0.46		
Angle (deg)							
Female	10	38.2	15.1	0.13	0.050	-	-
Male	10	36.2	18.0	0.20	0.53		
MA (mm)							
Female	10	21.1	8.7	0.20	0.91	0.40	0.14
Male	10	15.9	6.5	0.20	0.20		
LY30 (%)							
Female	10	29.2	25.1	0.039	0.11	-	-
Male	10	36.4	23.5	0.20	0.70		
LY60 (%)							
Female	10	49.5	24.8	0.16	0.34	0.82	0.43
Male	10	58.3	23.7	0.20	0.18		

Table 5.10 Preliminary statistical analyses for the calculation of normal ranges for the TEG with TPA on citrated PPP, divided by sex

Before establishing the normal ranges, the six TEG parameters were checked again to confirm their normal distribution in the overall population, including both males and females. All tests for normality gave $p>0.05$, except for the K time (Kolmogorov-Smirnov test $p=0.011$, Shapiro-Wilk test $p=0.008$). Therefore, given the fact that the angle and LY30 were not normally distributed in the two sexes and that the K time was not normally distributed in the overall population, these three normal ranges were calculated differently. For the R time, the MA, and the LY60, the normal ranges were calculated using the formula mean ± 1.96 * SD. For the K time, the angle and the LY30, the normal ranges were calculated as 2.5^{th} percentile to 97.5^{th} percentile (Table 5.11).

	N. of tests	Results, mean (SD) or median (IQR)	Normal ranges	
			Lower limit	Upper limit
R time (min)	20	13.0 (2.6)	7.9	18.1
K time (min)	9	2.8 (2.4-6.4)	2.1	10.7
Angle (deg)	20	33.9 (24.7-54.1)	10.3	60.9
MA (mm)	20	18.5 (7.9)	3.0	34.0
LY30 (%)	20	31.3 (11.1-55.2)	1.5	76.9
LY60 (%)	20	53.9 (24.0)	6.9	100

Table 5.11 Normal ranges for the TEG with TPA on citrated PPP

5.4.4.2 Effect of TPA addition on the normal PPP

In order to evaluate the effect of TPA addition, the results of the native TEG performed on the normal PPP and the TEG with the addition of TPA performed on the same day on the same normal PPP were compared. An example of the effect of TPA on the TEG curve is shown in Figure 5.13.

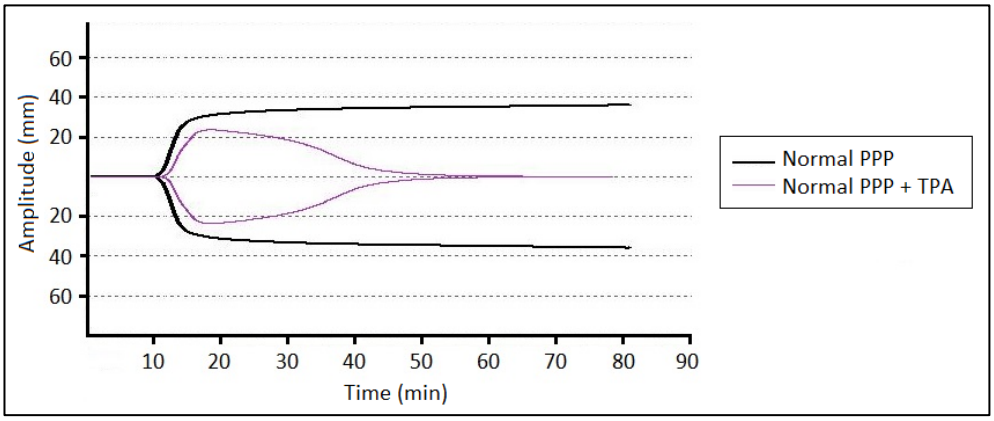


Figure 5.13 TEG with TPA performed on normal PPP

Thirty-two pairs of results were available (Table 5.12). Since there is no lysis in PPP without TPA addition, in the native TEG percent lysis at 30 min and 60 min after MA

(LY30 and LY60) was always 0, fibrinolytic status at 30 min and 60 min after MA (CL30 and CL60) was always 100%, the LTE was always >3 hours, and the amplitude at 30 min and 60 min after MA was always the same as the MA. The MA, G parameter, E parameter, TPI, and CI were normally distributed in both groups and therefore compared using a parametric test (paired samples t-test), while the other parameters were compared using a non-parametric test (Wilcoxon matched-pairs signed-ranks test).

With the exception of the R time and the time to the split point (SP), all the other parameters were significantly modified by the addition of TPA. For instance, K time was significantly longer, angle and MA were significantly reduced.

	Native TEG (n=32)		TEG with TPA (n=32)		P value
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**	
Main TEG parameters					
R time (min)	13.05 (2.64)	12.40 (11.25-14.20)	12.99 (1.90)	12.95 (11.90-14.70)	0.59
K time (min)	2.78 (0.66)	2.65 (2.25-3.20)	4.44 (3.24)	3.60 (3.10-4.40)	<0.001
Angle (deg)	51.77 (7.18)	53.75 (47.40-56.55)	42.92 (10.55)	45.85 (37.65-50.60)	<0.001
MA (mm)	32.44 (1.93)	32.00 (30.95-34.10)	27.07 (3.75)	27.00 (23.95-29.65)	<0.001
LY30 (%)	0	0	26.80 (19.76)	20.15 (12.95-46.55)	<0.001
LY60 (%)	0	0	51.39 (21.80)	54.45 (31.60-73.65)	<0.001
Secondary TEG parameters					
SP (min)	11.73 (2.27)	11.20 (10.20-13.15)	11.22 (1.71)	11.45 (10.25-12.30)	0.39
TMA (min)	23.23 (3.72)	22.55 (20.45-24.85)	21.08 (5.14)	19.70 (17.60-23.60)	0.010
G parameter (dyn/cm^2)	2.42 (0.21)	2.35 (2.20-2.60)	1.88 (0.36)	1.85 (1.60-2.10)	<0.001
E parameter (dyn/cm^2)	48.14 (4.25)	47.00 (44.80-51.70)	37.48 (7.21)	37.05 (31.55-42.15)	<0.001
TPI (/sec)	9.16 (2.26)	9.25 (7.65-10.60)	5.07 (1.87)	4.70 (3.75-6.70)	<0.001
CI	-4.05 (0.73)	-3.80 (-4.40 to -3.60)	-4.65 (0.84)	-4.60 (-5.50 to -3.95)	<0.001
A30 (mm)	32.44 (1.93)	32.00 (30.95-34.10)	12.39 (9.04)	11.80 (1.95-19.70)	<0.001
CL30 (%)	100	100	43.83 (30.53)	44.00 (8.25-70.40)	<0.001
A60 (mm)	32.44 (1.93)	32.00 (30.95-34.10)	4.23 (5.50)	1.05 (0.10-7.00)	<0.001
CL60 (%)	100	100	15.29 (20.50)	4.25 (0.06-23.30)	<0.001
LTE (min)	>3h	>3h	51.62 (21.10)	49.60 (29.80-66.90)	-

Table 5.12 TEG with TPA: effect on the normal PPP

5.4.4.3 Effect of TPA addition on the anticoagulated plasma

The different anticoagulant concentrations were tested with TPA 10 times (with the exception of danaparoid 0.33 U/ml, for which only nine results were available). The K time was undefined when the MA was < 20 mm, which occurred in some samples with INR 3.24 (n=4), INR 4.11 (n=4), apixaban 89 ng/ml (n=1), apixaban 128 ng/ml (n=2), edoxaban 51 ng/ml (n=7), edoxaban 85 ng/ml (n=9), rivaroxaban 118 ng/ml (n=5), rivaroxaban 174 ng/ml (n=5), argatroban 3.10 μg/ml (n=8), bivalirudin 13.8 μg/ml (n=6), dabigatran 92 ng/ml (n=5), dabigatran 148 ng/ml (n=4), and in all samples with the following concentrations argatroban 0.53 μg/ml, bivalirudin 5.9 μg/ml, danaparoid 0.33 U/ml, enoxaparin 0.35 U/ml, fondaparinux 0.64 μg/ml. Results are reported in Table 5.13 and were also expressed as ratio to the normal PPP with TPA addition run as baseline on the same day in Appendix D (Table D5).

		R time (min)	K time (min)	Angle (deg)	MA (mm)	LY30 (%)	LY60 (%)
Normal ranges		***7.9-18.1***	***2.1-10.7***	***10.3-60.9***	***3.0-34.0***	***1.5-76.9***	***6.9-100***
Warfarinised plasma							
INR 2.22		10.77 (1.19)	3.03 (0.96)	50.19 (8.99)	31.55 (3.82)	37.08 (22.94)	61.18 (19.08)
INR 3.24		21.01 (3.13)	6.52 (3.10)	26.64 (11.99)	20.67 (3.44)	60.75 (17.51)	76.01 (15.62)
INR 4.11		19.24 (2.81)	9.35 (1.84)	20.91 (5.23)	20.16 (2.90)	37.73 (16.94)	57.66 (19.04)
Direct factor Xa inhibitors							
Apixaban	89 ng/ml	15.60 (3.07)	7.56 (3.86)	32.30 (9.26)	25.10 (3.69)	41.95 (22.68)	59.85 (24.08)
	128 ng/ml	16.22 (3.10)	9.45 (3.39)	25.37 (7.49)	22.43 (3.69)	33.46 (23.63)	54.27 (20.72)
Edoxaban	51 ng/ml	21.71 (5.76)	6.93 (0.90)	28.01 (3.52)	18.10 (4.81)	58.21 (26.80)	74.32 (23.21)
	85 ng/ml	22.72 (4.01)	6.8 (NC)	19.49 (6.69)	14.57 (3.39)	72.02 (16.36)	83.68 (13.78)
Rivaroxaban	118 ng/ml	18.56 (3.73)	4.9 (1.68)	29.28 (14.74)	21.47 (8.30)	43.36 (20.94)	67.30 (13.70)
	174 ng/ml	22.07 (4.18)	9.16 (1.79)	24.12 (6.23)	18.50 (4.74)	48.80 (30.00)	64.95 (28.87)
Direct thrombin inhibitors							
Argatroban	0.53 µg/ml	22.64 (4.88)	-	27.89 (6.70)	14.52 (2.47)	82.52 (5.87)	91.20 (2.71)
	3.10 µg/ml	39.79 (7.38)	6.95 (3.32)	16.48 (8.18)	11.69 (9.78)	72.16 (30.02)	81.58 (24.99)
Bivalirudin	5.9 µg/ml	42.76 (7.88)	-	19.34 (8.11)	12.98 (4.29)	61.84 (19.99)	80.97 (9.91)
	13.8 µg/ml	50.72 (17.48)	9.23 (0.68)	20.16 (8.49)	17.92 (7.40)	28.25 (26.66)	52.11 (26.51)
Dabigatran	92 ng/ml	25.09 (5.09)	5.26 (1.26)	38.35 (8.54)	19.78 (3.32)	67.99 (11.69)	82.82 (9.60)
	148 ng/ml	30.81 (6.83)	6.12 (3.15)	35.96 (12.17)	20.54 (3.96)	74.15 (16.79)	86.86 (8.30)
Indirect factor Xa inhibitors							
Danaparoid 0.33 U/ml		43.40 (14.85)	-	4.89 (3.05)	4.92 (2.10)	52.64 (16.82)	73.19 (12.25)
Enoxaparin 0.35 U/ml		31.35 (9.66)	-	8.16 (3.28)	7.34 (4.20)	64.26 (17.26)	79.93 (11.98)
Fondaparinux 0.64 µg/ml		32.53 (9.14)	-	8.31 (3.21)	6.39 (2.95)	70.75 (10.67)	84.45 (4.61)

Table 5.13 Results of the different anticoagulant concentrations on the TEG with TPA
Results are reported as mean (SD) of 10 measurements.

Lysis parameters: LY30 and LY60

The mean results of the LY30 and LY60 did not show any progression in the warfarinised plasma with increasing INR values, neither when they were expressed as mean LY30 % and LY60 % (Table 5.13), nor when they were expressed as LY30 ratio and LY60 ratio (Table D5 in Appendix D).

For the direct factor Xa inhibitors, the LY30 % did not show any consistent trend (decreased for apixaban, increased for edoxaban and rivaroxaban), while the LY30 ratio showed increasing values with increasing concentrations. Both the mean LY60 % (decreased for apixaban and rivaroxaban, increased for edoxaban) and the LY60 ratio (decreased for edoxaban, increased for apixaban and rivaroxaban) did not show any consistent trend with increasing concentrations.

For the direct thrombin inhibitors, both the LY30 % (decreased for argatroban and bivalirudin, increased for dabigatran) and the LY30 ratio (decreased for bivalirudin, increased for argatroban and dabigatran) did not show any consistent trend with increasing concentrations. Similarly, both the LY60 % (decreased for argatroban and bivalirudin, increased for dabigatran) and the LY60 ratio (decreased for argatroban and bivalirudin, increased for dabigatran) did not show any consistent trend with increasing concentrations.

Finally, for the indirect factor Xa inhibitors only the prophylactic concentrations could be evaluated, since higher concentrations were producing flat traces. Therefore, no trend could be shown with anticoagulant concentrations.

With the exception of bivalirudin, there was no significant correlation between the anticoagulant concentrations and the LY30 and the LY60 (Table 5.14). Finally, box plots representing the median value and the interquartile range were created and showed wide variability between the different TEG runs (Figures 5.14-5.17).

Anticoagulant	N. of test*	LY30: correlation coefficient (p value)	LY60: correlation coefficient (p value)
Warfarin**	30	0.01 (0.96)	-0.06 (0.77)
Direct factor Xa inhibitors			
Apixaban	20	-0.21 (0.38)	-0.12 (0.61)
Edoxaban	20	0.33 (0.16)	0.31 (0.18)
Rivaroxaban	20	0.12 (0.61)	0.10 (0.66)
Direct thrombin inhibitors			
Argatroban	20	0.07 (0.77)	-0.01 (0.97)
Bivalirudin	20	-0.56 (0.011)	-0.62 (0.003)
Dabigatran	20	0.42 (0.07)	0.39 (0.09)
Indirect factor Xa inhibitors* **			
Danaparoid	9	NC	NC
Enoxaparin	10	NC	NC
Fondaparinux	10	NC	NC

Table 5.14 Correlation between anticoagulant concentrations and the LY30 and LY60 on the TEG with TPA

* Each concentration has been tested 10 times

** For the warfarinised plasma, the INR values have been considered instead of the concentrations.

*** The indirect factor Xa inhibitors were not computable because only the thromboelastograms of the lowest concentrations were available

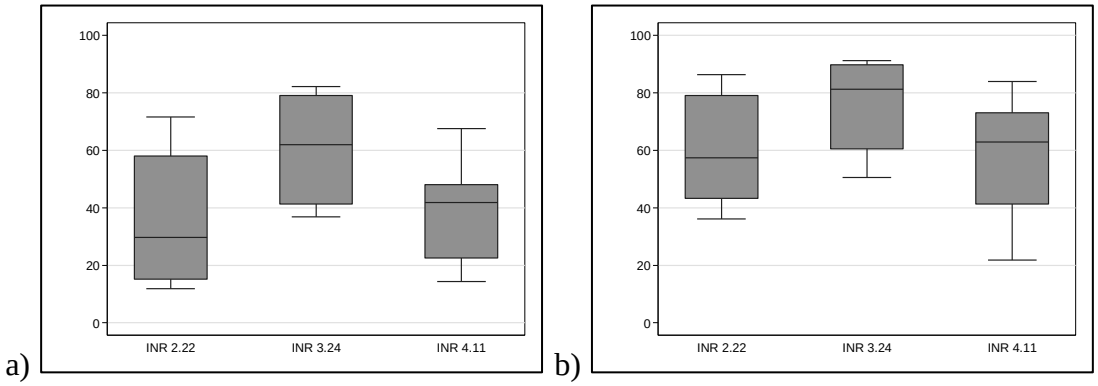


Figure 5.14 Box plots of the lysis parameters of the warfarinised plasma: LY30 (a) and LY60 (b)

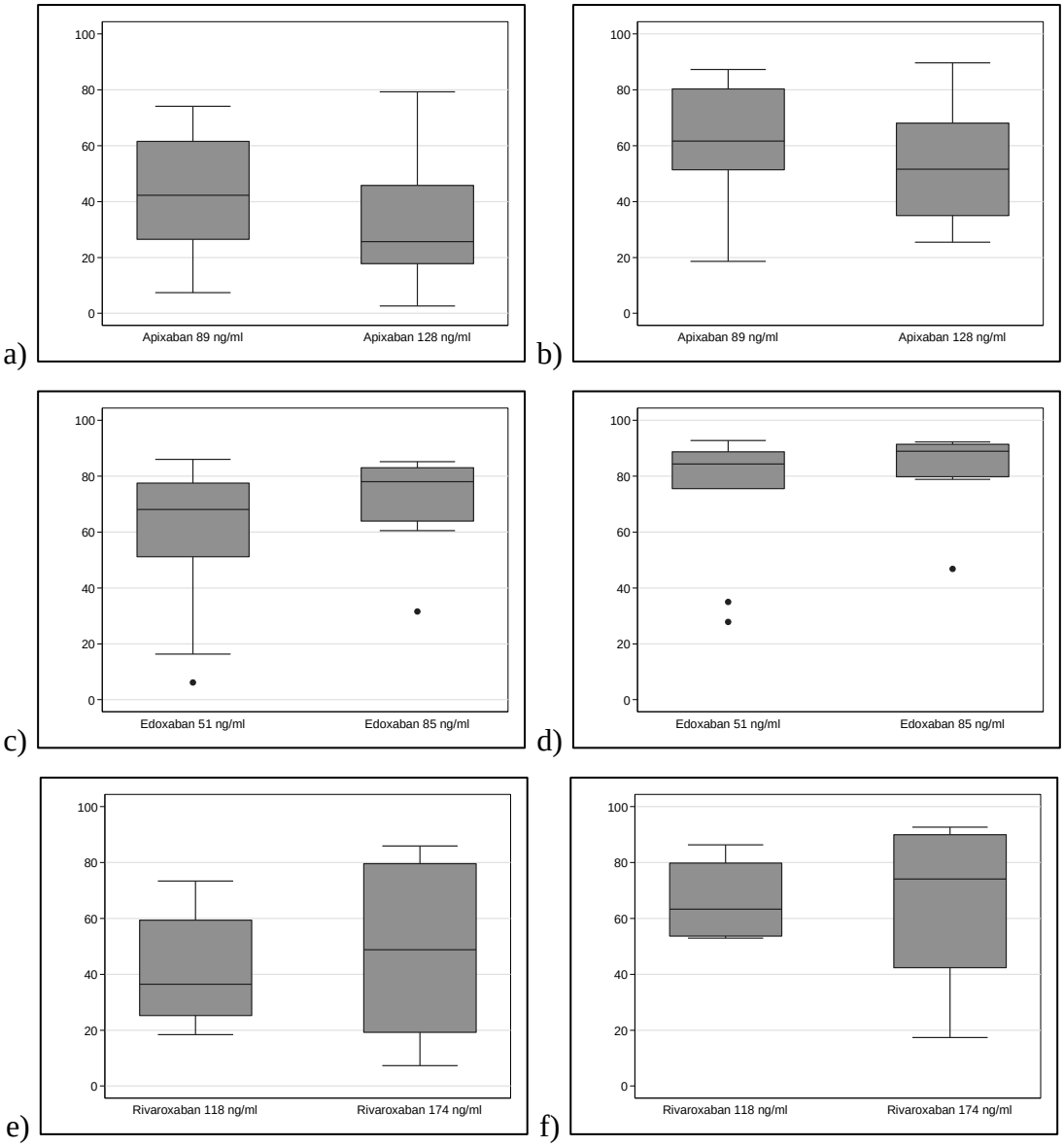


Figure 5.15 Box plots of the lysis parameters of the plasma spiked with the direct factor Xa inhibitors: apixaban LY30 (a) and LY60 (b), edoxaban LY30 (c) and LY60 (d), rivaroxaban LY30 (e) and LY60 (f)

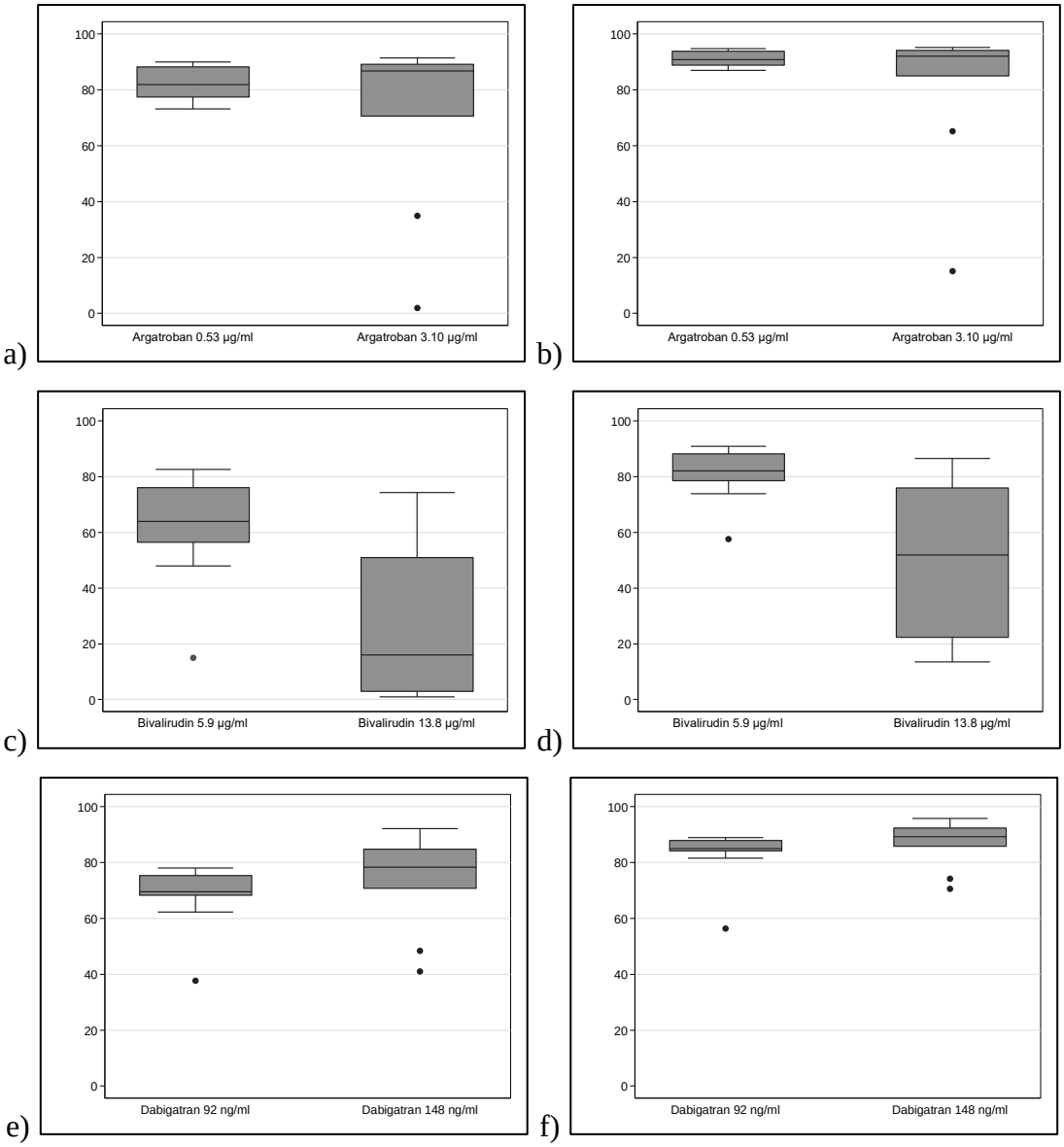
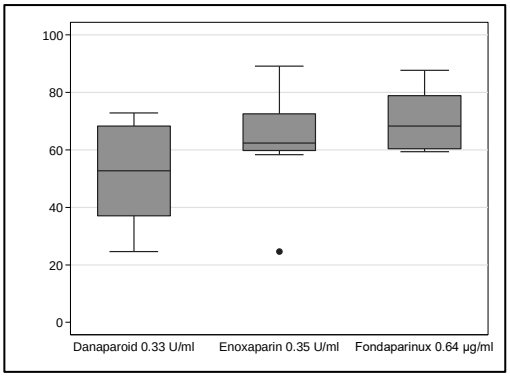


Figure 5.16 Box plots of the lysis parameters of the plasma spiked with the direct thrombin inhibitors: argatroban LY30 (a) and LY60 (b), bivalirudin LY30 (c) and LY60 (d), dabigatran LY30 (e) and LY60 (f)

a) 

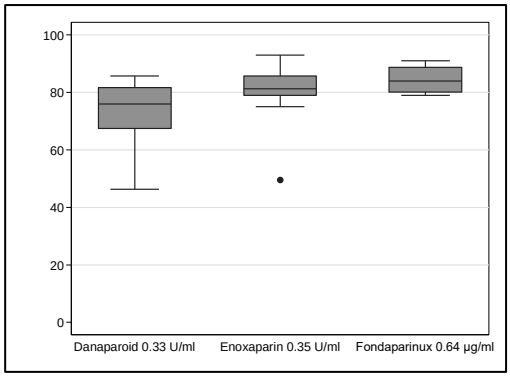
b) 

Figure 5.17 Box plots of the lysis parameters of the plasma spiked with the indirect factor Xa inhibitors: LY30 (a) and LY60 (b)

Maximum amplitude

Given the wide variability in the lysis parameters, it was decided to perform further analysis on the MA, which appeared to be the most consistent parameter. A ratio between the mean MA of the TEG with TPA and the mean of the native TEG (reported in paragraph 5.4.3) was calculated for each anticoagulant concentration. Results are reported in Table 5.15 and showed that, with the exception of bivalirudin, there was a decrease in the MA ratio with increasing anticoagulant concentrations. These results suggest that clot strength when challenged by TPA addition (as represented by the decrease in the MA with TPA compared to the baseline MA) decreased with increasing anticoagulant concentrations, suggesting that these clots are less stiff and already subjected to a certain degree of lysis.

Results are also reported in decreasing MA ratio order in Figure 5.18. The MA of the normal PPP when challenged by TPA was 83% of the baseline MA of the normal PPP, similar to the warfarinised plasma with INR 2.22. Results between 70% and 60% were obtained, in decreasing order, by dabigatran 92 ng/ml, apixaban 128 ng/ml, rivaroxaban 174 ng/ml and edoxaban 51 ng/ml. Results between 59% and 50% were

obtained, in decreasing order, by bivalirudin 13.8 μg/ml, dabigatran 148 ng/ml, warfarin INR 3.24 and INR 4.11, edoxaban 85 ng/ml. The indirect factor Xa inhibitors, even though they were tested at prophylactic concentrations, were providing the lowest ratios (between 26% and 18%).

Plasma		**MA with TPA, mean (SD)**	**MA without TPA, mean (SD)**	**MA ratio**
Normal PPP		27.07 (3.75)	32.44 (1.93)	0.83
Warfarinised PPP	INR 2.22	31.55 (3.82)	37.80 (2.83)	0.83
	INR 3.24	20.67 (3.44)	37.05 (0.49)	0.56
	INR 4.11	20.16 (2.90)	38.05 (1.34)	0.53
Apixaban	89 ng/ml	25.10 (3.69)	31.10 (2.55)	0.81
	128 ng/ml	22.43 (3.69)	33.40 (6.65)	0.67
Edoxaban	51 ng/ml	18.10 (4.81)	30.25 (5.44)	0.60
	85 ng/ml	14.57 (3.39)	27.85 (3.89)	0.52
Rivaroxaban	118 ng/ml	21.47 (8.30)	29.20 (2.12)	0.74
	174 ng/ml	18.50 (4.74)	28.40 (0.57)	0.65
Argatroban	0.53 μg/ml	14.52 (2.47)	29.40 (0.85)	0.49
	3.10 μg/ml	11.69 (9.78)	32.30 (1.84)	0.36
Bivalirudin	5.9 μg/ml	12.98 (4.29)	30.95 (0.07)	0.42
	13.8 μg/ml	17.92 (7.40)	30.55 (0.35)	0.59
Dabigatran	92 ng/ml	19.78 (3.32)	29.00 (1.27)	0.68
	148 ng/ml	20.54 (3.96)	36.10 (2.97)	0.57
Danaparoid 0.33 U/ml		4.92 (2.10)	27.10 (5.94)	0.18
Enoxaparin 0.35 U/ml		7.34 (4.20)	28.20 (0.99)	0.26
Fondaparinux 0.64 μg/ml		6.39 (2.95)	27.85 (0.07)	0.23

Table 5.15 Results of the MA on the TEG with and without TPA for the different anticoagulant concentrations

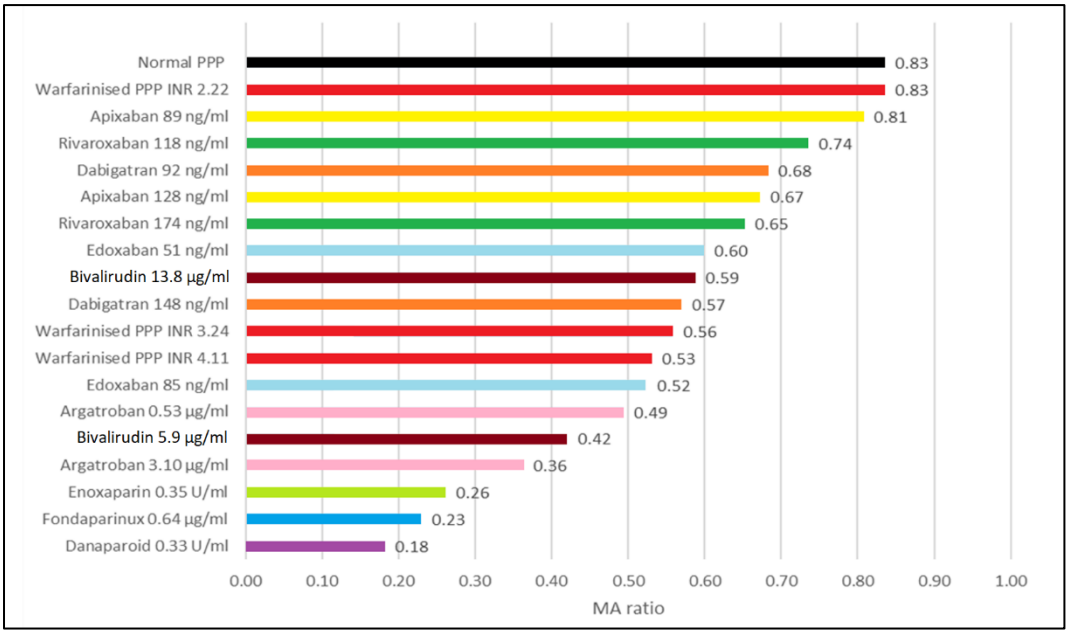


Figure 5.18 Results of the MA ratio of the different anticoagulant concentrations
Different concentrations of the same anticoagulant drug have been plotted using the same colour.

5.5 Discussion

This study analysed the profile of several anticoagulants, with a particular focus on the DOACs, using different assays that can assess the coagulation overall and the fibrinolysis. The effect of increasing anticoagulant concentrations was initially evaluated on routine coagulation assays, such as the APTT and the PT/INR. As previously reported (Exner, Ahuja, et al., 2019; Mani, 2014), the direct thrombin inhibitor dabigatran showed a greater effect on the APTT, while the direct factor Xa inhibitors had a more pronounced effect on the PT. Although the APTT and the PT/INR should not be used to estimate the plasma concentrations of the DOACs, the guidelines of the British Committee for Standards in Haematology recommend that each laboratory should be aware of the sensitivity of its own APTT and PT/INR to the presence of the DOACs (S. Kitchen et al., 2014). The APTT was quite sensitive to dabigatran, being raised above the normal ranges with dabigatran concentration < 50

ng/ml. The PT was raised above the normal ranges with concentrations of rivaroxaban and edoxaban slightly >50 ng/ml, while the sensitivity for apixaban was less pronounced. Although the APTT and the PT/INR were measured only once and on pooled plasma spiked *in vitro* with the DOAC, these results confirmed previous reports on the different sensitivity of the PT to the direct factor Xa inhibitors in patients plasma (Patel et al., 2015; Patel, Chitongo, et al., 2019; Patel et al., 2013).

The different classes of anticoagulant drugs showed diverse patterns when tested on the CAT. The direct thrombin inhibitors had a more pronounced effect on the lag time, with thrombin generation curves shifted to the right, while the direct and indirect factor Xa inhibitors had a more pronounced effect on the peak, with flattened thrombin generation curves. These findings confirmed the results of a previous study which spiked normal pooled plasma with apixaban, rivaroxaban and dabigatran and obtained similar curves (Rigano et al., 2018). However, when the same spiking experiment was repeated on different normal plasma samples, high inter-individual variability was observed (Rigano et al., 2018).

The CAT was more sensitive to the presence of the DOACs than the routine coagulation assays (APTT and PT) and was also more sensitive than the chromogenic anti-Xa or the chronometric dTT assays, specifically calibrated to evaluate the DOAC concentrations. In fact, the lag time on the CAT was already prolonged above the normal ranges at the lowest concentrations that were tested (apixaban 4 ng/ml, edoxaban 0 ng/ml, rivaroxaban 22 ng/ml, dabigatran 0 ng/ml), concentrations that were giving results below the lower limit of quantification for the anti-Xa and dTT assays (< 30 ng/ml). The ETP was below the normal ranges for all the tested warfarinised samples and from DOACs concentrations 50-100 ng/ml upwards.

The paradoxical rise of the peak with low concentrations of dabigatran and bivalirudin has been observed before with the direct thrombin inhibitors, whose activity is independent from AT (Samama et al., 2007; Xu et al., 2013). A possible explanation lies in the suppression of the negative feedback by the natural anticoagulant protein C, which is activated by the complex thrombin-thrombomodulin (Furugohri et al., 2011); however, the protein C pathway was not measured in these experiments. A more plausible explanation is the interaction of the direct thrombin inhibitors with the α_2-macroglobulin-thrombin complex used as calibrator in the CAT, which is erroneously interpreted as increased thrombin generation (Wagenvoord et al., 2010). Similarly to the current study, a paradoxical increase of the peak and the ETP at low concentrations of the direct thrombin inhibitors has been previously reported (Gribkova et al., 2016; Wagenvoord et al., 2010).

The CAT was also very sensitive to the presence of the indirect factor Xa inhibitors, which showed a greater reduction of the peak compared to the direct factor Xa inhibitors, even at prophylactic concentrations. This finding can be explained by their mechanism of action, since their inhibition of factor Xa is mediated by AT (Garcia et al., 2012).

Sensitivity of the native TEG to the different classes of anticoagulant drugs was widely variable. Differences between the CAT and the native TEG results could be due to the different assay techniques, since the native TEG evaluated mainly the intrinsic pathway, while the CAT with the addition of TF evaluated mainly the extrinsic pathway of the coagulation cascade. In the warfarinised plasma a positive correlation was observed between increasing INR values and the R time; however, the results were within or just above the normal ranges, for the tested INR values up to 4.11. These findings were in line with a previous study showing normal TEG results in 45%

of patients on warfarin with INR values between 1.6 and 4.2 (Dunham et al., 2014). With the exception of apixaban, the R time appeared to be the TEG parameter mostly correlated with the presence of the DOACs. It was already prolonged above the normal ranges for edoxaban concentration 85 ng/ml, rivaroxaban 55 ng/ml, dabigatran 44 ng/ml. With regards to apixaban, most of the results up to concentration 266 ng/ml were within the normal ranges. A previous study evaluating apixaban concentrations 250 ng/ml, 500 ng/ml and 1000 ng/ml reported a significant prolonged R time only for the highest concentration (Dias et al., 2015).

All the MA values were within the normal ranges, thus showing the lack of interference of the DOACs with the fibrin component of the clot strength, as previously reported (Dias et al., 2015). The TEG was very sensitive to the presence of the indirect factor Xa inhibitors, which were giving significantly prolonged R time and K time, and significantly decreased angle, at prophylactic concentrations and were giving flat curves at therapeutic and over-therapeutic concentrations. A previous study showed that all standard TEG parameters were affected by low concentrations of UFH, dalteparin and danaparoid when spiked on CWB (Coppell et al., 2006). However, the MA in the current study was not significantly affected, which can be partly explained by the fact that PPP was tested on the TEG, instead of CWB, therefore the MA was dependent more on fibrinogen than on platelets.

The most innovative part of this study was the TEG with the addition of TPA, in order to assess the fibrin clot resistance to fibrinolysis and, therefore, the final clot strength. There were only a few published studies using *in vitro* TEG-TPA models and they were mainly in different contexts, such as healthy volunteers (Foley et al., 2012; Genét et al., 2012; Godier et al., 2017) or haemophiliac patients (Dargaud et al., 2011). Only one study evaluated the effect of TPA addition on thrombin inhibitors (Xu et al., 2013),

while warfarin and the factor Xa inhibitors were never tested on the TEG with TPA before. In addition, several variations of the TEG-TPA assay have been reported. While the above mentioned studies were all performed on CWB, different concentrations of TPA have been tested and some authors used kaolin as activator (Genét et al., 2012; Godier et al., 2017), while others used TF (Dargaud et al., 2011; Foley et al., 2012; Xu et al., 2013). After several trials (reported in paragraph 2.14.3), an *in vitro* TEG-TPA model was developed, which consisted of native TEG (to evaluate the contact activation pathway), citrated PPP (to reduce inter-subject variability) and TPA diluted in HEPES buffer to final plasma concentration 0.52 μg/ml.

Since high variability in the lysis parameters was already reported when using TPA, it was suggested to dilute TPA in a solution of 0.2% saline/bovine serum albumin, to use TF at concentration 1/500 and to mix the TPA with the whole blood before pipetting in the TEG cup (Kupesiz et al., 2010). Despite using a different *in vitro* TEG-TPA model, in the current study the addition of TPA to normal PPP resulted in no effect on the R time, a significant prolongation of the K time and a significant reduction of the angle and the MA, similarly to a previous report (Genét et al., 2012). Since an acceptable degree of variability of the MA with TPA was observed (intra-assay CV 7.7%, inter-assay CV 11.6%), further analyses were performed on this parameter, which represents the fibrin clot resistance towards fibrinolysis.

Several interesting findings emerged when analysing the different classes of anticoagulants. The indirect factor Xa inhibitors, despite being tested at prophylactic concentrations, showed the most pronounced effect on the MA obtained with TPA. Considering that the mean MA of the PPP was 27.07 mm, the mean MA obtained with danaparoid 0.33 U/ml was 4.92 mm, with enoxaparin 0.35 U/ml was 7.34 mm, and

with fondaparinux 0.64 μg/ml was 6.39 mm. There are some reports in the literature showing that LMWH is associated with the formation of thin fibrin fibres (Collen et al., 2000) and with changes in the nanostructure of the fibrin fibres (Yeromonahos et al., 2012), therefore the important effect of LMWH on the MA obtained in the current TEG-TPA model could be due to a different clot structure. However, thin fibres usually form a tight fibrin network with small pores, which is associated with decreased influx of fibrinolytic enzymes into the clot and reduced fibrinolysis (He et al., 2010), but no significant differences were observed in the lysis parameters in the current study. Whether a similar effect on fibrin clot structure could also be obtained with the other indirect factor Xa inhibitors has not been demonstrated yet, since in the study by Yeromonahos et al. (2012) fondaparinux was not associated with any significant modification in the fibrin fibres, while the study by He et al. (2010) reported increased porosity of the fibrin network formed with danaparoid.

A reduction of the MA obtained with TPA was observed with increasing concentrations of most anticoagulants, with the exception of bivalirudin and dabigatran. In fact, the mean MA obtained with bivalirudin 5.9 μg/ml was 12.98 mm, while with bivalirudin 13.8 μg/ml it was 17.92 mm. Similarly, the mean MA obtained with dabigatran 92 ng/ml was 19.78 mm, while with dabigatran 148 ng/ml it was 20.54 mm. There are some reports that the clot formed in the presence of dabigatran has thick fibres (Ammollo et al., 2010) and that bivalirudin forms a fibrin network with large pores (He et al., 2010). However, in the study by He et al. (2010), argatroban showed a similar effect, but the current TEG-TPA model showed a decrease in the MA with increasing concentrations (the mean MA obtained with argatroban 0.53 μg/ml was 14.52 mm, while with argatroban 3.10 μg/ml was 11.69 mm). Thick fibres usually form a more permeable fibrin network with large pores, which is associated

with higher susceptibility to fibrinolysis (He et al., 2010). Different experimental conditions in the above-mentioned studies make it difficult to compare the results and ongoing future studies will elucidate these results and the underlying mechanism.

Finally, when considering the MA ratio as an index of the final clot strength, it was noted that the addition of TPA did not significantly decrease the final clot strength of the warfarinised plasma with INR 2.22, which was similar to the normal unspiked PPP (both MA ratio 0.83). However, the final clot strength of the warfarinised plasma with INR 3.24 and 4.11 was lower than apixaban 89 ng/ml and 128 ng/ml, dabigatran 92 ng/ml and 148 ng/ml, rivaroxaban 118 ng/ml and 174 ng/ml. These findings suggested that the clot strength could be better preserved in plasma containing the DOACs vs. warfarinised plasma. However, these results need to be interpreted with caution, due to the wide range of plasmatic concentrations after DOAC administration. The reported Cmax for apixaban 5 mg BID was 132 ng/ml (5^{th}-95^{th} percentile 59-302 ng/ml), for dabigatran 150 mg BID was 175 ng/ml (25^{th}-75^{th} percentile 117-275 ng/ml) and for rivaroxaban 20 mg OD was 270 ng/ml (5^{th}-95^{th} percentile 189-419 ng/ml) (Douxfils et al., 2018). Therefore, these results are hypothesis generating only and would need further confirmation through the analysis of fibrin clot structure or through modification of the TEG-TPA *in vitro* model.

This study has some limitations that need to be acknowledged. First, the CAT and the native TEG were performed only in duplicate, and this precluded the possibility to fit a regression line to correlate CAT and native TEG parameters with the actual anticoagulant concentrations. However, the main aim of this study was to evaluate the clot strength using the TEG with TPA, which was the most innovative part. Second, the wide CVs of the TEG with TPA might have hampered the possibility to detect significant differences in the lysis parameters among the different anticoagulant drugs.

Perhaps the choice of a different TEG-TPA *in vitro* model or the use of TEG activators (such as kaolin or TF) might have resulted in more reproducible results. Third, while the range of concentrations of the DOACs covered mainly to the therapeutic range, bivalirudin and argatroban were spiked at several supra-therapeutic concentrations, thus limiting the comparison of results.

The main strengths of this study are the large number of oral and parenteral anticoagulants, pertaining to four different pharmacodynamic classes, that have been tested at the same time under the same experimental conditions. Furthermore, it was the first time that the different anticoagulants have been analysed on the TEG performed on citrated pooled normal PPP, instead of CWB, to eliminate inter-subject variability. Finally, all the experiment on the TEG with TPA have been repeated 10 times, to account for the wide CVs of this assay, and further analyses were performed on the MA, which was the most consistent parameter.

5.6 Conclusion

The routine coagulation assays APTT and PT showed limited utility for DOAC detection. They may suggest the DOAC presence when they are prolonged, even if slightly above the normal ranges, but they cannot exclude the DOAC presence when normal. The CAT was more sensitive to the DOACs than the routine coagulation assays and the specific chromogenic anti-Xa or chronometric DTT assays. The TEG was insensitive to apixaban, while a prolongation of the R time appeared to be a good marker for the presence of edoxaban, rivaroxaban and dabigatran. Finally, there appeared to be some differences in the final clot strength among the different anticoagulants which would need confirmation in future studies.

Chapter 6 :

Reversal and Neutralisation of the Anticoagulant Drugs

6.1 Introduction

Bleeding is a common and feared complication of oral anticoagulant therapy, with a reported incidence of approximately 2% per year in RCTs, but that can reach more than 7% per year in real life data (Wiedermann & Stockner, 2008). Reversal strategies vary according to the different anticoagulants used. In case of a major bleeding complication during VKA treatment, FFP and PCC, together with vitamin K supplementation, are the currently available reversal strategies. However, RCTs in the context of emergency warfarin reversal are difficult to conduct and are subject to selection bias. Despite inconclusive results from studies directly comparing FFP and PCC, current guidelines suggest 4-factor PCC rather than FFP for VKA-associated major bleeding (Holbrook et al., 2012). PCCs are the standard of care in the UK, but in Malta these agents are not available and FFP is currently used for VKA reversal.

In case of a major bleeding complication during DOAC treatment, specific antidotes have been recently developed: idarucizumab for dabigatran (Pollack et al., 2015; Pollack et al., 2017) and andexanet alfa for the factor Xa inhibitors (Connolly et al., 2019; Connolly et al., 2016). Idarucizumab was recently licenced by the EMA (European Medicines Agency, 2015), whereas andexanet alfa so far has been licensed only by the US FDA (Food and Drug Administration, 2019). Furthermore, the cost of these specific reversal agents can be prohibitive for some countries. PCCs (either 3- or 4-factor PCC or activated PCC) and rVIIa are other reversal options for the DOACs, when the specific antidotes are not available, although lacking strong evidence (Garcia & Crowther, 2019).

Neutralisation of the anticoagulant effect can be performed *in vitro*, in order to reduce the impact of the different anticoagulants on coagulation assays. DOAC Stop® is a recently manufactured product with the aim to neutralise the effect of the DOACs in

plasma samples *in vitro* (Exner et al., 2018). This agent might allow thrombophilia testing without the need to stop the anticoagulant treatment. However, there are still some grey areas regarding its use. While there are some data that argatroban and lepirudin might also be removed by the DOAC Stop® (Exner, Ahuja, et al., 2019), its effect on fondaparinux, bivalirudin and warfarin has not been evaluated yet. Furthermore, there is only one study, so far, that utilised a global coagulation assay (the thrombin generation) to compare samples before- and after-DOAC Stop® treatment (Kopatz et al., 2018).

In this chapter different reversal and neutralising agents were studied:

1) The effect of FFP for VKA reversal, by analysing *ex vivo* plasma from patients with bleeding events during VKA treatment and receiving FFP for reversal;
2) The effect of different reversal agents (i.e. FFP, PCC, activated PCC, rVIIa) for DOAC reversal, by analysing plasma spiked *in vitro* with the DOACs and with different concentrations of the reversal agents;
3) The effect of DOAC Stop® *in vitro* on a broad range of oral and parenteral anticoagulants and on a broad range of clotting and global coagulation assays.

6.2 Warfarin-related bleeding events and the effect of fresh frozen plasma

6.2.1 Aim

The aims of this study were:

1) to evaluate the use of the CAT in patients with warfarin-related bleeding, given the hypothesis that bleeding patients might have a different thrombin generation profile compared to not-bleeding patients;
2) to assess the effect of FFP when used for warfarin reversal, by analysing the thrombin generation curves before and after FFP infusion.

6.2.2 Methods

Between July 2015 and January 2018, 30 random samples analysed in the Coagulation Laboratory at Mater Dei Hospital (Msida, Malta) and arriving from the Accident and Emergency Department with a request for INR measurement because of a bleeding event during warfarin treatment, were further processed. After INR measurement, the remaining citrated plasma was centrifuged again and the supernatant was divided into 300 µl aliquots and stored at -80°C until the analysis. Some of these patients received FFP for warfarin reversal and, as part of routine clinical monitoring, their INR was checked again after the infusion. These citrated samples, when available, were processed in the same way and stored. However, due to difficulties in identifying bleeding patients, time from blood collection to storage was widely variable (range 1-24 hours). Frozen samples were shipped to Sheffield (UK) in dry ice and the CAT was performed at TF 5pM (paragraph 2.13), using one calibrator well and two test wells. Before and after FFP samples were always run in the same plate. All samples were analysed in May 2019.

This study was reviewed and approved by the University of Malta Research and Ethics Committee (protocol 21/2015, Appendix B). Due to the fact that this study consisted in a collection of blood samples routinely performed as standard of care, without any interference with routine clinical practice and without any direct contact between the researchers and the patients, the local Ethics Committee waived the need for patients' informed consent. The sample size was chosen as a pilot study, due to the lack of data in literature on the CAT results before and after FFP infusion.

Continuous variables were expressed as mean (SD) or median (IQR); categorical variables were expressed as counts and percentages. Normality was evaluated using the Wilk-Shapiro test. Continuous variables were compared using the Student's t-test for normally distributed variables or the non-parametric Mann-Whitney U test for not-normally distributed variables; categorical variables were compared using the Chi square or Fisher's exact tests, as appropriate. Results of the CAT in patients with bleeding events were compared with a cohort of warfarin patients without bleeding events, for whom thrombin generation results were available (described in Chapter 3, paragraph 3.4.3). However, samples described in Chapter 3 were analysed in 2016, while samples of the current study were analysed in 2019 with a different lot of CAT reagents. High lot-to-lot variation has been reported in the literature for several laboratory assays, and is a potential source of analytical errors (S. Thompson & Chesher, 2018). Thus, the CAT results were expressed as a ratio to the FFP QC plasma results, since this QC was run in every plate and pertained to the same batch.

The non-parametric Wilcoxon matched-pairs signed-ranks test was used to compare the results before and after FFP infusion. Further analyses were performed stratifying by INR values. Normalisation of the thrombin generation curve was defined as values within the normal ranges (reported in paragraph 5.4.2.1) after VKA reversal.

6.2.3 Results

6.2.3.1 A preliminary experiment *in vitro* on warfarinised plasma

A preliminary experiment was performed using a pool of plasma from patients on warfarin treatment with over-therapeutic INR (> 5.0) in February 2016. A 4-factor PCC (Beriplex® P/N, lot 32660111C) was tested in one plate at the following concentrations: 0 U/ml; 0.125 U/ml (corresponding to ~ 5 U/kg); 0.25 U/ml (~ 10 U/kg); 0.375 U/ml (~ 15 U/kg); 0.5 U/ml (~ 20 U/kg); 0.75 U/ml (~ 30 U/kg); 1 U/ml (~ 40 U/kg). FFP was tested in another plate at the following concentrations: 0 µl/ml; 250 µl/ml (corresponding to ~ 10 ml/kg); 500 µl/ml (~ 20 ml/kg); 750 µl/ml (~ 30 ml/kg); 1000 µl/ml (~ 40 ml/kg).

Results are reported as mean (SD) of the two test wells and as a ratio against the results of the FFP QC plasma run in every plate (Table 6.1). The thrombin generation curves with the different concentrations are shown in Figure 6.1. Both the 4-factor PCC and the FFP resulted in a progressive normalisation of the thrombin generation curve with increasing concentrations; however, a different pattern of normalisation was observed with the two reversal agents:

- 4-factor PCC: the lag time and the time to peak were within the normal ranges from concentration 0.125 U/ml upwards, while the peak was normalised from concentration 0.375 U/ml upwards. The ETP, the most relevant parameter of the CAT, was still reduced with concentrations 0.125 U/ml and 0.25 U/ml, normalised with concentrations 0.375 U/ml and 0.5 U/ml, and excessively increased with concentrations 0.75 U/ml and 1 U/ml, therefore suggesting hypercoagulability.

- FFP: the time to peak was within the normal ranges from concentration 250 µl/ml upwards, the lag time from 500 µl/ml upwards, while the peak and the ETP from 750 µl/ml. None of these results suggested hypercoagulability.

	Lag time (min)	**ETP (nM*min)**	**Peak (nM)**	**Time to peak (min)**	**Lag time**	**ETP**	**Peak**	**Time to peak**
Normal ranges	***2.2-4.5***	***1540-2978***	***241-444***	***4.7-8.3***	**Ratio to normal plasma**			
4-factor PCC								
0 U/ml	10.50 (0.17)	372.5 (17.5)	44.35 (1.37)	14.83 (0.17)	2.74	0.16	0.19	1.71
0.125 U/ml	4.50 (0.17)	988.5 (28.5)	135.78 (1.34)	7.50 (0.17)	1.17	0.43	0.57	0.87
0.25 U/ml	3.83 (0.17)	1451.5 (53.5)	192.91 (1.78)	6.83 (0.17)	1.00	0.64	0.82	0.79
0.375 U/ml	3.50 (0.17)	1915.5 (95.5)	248.00 (1.37)	6.50 (0.17)	0.91	0.84	1.05	0.75
0.50 U/ml	3.67 (0)	2497.0 (77.0)	310.08 (1.58)	6.67 (0)	0.96	1.10	1.31	0.77
0.75 U/ml	3.67 (0)	3686.5 (34.5)	409.70 (4.07)	6.83 (0.17)	0.96	1.62	1.73	0.79
1.0 U/ml	3.67 (0)	4602.5 (146.5)	444.46 (5.81)	7.17 (0.17)	0.96	2.02	1.88	0.83
Fresh frozen plasma								
0 µl/ml	10.00 (0)	382.5 (5.5)	58.07 (1.41)	14.00 (0)	2.61	0.21	0.29	1.59
250 µl/ml	4.67 (0)	731.5 (34.5)	130.99 (3.67)	7.67 (0)	1.22	0.41	0.65	0.87
500 µl/ml	4.17 (0.17)	1144.5 (25.5)	196.31 (1.42)	7.00 (0)	1.09	0.64	0.98	0.79
750 µl/ml	4.00 (0)	1896.0 (16.0)	305.24 (5.93)	6.83 (0.17)	1.04	1.06	1.52	0.77
1000 µl/ml	3.67 (0)	2357.5 (28.5)	341.77 (2.54)	6.83 (0.17)	0.96	1.32	1.71	0.77

Table 6.1 The effect of increasing concentrations of different reversal agents on over-therapeutic warfarin, measured using the CAT
Results are reported as mean (SD) of two measurements.

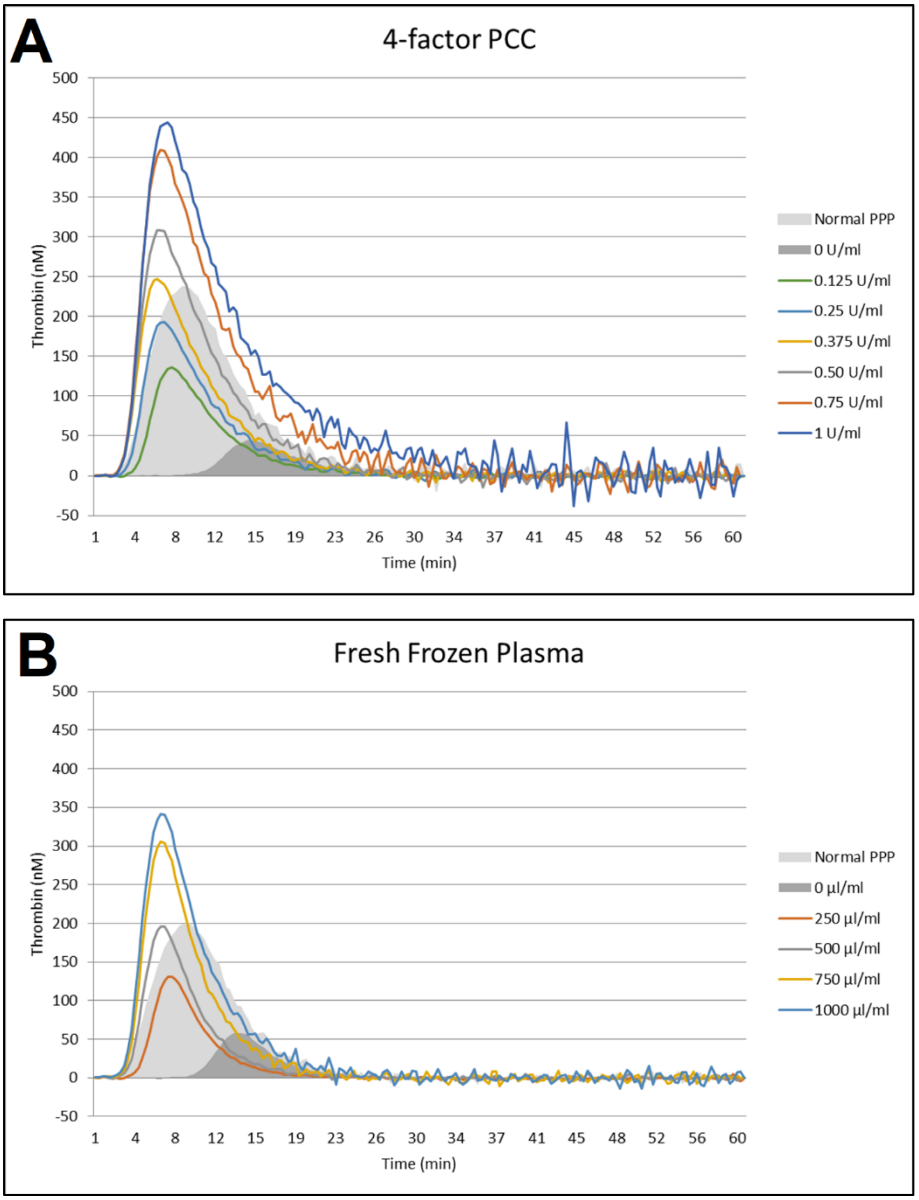


Figure 6.1 Thrombin generation curves with increasing concentrations of different reversal agents on over-therapeutic warfarin: 4-factor prothrombin complex concentrates (A), fresh frozen plasma (B)

6.2.3.2 Patients with warfarin-related bleeding events

Samples from 30 patients with a bleeding event during warfarin treatment were identified and processed as described above. Mean age was 74.2 (± 9.1) years; 19 (63.3%) were males; 22 (73.3%) were anticoagulated because of AF. The most common sites of bleeding were gastrointestinal (8 patients, 26.7%), haematuria (7 patients, 23.3%) and intracranial haemorrhage (5 patients, 16.7%). Other sites of

bleeding were: epistaxis (n=2), rectus sheath haematoma (n=2), soft tissues (n=2), oral cavity (n=1), intraperitoneal (n=1), retroperitoneal (n=1), hemarthrosis (n=1).

Compared to 59 patients on warfarin without bleeding, bleeding patients were significantly older (mean age 74.2 ± 9.1 vs. 68.3 ± 11.4 years, p=0.016) and had higher median INR (3.16 vs. 2.30, p=0.002). Comparison of the CAT results showed that the lag time and the time to peak were prolonged in bleeding patients, while the peak and the velocity index were reduced (Table 6.2). However, when the results were expressed as ratio to normal plasma, only the lag time was significantly different between the two groups.

	Bleeding patients (n=30)	**Not-bleeding patients (n=59)**	**p value**
INR	3.16 (2.25-4.23)	2.30 (1.94-2.69)	0.002
Thrombin generation results			
Lag time (min)	9.55 (6.58-13.00)	6.00 (5.17-7.17)	<0.001
ETP (nM*min)	457.5 (308.5-717.5)	547.5 (419.0-722.5)	0.094
Peak (nM)	67.87 (50.99-107.68)	91.49 (72.51-121.20)	0.008
Time to peak (min)	12.88 (9.25-16.17)	8.83 (7.67-10.17)	<0.001
Velocity index (nM/min)	21.46 (16.06-33.04)	31.34 (25.22-47.91)	0.002
Thrombin generation results (ratio to normal plasma)			
Lag time	2.27 (1.60-3.13)	1.63 (1.45-2.08)	0.009
ETP	0.30 (0.21-0.48)	0.31 (0.24-0.41)	0.89
Peak	0.45 (0.32-0.73)	0.45 (0.35-0.65)	0.93
Time to peak	1.19 (0.90-1.55)	1.02 (0.88-1.18)	0.078
Velocity index	0.98 (0.68-1.46)	0.85 (0.66-1.21)	0.32

Table 6.2 CAT results in warfarin patients with and without bleeding events
Results are reported as median (IQR) and compared using the Mann-Whitney U test.

Since the significant difference in the median INR values between the two groups could partly explain the differences in the CAT results, bleeding and not-bleeding patients were stratified into seven groups based on the INR values: INR ≤ 1.5 (n=2),

INR >1.5-2.0 (n=19), INR >2.0-2.5 (n=28), INR >2.5-3.0 (n=15), INR >3.0-4.0 (n=12), INR >4.0-5.0 (n=7), INR >5.0 (n=6). Apart from the group INR ≤ 1.5, which consisted only of not-bleeding patients, and the group INR >5.0, which consisted only of bleeding patients, in all the other groups median INR values were not significantly different between bleeding and not-bleeding patients. Stratification according to INR values corrected the differences between the two groups in the lag time (Figure 6.2) and the time to peak (Figure 6.3), and showed instead some differences in the ETP and the peak. The ETP was actually higher in bleeding patients, although this result was statistically significant only in the INR categories >2.5-3.0 and >4.0-5.0 (Figure 6.4). Similarly, the peak thrombin was higher in bleeding patients, although this result was statistically significant only in the INR categories >2.0-2.5, >2.5-3.0 and >4.0-5.0 (Figure 6.5). These findings suggested an increase in thrombin generation in order to compensate for the haemorrhagic event.

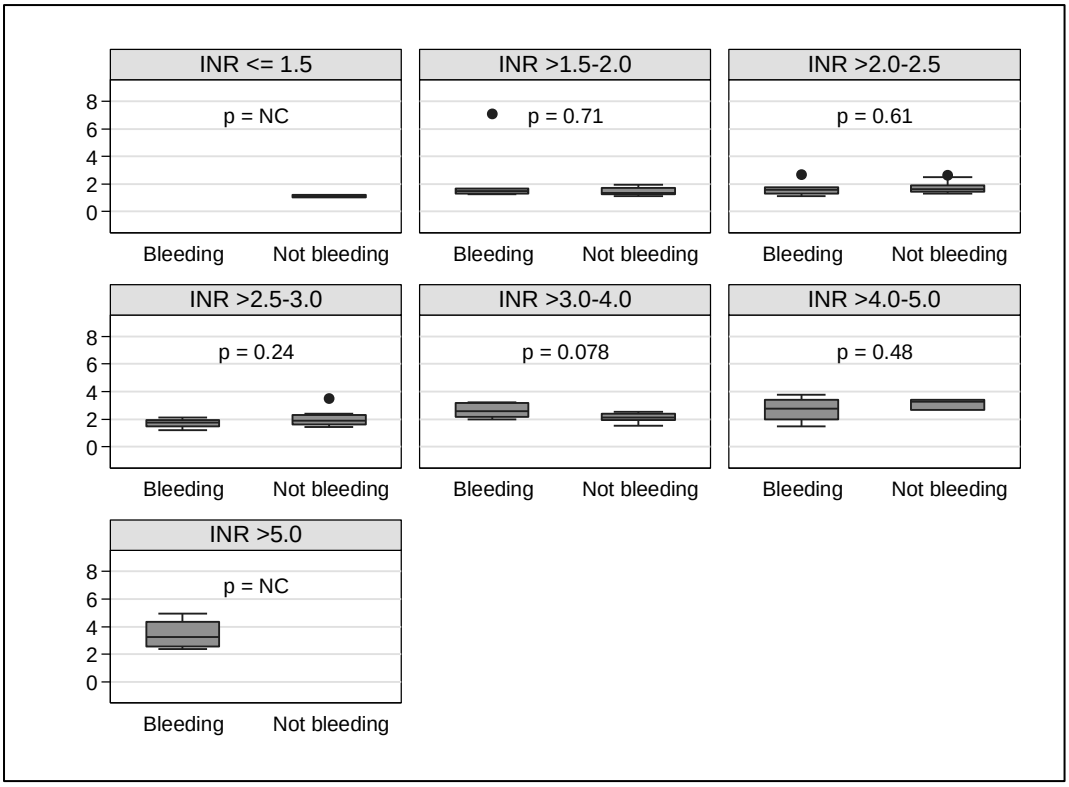


Figure 6.2 Lag time in patients with and without bleeding events, stratified by INR values

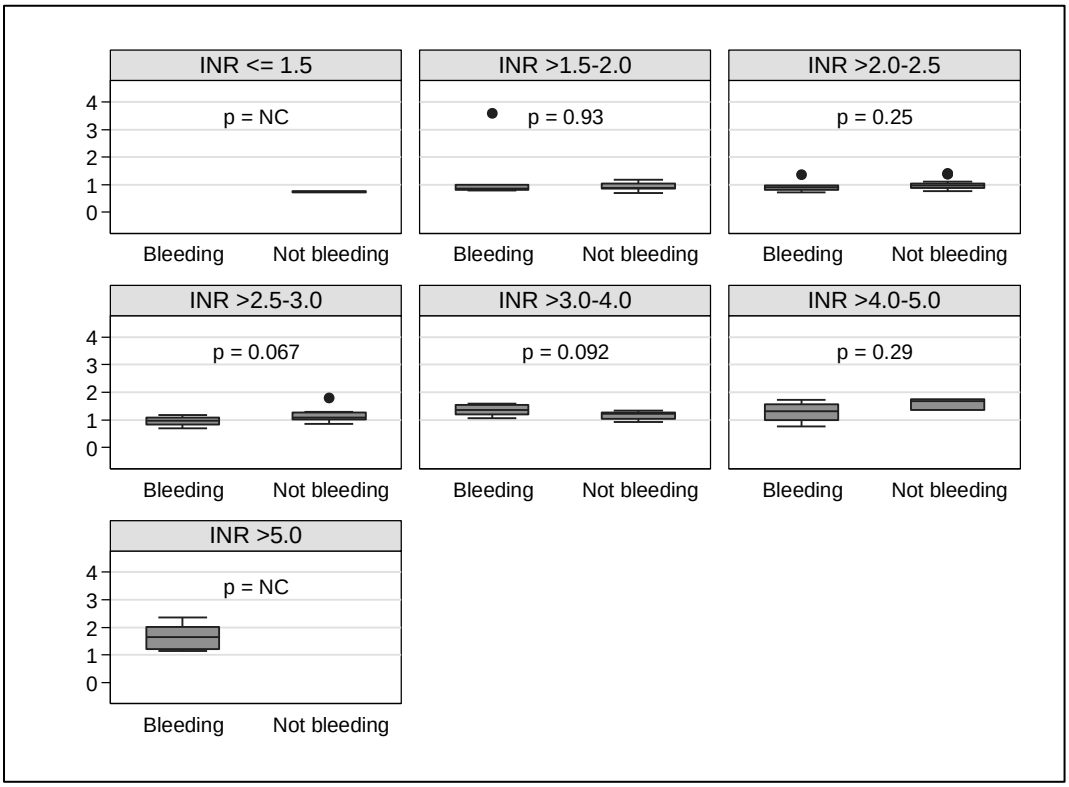


Figure 6.3 Time to peak in patients with and without bleeding events, stratified by INR values

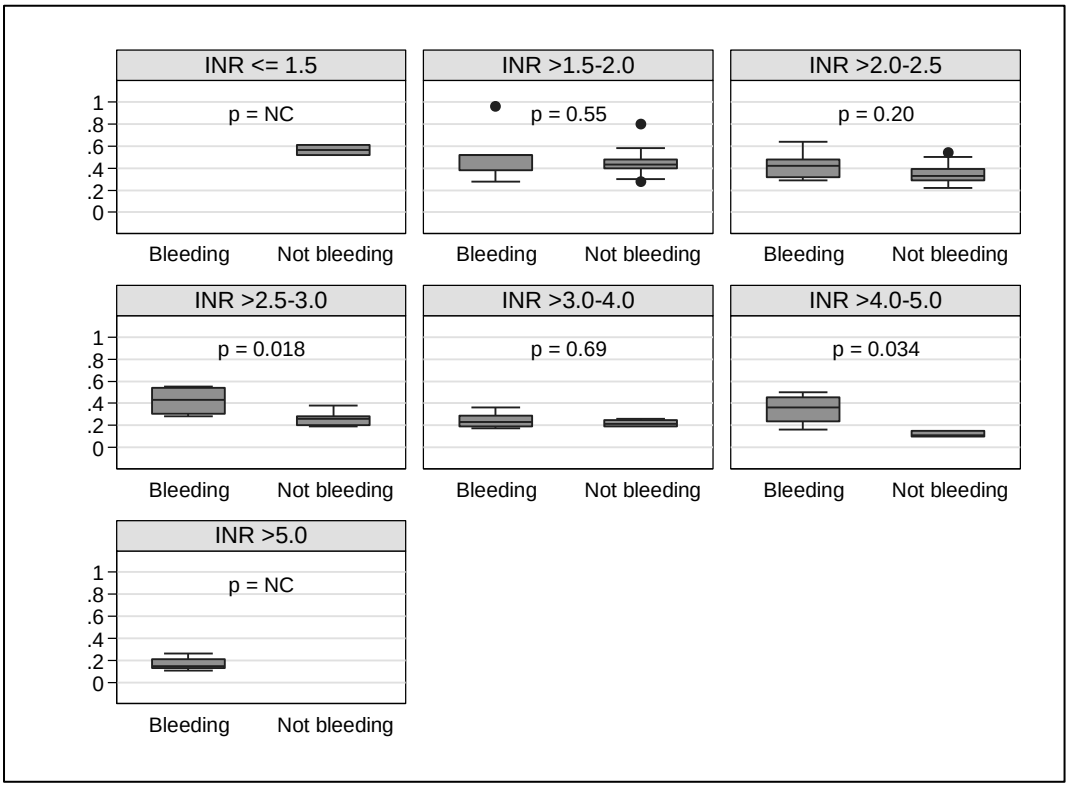


Figure 6.4 ETP in patients with and without bleeding events, stratified by INR values

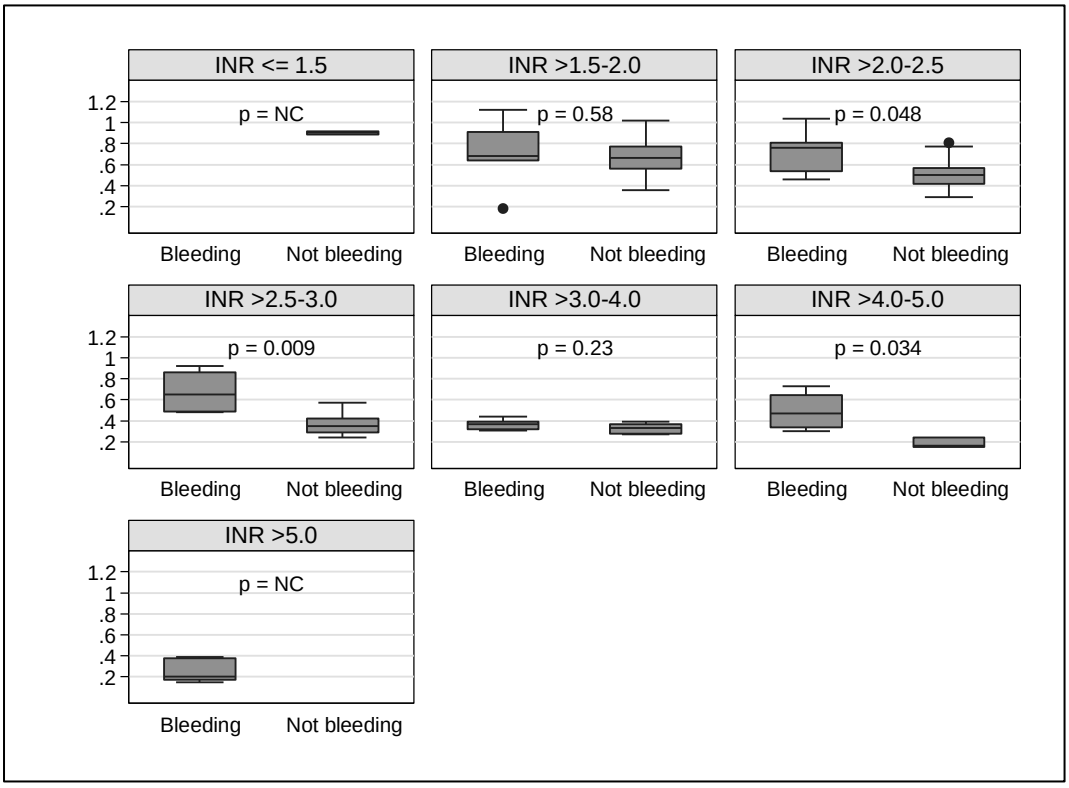


Figure 6.5 Peak thrombin in patients with and without bleeding events, stratified by INR values

6.2.3.3 The effect of fresh frozen plasma for warfarin-reversal

Fourteen patients received FFP for warfarin-reversal. Mean age was 75.1 (± 9.9) years; eight (57.1%) were males; 11 (78.6%) were anticoagulated because of AF. The most common sites of bleeding were gastrointestinal (five patients, 35.7%), intracranial haemorrhage (four patients, 28.6%) and rectus sheath hematoma (two patients, 14.3%). Other sites of bleeding were: intraperitoneal (n=1), retroperitoneal (n=1), and hemarthrosis (n=1).

After FFP administration there was a significant reduction of the median INR (from 4.17 to 1.51, p=0.001). However, seven out of 14 (50%) INR values were still above the cut-off of 1.50. After FFP administration there was also a significant reduction of all CAT parameters (Table 6.3). However, the ETP and the peak were still below the established normal ranges, the lag time was normalised in 50% and the time to peak in 64.3% of cases.

The samples were subsequently stratified by the INR values before FFP infusion: >2.0-3.0 (n=3), >3.0-4.0 (n=3), >4.0-5.0 (n=3), >5.0 (n=5). There was no correlation between these categories and the normalisation of the lag time (p=1.0) or the time to peak (p=0.66).

	Before FFP (n=14)	**After FFP (n=14)**	**p value**
INR	4.17 (3.33-5.33)	1.51 (1.24-1.69)	0.001
Thrombin generation results			
Lag time (min)	10.84 (7.50-14.17)	4.50 (3.67-6.67)	0.001
ETP (nM*min)	342.5 (229.5-585.5)	899.5 (747.0-1014.0)	0.001
Peak (nM)	53.11 (41.57-84.10)	179.60 (134.19-195.02)	0.001
Time to peak (min)	13.67 (10.67-17.50)	7.25 (6.33-9.67)	0.001
Velocity index (nM/min)	19.92 (14.72-28.03)	62.44 (46.24-85.02)	0.001

Table 6.3 CAT results before and after FFP infusion in patients with warfarin-related bleeding events
Results are reported as median (IQR) and compared using the Wilcoxon Signed-Rank test.

6.2.4 Discussion

This study evaluated the use of the thrombin generation in patients with warfarin-related bleeding events. The estimate of the bleeding risk in anticoagulated patients is crucial, in order to guide the intensity and duration of the treatment. The risk of bleeding increases in parallel with the INR (Hylek et al., 2003; Palareti & Cosmi, 2009), although bleeding complications can occur even in patients with INR values within the target range (Palareti et al., 1996; Riva et al., 2014). Thrombin generation, being a test that provides a global assay of coagulation, can potentially identify a haemorrhagic tendency and help the management of anticoagulated patients, but its application to the prediction of bleeding complications in anticoagulated patients has not been extensively studied yet. A previous study enrolled 129 patients on warfarin during a routine follow-up at the anticoagulation clinics. Among them, 26 developed bleeding events during a mean follow-up of 15.5 months (Bloemen et al., 2017). No difference was found in the CAT performed on PPP, while the CAT performed on whole blood showed that bleeders had lower median ETP (182.5 nM*min vs. 256 nM*min, p=0.002) (Bloemen et al., 2017). However, in this study, blood samples were collected at the time of enrolment and not at the time of bleeding. Another study enrolling phenprocoumon-treated patients undergoing VKA reversal showed that 57 patients with bleeding had a significantly lower median ETP than 29 patients in need of preoperative prophylaxis (230 nM*min vs. 321 nM*min, p=0.03) (Herpers et al., 2015). Finally, a study enrolling 341 patients on VKA and INR in the range 2.0-3.0 (28 with bleeding, 13 with thrombosis and 300 admitted to Emergency Department for other reasons) showed that bleeders had significantly lower mean ETP on the CAT, compared to the other two categories (333 nM*min in the bleeding group vs. 441

nM*min in the thrombosis groups, p=0.037; vs. 436 nM*min in the warfarin control group, p<0.001) (Dargaud et al., 2013).

In the present study, the median ETP was slightly lower (457.5 nM*min in bleeding patients vs. 547.5 nM*min in not-bleeding patients, p=0.094). However, when the results were considered as ratio of the normal plasma and stratified by INR categories, the ETP was higher in bleeders, although statistically significant only in the INR categories >2.5-3.0 and >4.0-5.0. These results suggested an increase in thrombin generation in order to compensate for the haemorrhagic event.

The effect of FFP for warfarin reversal was subsequently evaluated and it was found that both the INR and the CAT results were only partially normalised, suggesting that FFP reversal might not achieve a complete normalisation of the haemostatic balance. These findings confirmed a previous study on warfarinised plasma spiked *in vitro* with different concentrations of the reversal agents, showing that while PCC can reverse all the thrombogram parameters, FFP can improve only the lag time and the time to peak, representing the initial stages of coagulation (Gatt et al., 2009). So far, the effect of FFP reversal *ex vivo* has never been assessed using the thrombin generation assay.

The main limitations of this study are the small sample size and the widely variable time to storage.

Furthermore, there was lack of information on the clinical conditions of the patients (amount, duration and severity of bleeding, eventual comorbidities), on the concomitant use of other reversal strategies (such as vitamin K) and on patients' body weight. Therefore, it was not possible to evaluate the actual dose of FFP infused, although at Mater Dei Hospital (Msida, Malta) the protocol for warfarin reversal recommends a dose of 15 ml/kg. The ideal study to be performed in this setting should enrol patients directly from the Emergency Department at the time of bleeding, in

order to process and freeze the samples within two hours from blood collection. This study design was not feasible at Mater Dei Hospital and several different strategies were adopted in order to identify as soon as possible samples from patients with VKA-related bleeding. The list of FFP receivers from the Blood Bank and the list of INR samples analysed in the Coagulation laboratory were screened every morning, however this approach resulted in some delay between blood collection and storage. Nevertheless, these findings can be seen as confirmatory results on the limited efficacy of FFP administration in warfarin reversal.

6.3 Reversal of the direct oral anticoagulants *in vitro* using the Calibrated Automated Thrombin Generation

6.3.1 Aim

The aim of this study was to assess the effect of five different reversal agents (FFP, rVIIa, 3-factor PCC, 4-factor PCC, and activated PCC) on plasma spiked with the DOAC, measured using the CAT.

6.3.2 Methods

The remaining citrated plasma from normal samples analysed in the Coagulation Laboratory in Sheffield (UK) was collected and centrifuged again, in order to create a pool of PPP. It was divided into 15 ml aliquots and frozen at -80°C until use. On the days of the experiment, one aliquot of plasma was thawed in the water bath at 37°C and spiked with one of the DOAC, dabigatran (Sigma-Aldrich, USA), apixaban (MedChemExpress, USA), edoxaban (MedChemExpress, USA) and rivaroxaban (MedChemExpress, USA), at prophylactic or therapeutic concentrations. Concentrations of the DOAC were assessed using the appropriate tests, either the anti-Xa assay or the DTT (paragraphs 2.9-2.10). The anticoagulated plasma was subsequently spiked with different concentration of the five reversal agents and analysed using the CAT at TF 5pM, using one calibrator well and two test wells (paragraph 2.13). An unspiked plasma sample (not anticoagulated and not reversed) was run in every plate. Given the known interference between the thrombin inhibitors and the calibrator, the CAT for dabigatran was performed without this drug in the calibrator wells, which consisted instead of normal non-anticoagulated plasma spiked with the respective concentrations of the reversal agents. Final concentrations of the

reversal agents were calculated considering an average body weight of 70 kg with an average blood volume of five litres, of which 60% is plasma (three litres). All these experiments were performed in May 2019, using the following reversal agents:

- NovoSeven® 5 mg (NovoNordisk, UK; lot number FS60S35). Novoseven® contains eptacog alfa activated (rVIIa). According to the manufacturer's instructions, 5 mg of NovoSeven® in powder form were diluted with 5 ml of sterile distilled water, in order to obtain a concentration of 1 mg/ml. It was divided into small aliquots of 0.2 ml each and frozen at -80°C until use. On the days of the experiment, a further dilution 1:9 was performed in deionised water to obtain a concentration of 100 µg/ml. NovoSeven® was spiked into the anticoagulated plasma at the following final concentrations: 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, and 50 µg/ml. These concentrations were chosen based on the licensed dosage of NovoSeven® for patients with haemophilia A or B with inhibitors and active bleeding (range 90-270 µg/kg, which correspond to approximately 2.1-6.3 µg/ml) (European Medicines Agency, 2018e) and a previous VKA reversal study *in vitro* which evaluated NovoSeven® at supra-therapeutic doses (5 µg/ml, 10 µg/ml, 50 µg/ml) (Gatt, van Veen, Woolley, et al., 2008). In order to obtain these concentrations, the volume of the reversal agent spiked into the anticoagulated plasma ranged from 2.5% to 50% of the total final volume.
- FEIBA® 1000 U (Baxalta, Austria; lot number UNF2R085). FEIBA® is an activated PCC, which contains factors II, IX and X (mainly in non-activated form) and factor VII activated. According to the manufacturer's instructions, 1000 U of FEIBA® in powder form were diluted with 20 ml of sterile distilled water, in order to obtain a concentration of 50 U/ml. It was divided into small aliquots of 0.2 ml each and frozen at -80°C until use. On the days of the experiment, a further dilution

1:9 was performed in deionised water in order to obtain a concentration of 5 U/ml. FEIBA® was initially spiked into the anticoagulated plasma at the following concentrations: 0.2 U/ml, 0.5 U/ml, 0.8 U/ml, 1.2 U/ml, 1.8 U/ml, 2.4 U/ml. However, the highest concentrations were giving no calibrator fit, which can be due to substrate depletion before completion of the thrombin generation assay, as reported in a previous study (van Veen et al., 2009). Therefore, the range of concentrations was reduced as follows: 0.2 U/ml, 0.4 U/ml, 0.6 U/ml, 0.8 U/ml, 1.0 U/ml, 1.2 U/ml. These concentrations were chosen based on the licensed dosage of FEIBA® for patients with haemophilia A or B with inhibitors (range 50-100 U/kg, which correspond to approximately 1.2-2.3 U/ml) (Baxter International Inc, 2013) and the recommendations for the reversal of dabigatran-associated bleeding when specific antidotes are not available (activated PCC 50-80 U/kg) (Garcia & Crowther, 2019). In order to obtain these concentrations, the volume of the reversal agent spiked into the anticoagulated plasma ranged from 4% to 24% of the total final volume.

- Beriplex® P/N 500 U (CSL Behring, UK; lot number P100026267). Beriplex® P/N is a 4-factor PCC, which contains factors II, VII, IX and X and small quantities of proteins C and S. According to the manufacturer's instructions, 500 U of Beriplex® P/N in powder form were diluted with 20 ml of sterile distilled water, in order to obtain a concentration of 25 U/ml. It was divided into small aliquots of 0.2 ml each and frozen at -80°C until use. On the days of the experiment, a further dilution 1:4 was performed in deionised water in order to obtain a concentration of 5 U/ml. Similarly to FEIBA®, Beriplex® P/N was initially spiked at high concentrations, subsequently reduced to 0.2 U/ml, 0.4 U/ml, 0.6 U/ml, 0.8 U/ml, 1.0 U/ml, 1.2 U/ml. These concentrations were chosen based on the licensed dosage of Beriplex®

P/N for patients with bleeding during VKA treatment (range 25-50 U/kg, which correspond to approximately 0.6-1.2 U/ml) (CSL Behring UK Limited, 2017) and to the recommendations for the reversal of DOAC-associated bleeding when specific antidotes are not available (4-factor PCC 50 U/kg) (Garcia & Crowther, 2019).

- Uman Complex® 500 U (kindly donated by Kedrion Biopharma, Italy; lot number 511803). Uman Complex® is a 3-factor PCC, which contains factors II, IX and X. According to the manufacturer's instructions, 500 U of Uman Complex® in powder form were diluted with 20 ml of sterile distilled water, in order to obtain a concentration of 25 U/ml. It was divided into small aliquots of 0.2 ml each and frozen at -80°C until use. On the days of the experiment, a further dilution 1:4 was performed in deionised water in order to obtain a concentration of 5 U/ml. Similarly to FEIBA®, Uman Complex® was initially spiked at high concentrations, subsequently reduced to 0.2 U/ml, 0.4 U/ml, 0.6 U/ml, 0.8 U/ml, 1.0 U/ml, 1.2 U/ml. These concentrations were chosen based on the recommended dose for VKA reversal (range 25-50 U/kg, which correspond to approximately 0.6-1.2 U/ml) (Hull & Garcia, 2019) and to the recommendations for the reversal of DOAC-associated bleeding when specific antidotes are not available (3-factor PCC 50 U/kg) (Garcia & Crowther, 2019).
- FFP: frozen normal pooled plasma was used to mimic the effect of FFP. FFP was spiked into the anticoagulated plasma at the following final concentrations: 250 µl/ml, 500 µl/ml, 750 µl/ml. These concentrations were chosen based on the recommended dose for VKA reversal (range 15-30 ml/kg, which correspond to approximately 350-700 µl/ml) (Hull & Garcia, 2019) and a previous VKA reversal study *in vitro* which evaluated the same concentrations (Gatt, van Veen, Woolley,

et al., 2008). In order to obtain these concentrations, the volume of the reversal agent spiked into the anticoagulated plasma ranged from 25% to 75% of the total final volume.

For this study, normalisation of the thrombin generation results was defined as values, after reversal, within the ranges of the same unspiked plasma, which was run six times in different plates, calculated as mean ± 1.96 * SD. The percentage of normalisation of the ETP was calculated with reference to the ETP of the normal sample in the same plate. Only the ETP was considered because it is the most consistent parameter of the thrombin generation curve, representing the total amount of thrombin generated.

6.3.3 Results

6.3.3.1 Dabigatran

Normal PPP was spiked with dabigatran at concentration 230 ng/ml. A different pattern of normalisation was observed with the different reversal agents (Table 6.4):

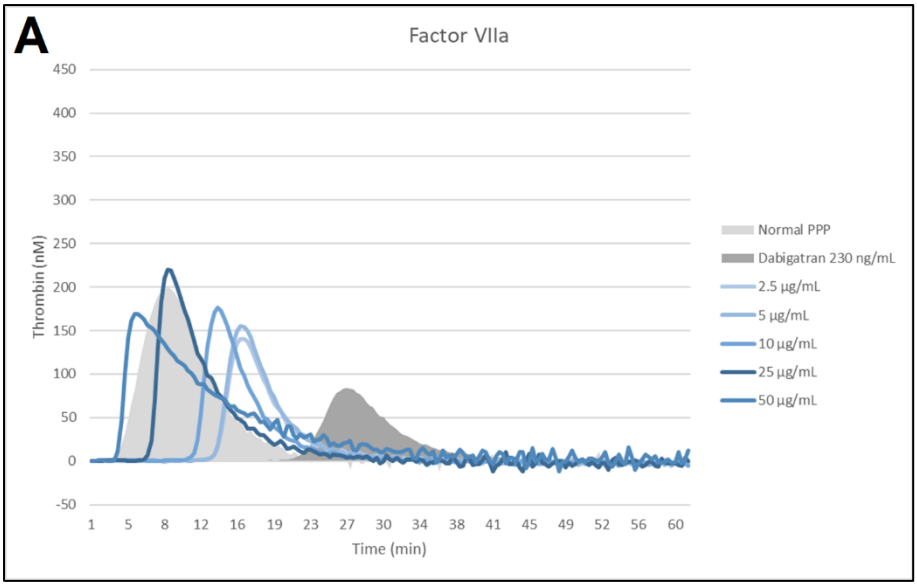
- rVIIa: the peak was normalised with concentrations from 2.5 µg/ml upwards; the time to peak was normalised with concentrations from 25 µg/ml upwards; the lag time and the ETP were normalised only with the highest concentration 50 µg/ml.
- FFP: the peak was normalised with concentrations from 500 µl/ml upwards; the ETP was normalised only with the highest concentration 750 µl/ml; none of the tested concentrations normalised the lag time and the time to peak.
- Activated PCC, 4-factor PCC and 3-factor PPC showed the same pattern: the peak was normalised with concentrations from 0.2 U/ml upwards; the ETP was not normalised with concentration 0.2 U/ml but it was already above the unspiked plasma ranges with concentration 0.5 U/ml, suggesting hypercoagulability. The

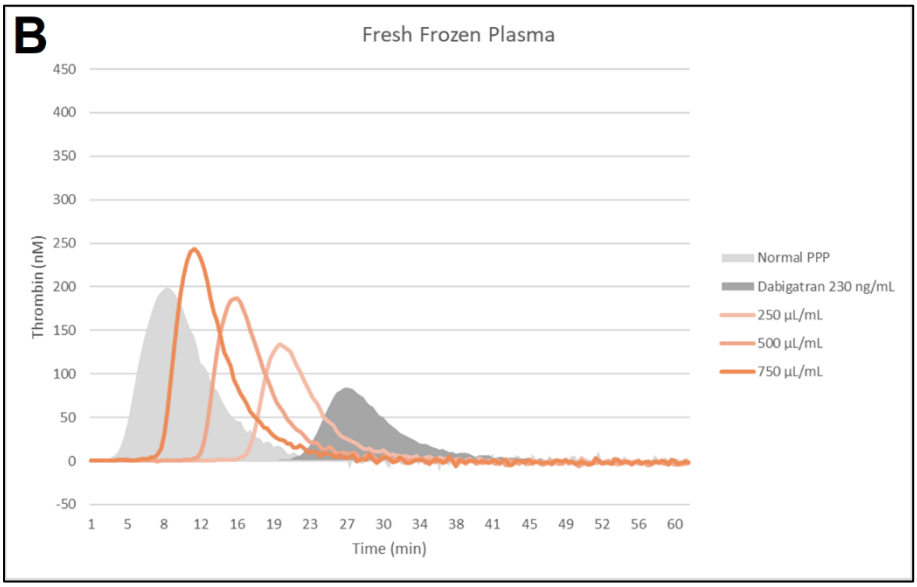
lag time and the time to peak were not normalised with concentrations up to 0.8 U/ml; however, concentrations from 1.2 U/ml upwards could not be evaluated because of the lack of calibrator fit.

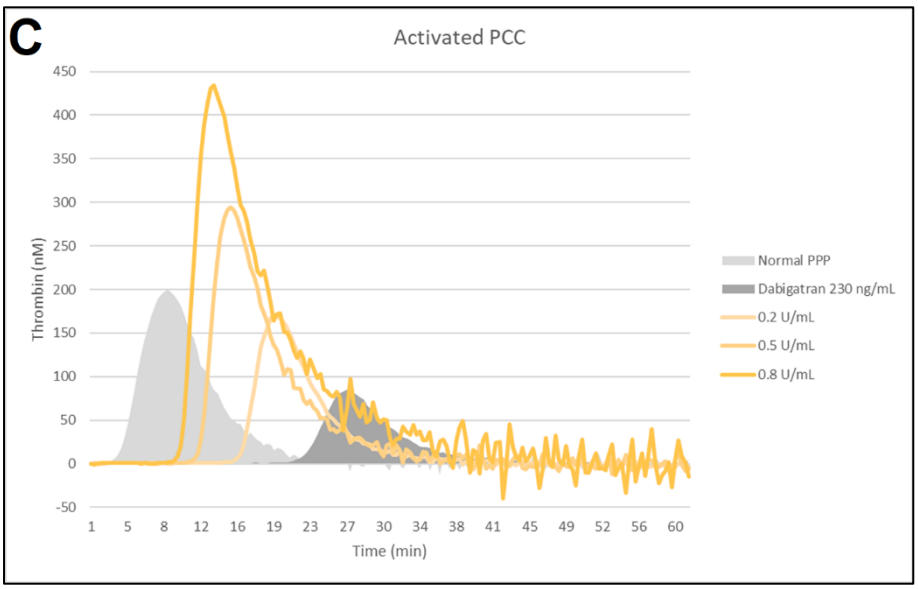
When observing the corresponding thrombin generation curves (Figure 6.6), it was evident that, among the different types of PCCs, the activated PCC was giving the highest peak, although the curve was still shifted to the right, while the 3-factor PCC showed the least pronounced peak.

	Lag time (min)	ETP (nM*min)	Peak (nM)	Time to peak (min)
Unspiked PPP ranges	**3.41-4.92**	**1423.6-1971.4**	**134.02-316.84**	**6.31-10.18**
Dabigatran 230 ng/ml	22.64 (0.33)	718.0 (0)	84.13 (3.12)	26.81 (0.17)
rVIIa				
2.5 µg/ml	13.46 (0.50)	832.5 (14.5)	140.69 (3.90)	16.13 (0.50)
5 µg/ml	13.62 (0)	894.0 (9.0)	155.37 (0.60)	16.13 (0.17)
10 µg/ml	11.79 (0.17)	968.0 (11.0)	175.88 (0.83)	13.96 (0)
25 µg/ml	7.11 (0.17)	1348.0 (15.0)	221.29 (1.64)	9.12 (0.17)
50 µg/ml	3.44 (0.17)	1899.0 (23.0)	168.86 (1.92)	5.44 (0.17)
Fresh frozen plasma				
250 µl/ml	16.96 (0.33)	888.0 (12.0)	133.47 (4.65)	20.13 (0.50)
500 µl/ml	12.45 (0.17)	1176.5 (6.5)	187.44 (1.06)	15.63 (0)
750 µl/ml	8.45 (0.17)	1537.5 (0.5)	243.11 (2.69)	11.62 (0)
Activated PCC				
0.2 U/ml	16.29 (0)	1236.0 (2.0)	170.42 (2.02)	19.63 (0)
0.5 U/ml	11.95 (0)	2265.5 (24.5)	294.37 (1.0)	14.96 (0)
0.8 U/ml	10.12 (0.17)	4069.0 (45.0)	432.11 (1.64)	13.12 (0.17)
1.2 U/ml	No calibrator fit			
1.8 U/ml	No calibrator fit			
2.4 U/ml	No calibrator fit			
4-factor PCC				
0.2 U/ml	16.79 (0.17)	1052.0 (30.0)	141.34 (2.09)	20.13 (0.17)
0.5 U/ml	13.29 (0.33)	2000.5 (120.5)	230.38 (12.06)	16.79 (0.17)
0.8 U/ml	10.79 (0.17)	4092.0 (20.0)	370.24 (0.66)	14.79 (0.17)
1.2 U/ml	No calibrator fit			
1.8 U/ml	No calibrator fit			
2.4 U/ml	No calibrator fit			
3-factor PCC				
0.2 U/ml	15.13 (0.17)	1165.0 (12.0)	154.03 (6.13)	18.80 (0.17)
0.5 U/ml	11.45 (0.17)	2135.0 (5.0)	229.11 (8.28)	15.29 (0.33)
0.8 U/ml	10.62 (0)	3658.5 (66.5)	214.43 (3.03)	17.63 (0.33)
1.2 U/ml	No calibrator fit			
1.8 U/ml	No calibrator fit			
2.4 U/ml	No calibrator fit			

Table 6.4 The effect of increasing concentrations of different reversal agents on therapeutic dabigatran, measured using the CAT
Results are reported as mean (SD) of two measurements.


A
Factor VIIa
Thrombin (nM)
Time (min)
Normal PPP
Dabigatran 230 ng/mL
2.5 µg/mL
5 µg/mL
10 µg/mL
25 µg/mL
50 µg/mL


B
Fresh Frozen Plasma
Thrombin (nM)
Time (min)
Normal PPP
Dabigatran 230 ng/mL
250 µL/mL
500 µL/mL
750 µL/mL


C
Activated PCC
Thrombin (nM)
Time (min)
Normal PPP
Dabigatran 230 ng/mL
0.2 U/mL
0.5 U/mL
0.8 U/mL

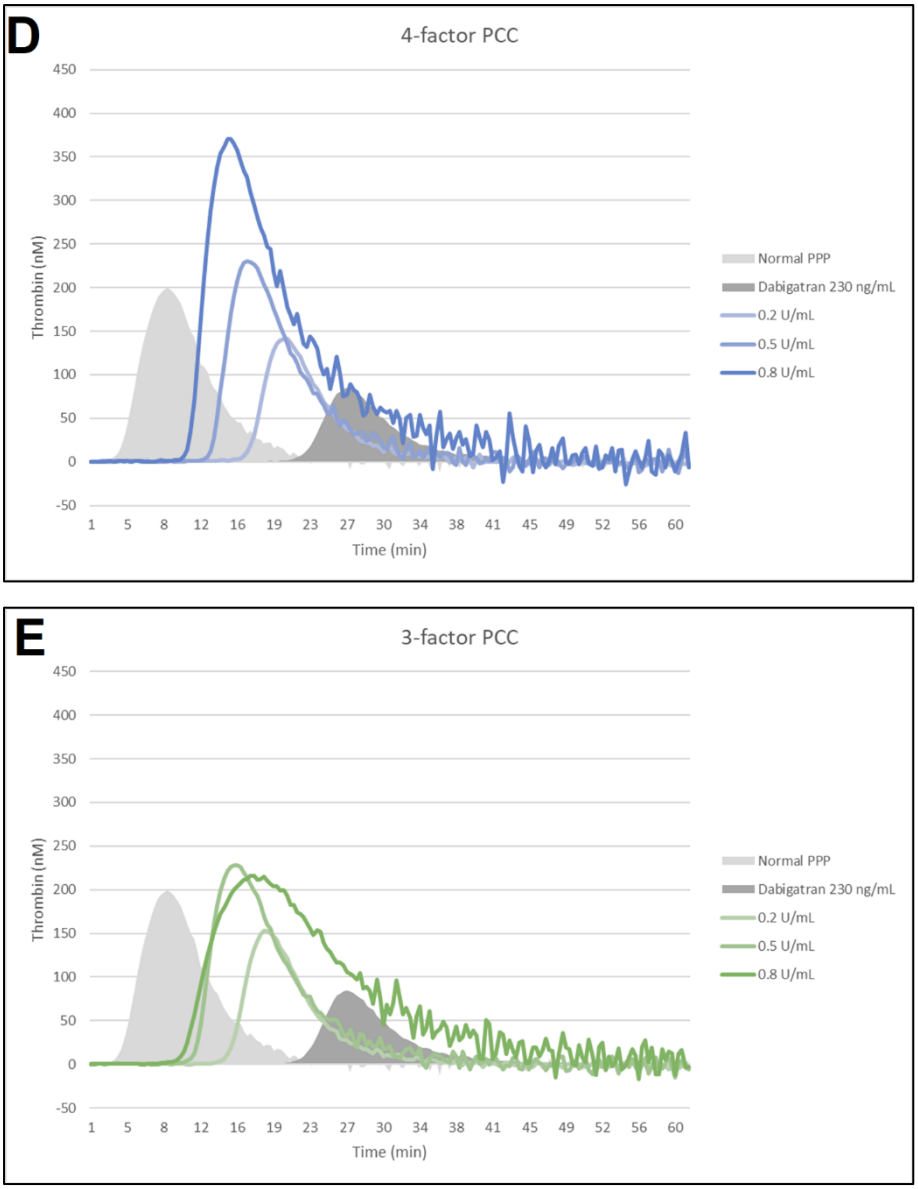


Figure 6.6 Thrombin generation curves with increasing concentrations of different reversal agents on therapeutic dabigatran: factor VIIa (A), fresh frozen plasma (B), activated PCC (C), 4-factor PCC (D), 3-factor PCC (E)

The curves obtained with high concentrations of activated PCC, 3-factor and 4-factor PCC (1.2 U/ml, 1.8 U/ml, 2.4 U/ml) could not be plotted because there was no calibrator fit.

6.3.3.2 Apixaban

Normal PPP was spiked with apixaban at concentration 225 ng/ml. A different pattern of normalisation was observed with the different reversal agents (Table 6.5):

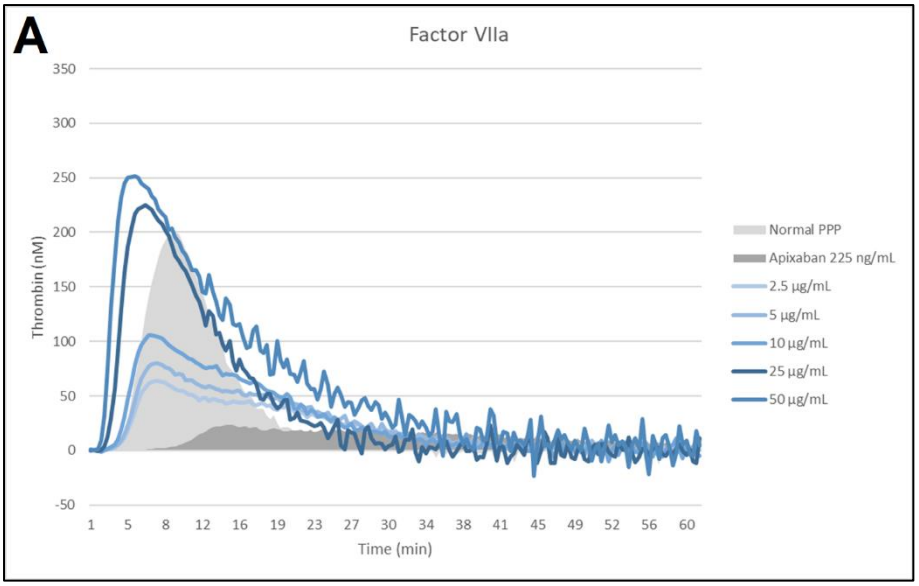
- rVIIa: the lag time and the time to peak were normalised with concentrations from 2.5 µg/ml upwards; the peak was normalised with concentrations from 25 µg/ml upwards; the ETP was normalised with concentration 10 µg/ml, but was above the unspiked PPP ranges with concentration 25 µg/ml, suggesting hypercoagulability.
- FFP: none of the tested concentrations normalised any of the four main CAT parameters (lag time, time to peak, ETP, peak).
- Activated PCC: the lag time was normalised only with concentration 0.8 U/ml; the peak was normalised with concentrations from 0.8 U/ml upwards; the time to peak was normalised with concentrations from 0.2 U/ml to 0.8 U/ml. The ETP was normalised with concentrations 0.2-0.4 U/ml, but was above the unspiked PPP ranges with higher concentrations, suggesting hypercoagulability.
- 4-factor PCC: none of the tested concentrations normalised the lag time, the time to peak and the peak. The ETP was normalised with concentration 0.4 U/ml, but was above the ranges with higher concentrations, suggesting hypercoagulability.
- 3-factor PCC: none of the tested concentrations normalised the lag time, the time to peak and the peak. The ETP could not be calculated for concentrations 0.6-0.8-1.0 U/ml because no tail could be identified in the thrombin generation curve after 120 minutes, while it was normalised with concentration 1.2 U/ml.

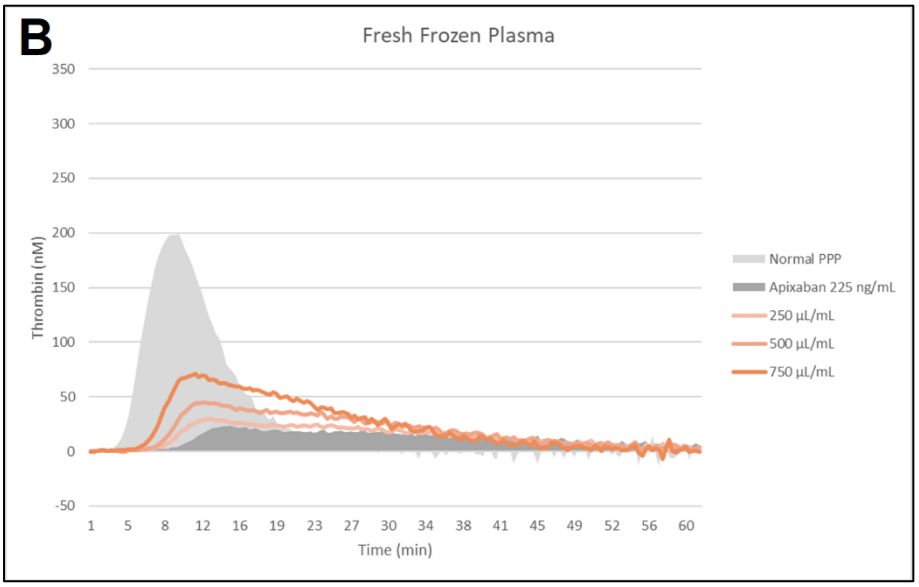
When observing the corresponding thrombin generation curves (the first 60 minutes are shown in Figure 6.7), it was evident that only the rVIIa corrected the lag time, the activated PCC almost reached the peak of the unspiked plasma, while the 3-factor and 4-factor PCC showed the less pronounced peaks.

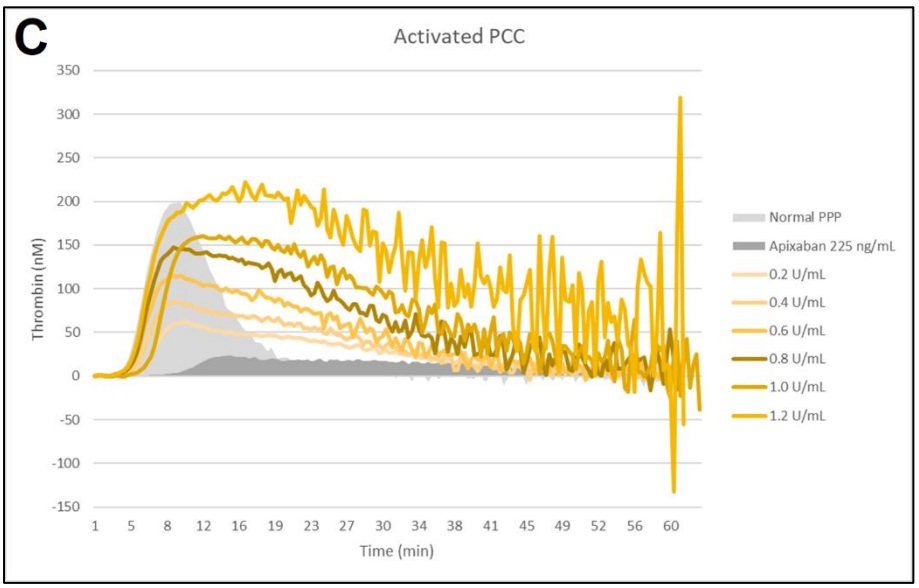
	Lag time (min)	ETP (nM*min)	Peak (nM)	Time to peak (min)
Unspiked PPP ranges	**3.41-4.92**	**1423.6-1971.4**	**134.02-316.84**	**6.31-10.18**
Apixaban 225 ng/ml	9.33 (0)	858.5 (0.5)	23.39 (0)	14.67 (0)
rVIIa				
2.5 µg/ml	4.00 (0)	1185.5 (31.5)	63.81 (0.49)	7.67 (0)
5 µg/ml	4.00 (0)	1404.0 (16.0)	80.04 (2.69)	7.33 (0)
10 µg/ml	3.67 (0)	1623.5 (39.5)	106.08 (0.90)	7.17 (0.17)
25 µg/ml	3.00 (0)	2426.5 (87.5)	224.30 (9.22)	6.33 (0)
50 µg/ml	2.33 (0)	3743.5 (103.5)	252.60 (3.78)	5.17 (0.17)
Fresh frozen plasma				
250 µl/ml	8.00 (0)	883.0 (12.0)	29.69 (0.35)	12.83 (0.17)
500 µl/ml	7.50 (0.17)	1156.5 (36.5)	44.79 (1.58)	12.17 (0.17)
750 µl/ml	6.67 (0)	1387.0 (4.0)	70.0 (0.69)	11.33 (0)
Activated PCC				
0.2 U/ml	5.50 (0.17)	1494.0 (19.0)	61.72 (1.40)	9.67 (0)
0.4 U/ml	5.00 (0)	1894.5 (38.5)	84.50 (1.0)	9.17 (0.17)
0.6 U/ml	5.00 (0)	2644.0 (3.0)	115.17 (0.51)	9.33 (0)
0.8 U/ml	4.83 (0.17)	3902.5 (93.5)	145.92 (11.41)	9.50 (0.17)
1.0 U/ml	5.00 (0)	4541.5 (33.5)	159.48 (0.26)	11.50 (0.17)
1.2 U/ml	5.17 (0.17)	9524.0 (NC)*	206.24 (5.13)	16.33 (0.33)
4-factor PCC				
0.2 U/ml	7.17 (0.17)	1138.0 (4.0)	37.94 (0.39)	12.17 (0.17)
0.4 U/ml	6.83 (0.17)	1497.5 (37.5)	40.94 (2.26)	13.50 (0.83)
0.6 U/ml	7.00 (0)	2066.5 (60.5)	56.47 (0.43)	18.50 (2.83)
0.8 U/ml	6.83 (0.17)	2175.5 (84.5)	57.10 (0.79)	20.50 (0.17)
1.0 U/ml	7.00 (0)	2647.0 (69.0)	64.61 (2.06)	21.50 (0.17)
1.2 U/ml	7.17 (0.17)	3192.0 (NC)*	78.18 (1.16)	25.50 (0.17)
3-factor PCC				
0.2 U/ml	7.83 (0.17)	1129.5 (24.5)	24.39 (0.38)	19.33 (3.67)
0.4 U/ml	7.83 (0.17)	1391.0 (39.0)	29.62 (0.23)	25.50 (0.17)
0.6 U/ml	7.67 (0)	No tail found**	32.19 (0.10)	37.83 (1.17)
0.8 U/ml	9.33 (0)	No tail found**	33.03 (0.02)	58.83 (1.50)
1.0 U/ml	9.33 (0.33)	No tail found**	41.30 (2.10)	58.67 (0.67)
1.2 U/ml	9.17 (0.17)	1632.0 (NC)*	49.03 (1.73)	54.67 (2.33)

Table 6.5 The effect of increasing concentrations of different reversal agents on therapeutic apixaban, measured using the CAT

Results are reported as mean (SD) of two measurements. *Only one curve was finished. ** The tail of the curve could not be identified during the running time of the thrombin generation assay, which was 120 minutes for this plate.


A
Factor VIIa
Thrombin (nM)
Time (min)
Normal PPP
Apixaban 225 ng/mL
2.5 µg/mL
5 µg/mL
10 µg/mL
25 µg/mL
50 µg/mL


B
Fresh Frozen Plasma
Thrombin (nM)
Time (min)
Normal PPP
Apixaban 225 ng/mL
250 µL/mL
500 µL/mL
750 µL/mL


C
Activated PCC
Thrombin (nM)
Time (min)
Normal PPP
Apixaban 225 ng/mL
0.2 U/mL
0.4 U/mL
0.6 U/mL
0.8 U/mL
1.0 U/mL
1.2 U/mL

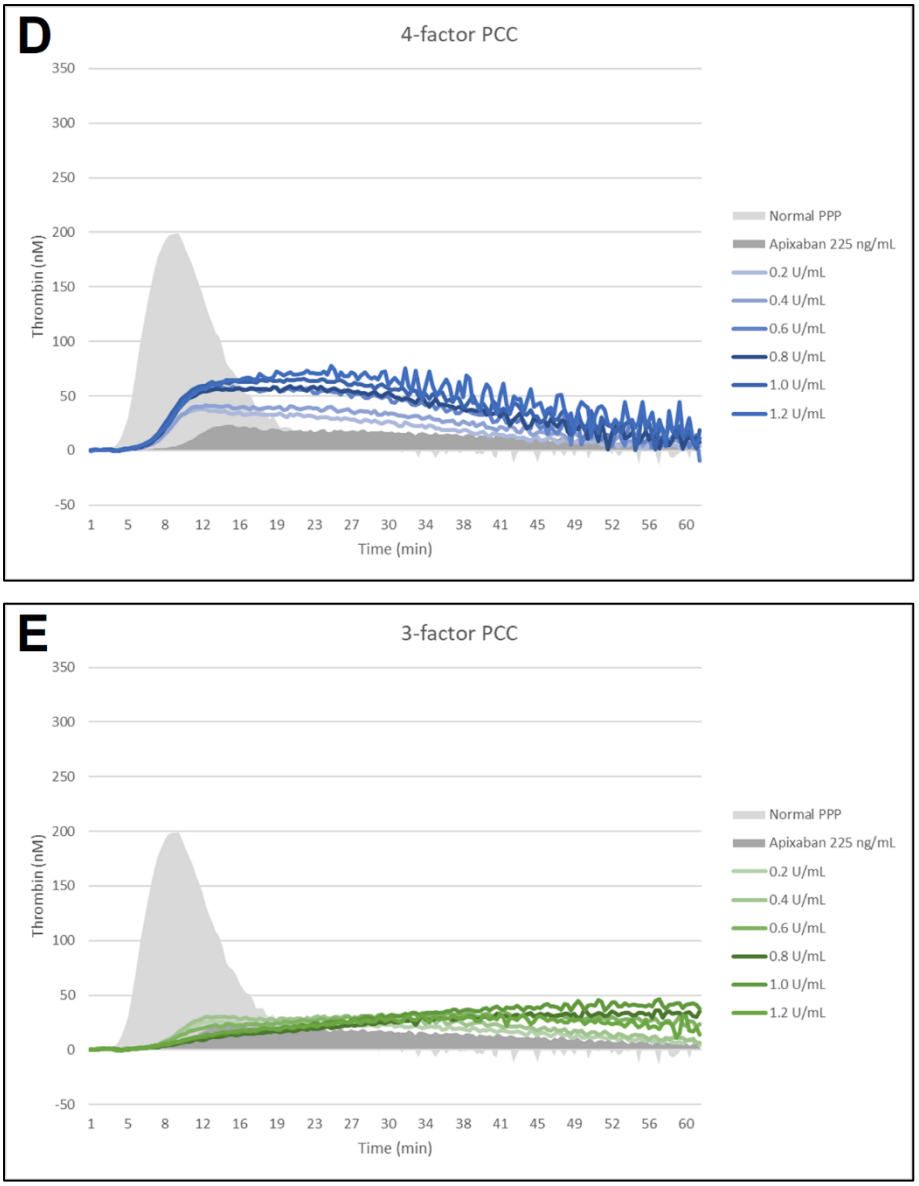


Figure 6.7 Thrombin generation curves with increasing concentrations of different reversal agents on therapeutic apixaban: factor VIIa (A), fresh frozen plasma (B), activated PCC (C), 4-factor PCC (D), 3-factor PCC (E)

The curve obtained with activated PCC at 1.2 U/ml showed a lot of background interference and the analyser gave a warning that TG was too high. The curves obtained with 3-factor PCC at 0.6 U/ml, 0.8 U/ml and 1.0 U/ml had no tail at 120 minutes.

6.3.3.3 Edoxaban

Normal PPP was spiked with edoxaban at concentration 192 ng/ml. A different pattern of normalisation was observed with the different reversal agents (Table 6.6):

- rVIIa: the lag time was normalised with concentrations from 2.5 µg/ml upwards; the peak was normalised with concentrations from 10 µg/ml upwards; the time to peak was normalised with concentrations from 25 µg/ml upwards. The ETP was normalised with concentration from 2.5 to 10 µg/ml, but was above the unspiked plasma ranges with concentration 25 µg/ml, suggesting hypercoagulability.
- FFP: none of the tested concentrations normalised the lag time, the time to peak and the peak; the ETP was normalised with the highest concentration 750 µl/ml.
- Activated PCC: the lag time and the time to peak were not normalised with concentrations up to 0.8 U/ml; however, concentrations from 1.0 U/ml upwards could not be evaluated because of the lack of calibrator fit. The peak was normalised with concentrations from 0.4 U/ml upwards. The ETP was normalised with concentration 0.2 U/ml, but was above the unspiked plasma ranges with concentration 0.4 U/ml, suggesting hypercoagulability.
- 4-factor PCC: the lag time and the time to peak were not normalised with concentrations up to 1.0 U/ml. However, the highest concentrations 1.2 U/ml could not be evaluated because of the lack of calibrator fit. The peak was normalised with concentrations from 0.6 U/ml upwards. The ETP was normalised with concentration 0.2 U/ml, but was above the unspiked plasma ranges with concentration 0.4 U/ml, suggesting hypercoagulability.
- 3-factor PCC: none of the tested concentrations normalised the lag time and the time to peak. The peak was normalised with concentrations from 0.4 U/ml upwards. The ETP was normalised with concentration 0.2 U/ml, but was above

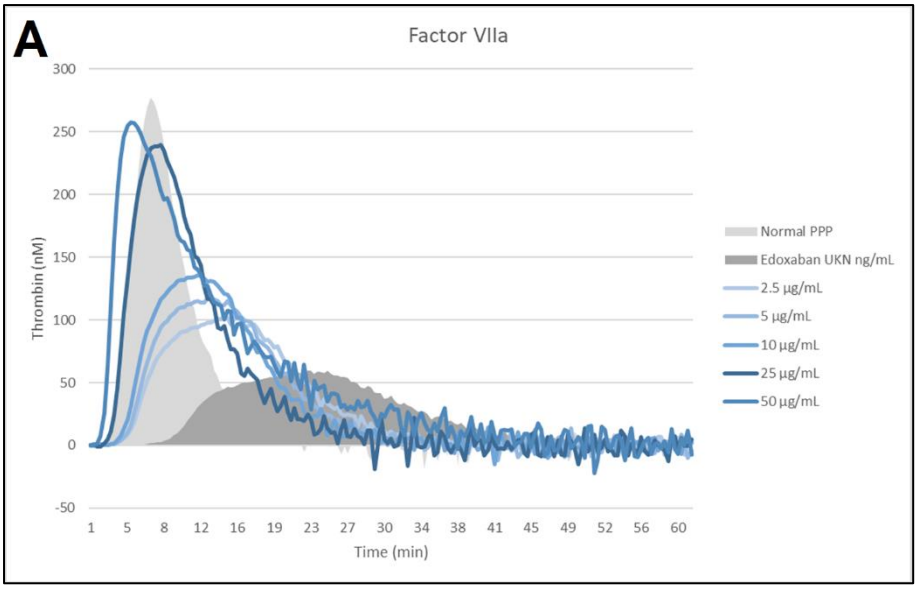
the unspiked plasma ranges with concentration 0.4 U/ml, suggesting hypercoagulability. The ETP could not be calculated for the concentrations of 1.2 U/ml because no tail could be identified in the thrombin generation curve after 90 minutes.

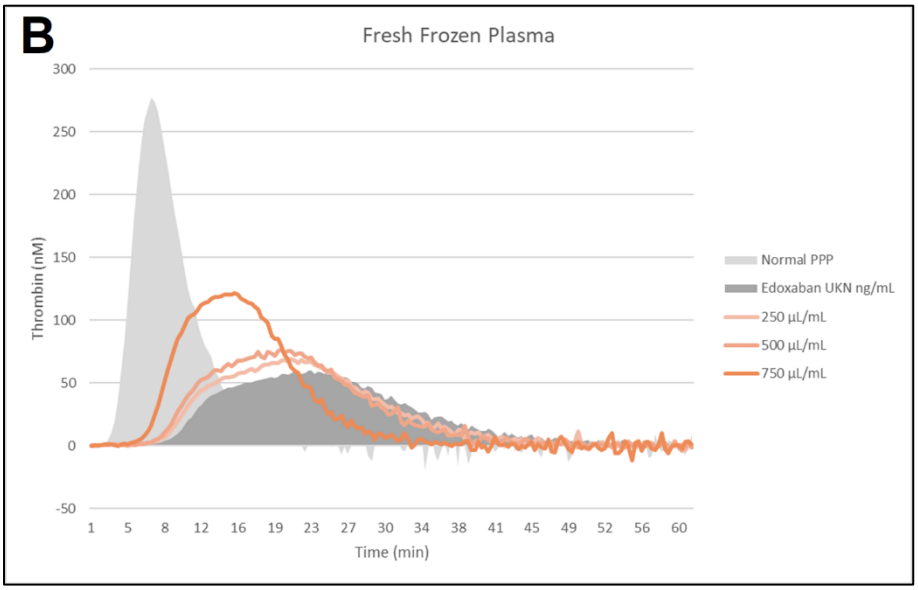
When observing the corresponding thrombin generation curves (the first 60 minutes are shown in Figure 6.8), it was evident that only the rVIIa corrected the lag time, the activated PCC and the 4-factor PCC almost reached the peak of the unspiked plasma, while the 3-factor PCC showed the less pronounced peak.

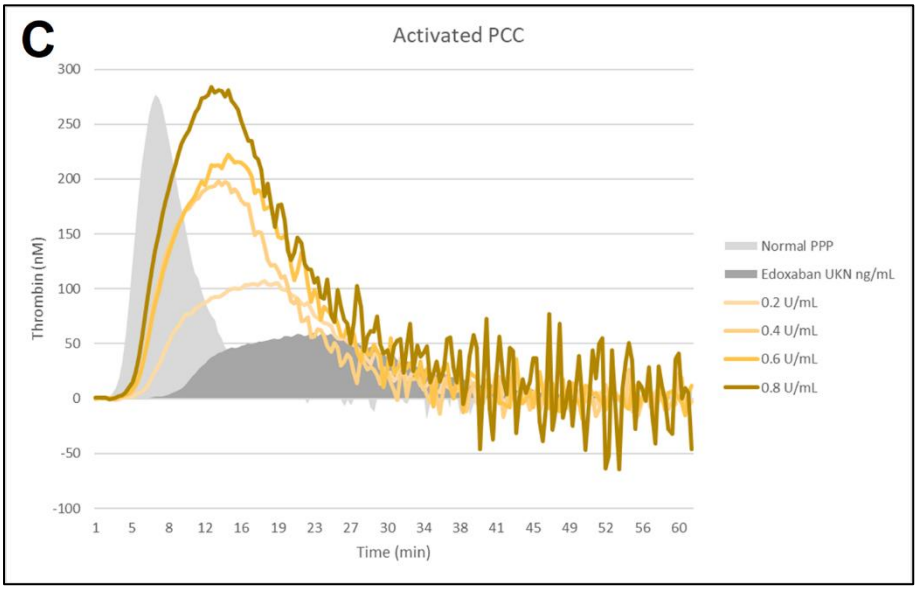
	Lag time (min)	ETP (nM*min)	Peak (nM)	Time to peak (min)
Unspiked PPP ranges	**3.41-4.92**	**1423.6-1971.4**	**134.02-316.84**	**6.31-10.18**
Edoxaban 192 ng/ml	9.67 (0.33)	1319.5 (59.5)	59.22 (7.40)	23.00 (1.33)
rVIIa				
2.5 µg/ml	4.83 (0.17)	1671.5 (30.5)	100.48 (3.53)	14.33 (0.33)
5 µg/ml	4.83 (0.17)	1696.0 (1.0)	116.53 (7.12)	12.50 (0.83)
10 µg/ml	4.67 (0)	1786.0 (11.0)	134.80 (3.88)	11.33 (0.33)
25 µg/ml	3.33 (0)	2312.0 (47.0)	239.85 (7.42)	7.67 (0)
50 µg/ml	2.17 (0.17)	3241.5 (207.5)	258.49 (2.60)	5.00 (0)
Fresh frozen plasma				
250 µl/ml	8.67 (0)	1347.5 (25.5)	68.09 (0.19)	21.00 (0)
500 µl/ml	8.33 (0)	1400.0 (10.0)	74.65 (0.96)	19.67 (0)
750 µl/ml	7.00 (0)	1614.0 (5.0)	120.63 (0.33)	14.50 (0.17)
Activated PCC				
0.2 U/ml	6.33 (0)	1948.5 (26.5)	105.23 (2.28)	17.33 (0)
0.4 U/ml	5.67 (0)	2803.5 (29.5)	196.10 (1.57)	13.50 (0.17)
0.6 U/ml	5.50 (0.17)	3461.5 (14.5)	218.40 (2.18)	14.17 (0.17)
0.8 U/ml	5.33 (0)	4569.0 (NC)*	281.49 (11.39)	13.67 (0.33)
1.0 U/ml	No calibrator fit			
1.2 U/ml	No calibrator fit			
4-factor PCC				
0.2 U/ml	8.67 (0)	1466.0 (14.0)	70.67 (0.98)	21.00 (0.33)
0.4 U/ml	8.33 (0)	2093.5 (54.5)	100.52 (2.09)	20.17 (0.17)
0.6 U/ml	8.00 (0)	2645.0 (94.0)	134.45 (6.56)	19.00 (0.33)
0.8 U/ml	7.50 (0.17)	3973.5 (104.5)	222.15 (4.85)	16.83 (0.17)
1.0 U/ml	7.33 (0)	4643.0 (NC)*	251.61 (10.36)	17.67 (0.33)
1.2 U/ml	No calibrator fit			
3-factor PCC				
0.2 U/ml	9.00 (0)	1718.0 (14.0)	83.76 (3.06)	21.50 (0.50)
0.4 U/ml	7.83 (0.17)	2471.5 (118.5)	145.85 (20.26)	18.00 (1.33)
0.6 U/ml	7.33 (0.33)	3138.0 (96.0)	178.94 (12.32)	17.33 (1.0)
0.8 U/ml	7.83 (0.17)	3838.0 (186.0)	178.08 (14.49)	20.17 (0.83)
1.0 U/ml	7.83 (0.17)	4019.5 (253.5)	145.39 (0.10)	23.33 (0.67)
1.2 U/ml	9.00 (NC)*	No tail found**	133.57 (NC)*	30.67 (NC)*

Table 6.6 The effect of increasing concentrations of different reversal agents on prophylactic edoxaban, measured using the CAT

Results are reported as mean (SD) of two measurements. * Only one curve was finished. ** The tail of the curve could not be identified during the running time of the thrombin generation assay, which was 90 minutes for this plate.


A
Factor VIIa
Thrombin (nM)
Time (min)
Normal PPP
Edoxaban UKN ng/mL
2.5 µg/mL
5 µg/mL
10 µg/mL
25 µg/mL
50 µg/mL


B
Fresh Frozen Plasma
Thrombin (nM)
Time (min)
Normal PPP
Edoxaban UKN ng/mL
250 µL/mL
500 µL/mL
750 µL/mL


C
Activated PCC
Thrombin (nM)
Time (min)
Normal PPP
Edoxaban UKN ng/mL
0.2 U/mL
0.4 U/mL
0.6 U/mL
0.8 U/mL

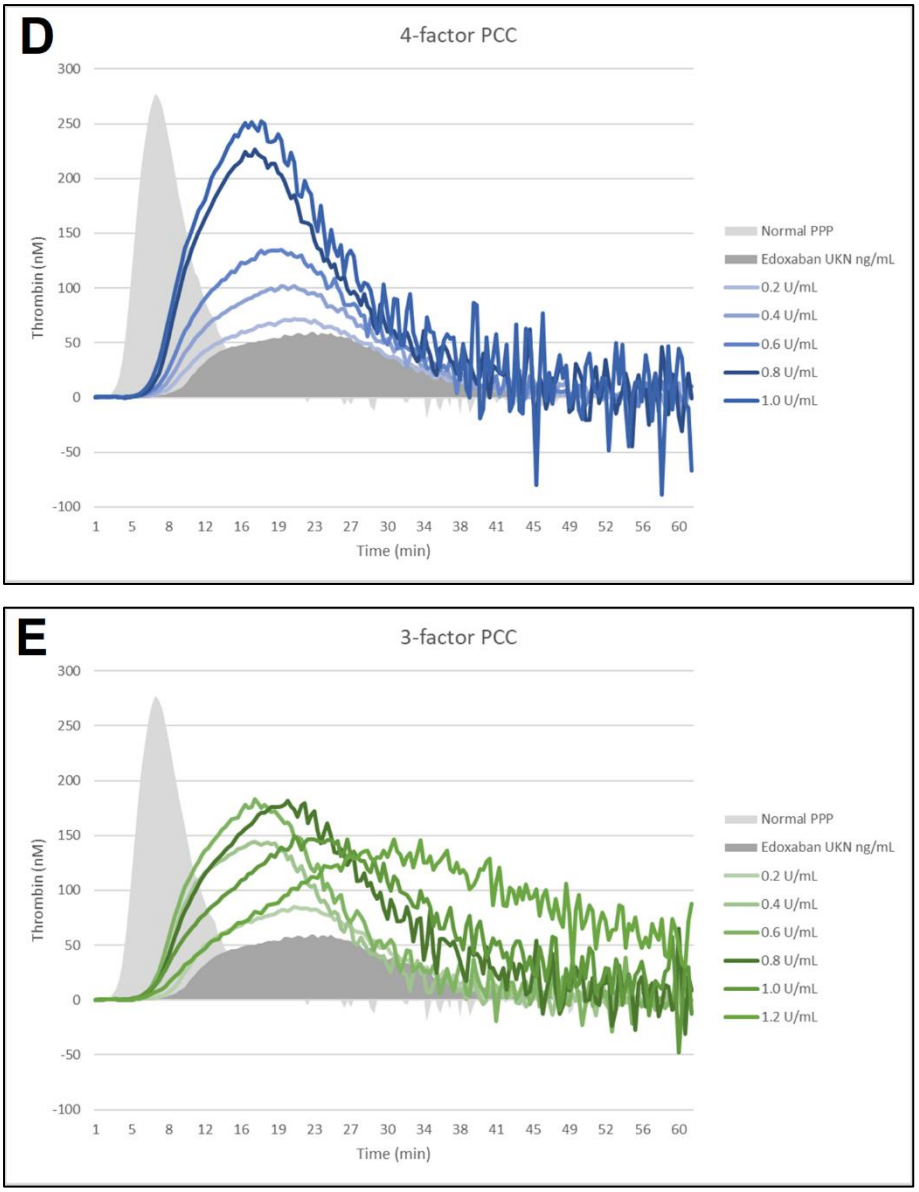


Figure 6.8 Thrombin generation curves with increasing concentrations of different reversal agents on prophylactic edoxaban: factor VIIa (A), fresh frozen plasma (B), activated PCC (C), 4-factor PCC (D), 3-factor PCC (E)

The curves obtained with high concentrations of activated PCC (1.2 U/ml, 1.0 U/ml) and 4-factor PCC (1.2 U/ml) could not be plotted because there was no calibrator fit. The curves obtained with activated PCC at 0.8 U/ml and 4-factor PCC at 1.0 U/ml showed a lot of background interference and the analyser gave a warning that TG was too high. The curve obtained with 3-factor PCC at 1.2 U/ml had no tail at 90 minutes.

6.3.3.4 Rivaroxaban

Normal PPP was spiked with rivaroxaban at concentration 102 ng/ml. A different pattern of normalisation was observed with the different reversal agents (Table 6.7):

- rVIIa: the lag time and the time to peak were normalised from concentration 2.5 µg/ml upwards; the peak was normalised from concentration 5 µg/ml upwards. The ETP was normalised with concentrations from 2.5 to 10 µg/ml, but was above the unspiked plasma ranges with higher concentrations, suggesting hypercoagulability.
- FFP: none of the tested concentrations normalised the lag time, the time to peak and the peak; the ETP was normalised with concentrations from 500 µl/ml upwards.
- Activated PCC: the lag time and the peak were normalised with concentrations from 0.4 U/mL upwards. None of the tested concentrations up to 0.8 U/ml normalised the time to peak; however, concentrations from 1.0 U/ml upwards could not be evaluated because of lack of calibrator fit. The ETP was already above the unspiked plasma ranges with the lowest concentration 0.2 U/ml, suggesting hypercoagulability.
- 4-factor PCC: none of the tested concentrations normalised the lag time and the time to peak. The peak was normalised from concentration 0.6 U/ml upwards. The ETP was normalised with concentration 0.2 U/ml, but became above the unspiked plasma ranges with higher concentrations, suggesting hypercoagulability. However, the concentration 1.0 U/ml could not be evaluated because of the lack of calibrator fit, while the ETP could not be calculated for the concentration 1.2 U/ml because this curve was not finished at 120 minutes.

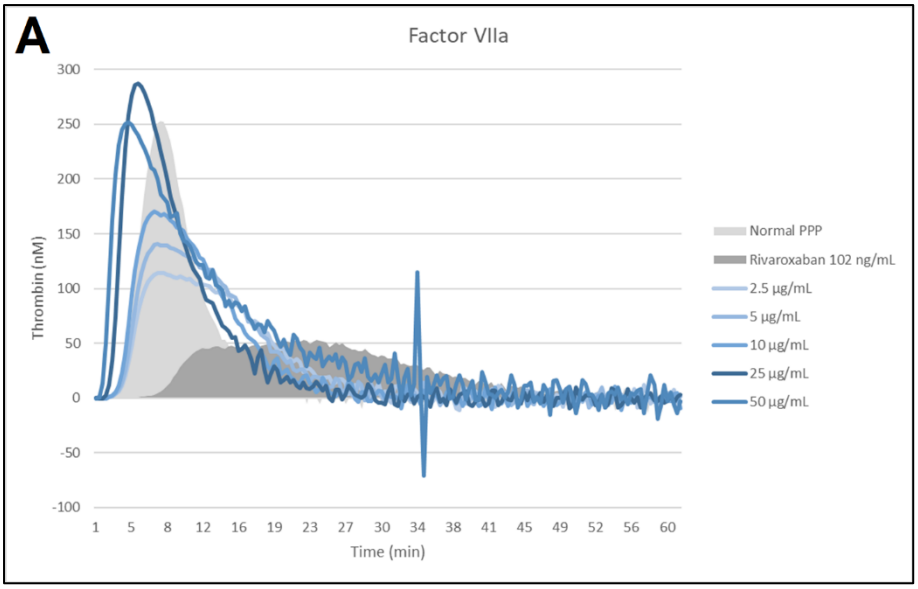
- 3-factor PCC: none of the tested concentrations normalised the lag time and the time to peak. The peak was normalised with concentration 0.8 U/ml; however, concentrations from 1.0 U/ml upwards could not be evaluated because of the lack of calibrator fit. The ETP was normalised with concentration 0.2 U/ml, but became above the unspiked plasma ranges with higher concentrations, suggesting hypercoagulability.

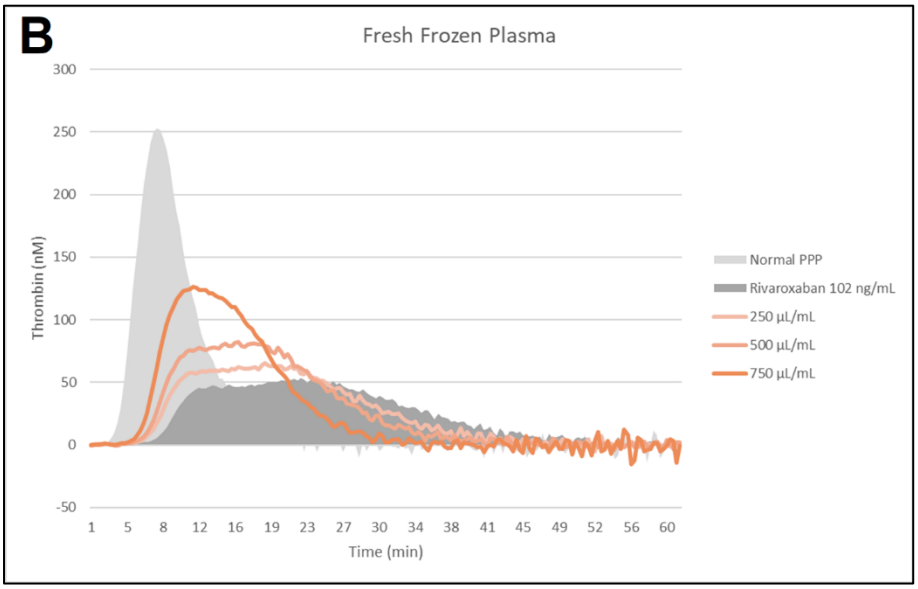
When observing the corresponding thrombin generation curves (the first 60 minutes are shown in Figure 6.9), it was evident that the rVIIa and the activated PCC corrected the lag time, the activated PCC and the 4-factor PCC almost reached the peak of the unspiked plasma, while the 3-factor showed the less pronounced peak.

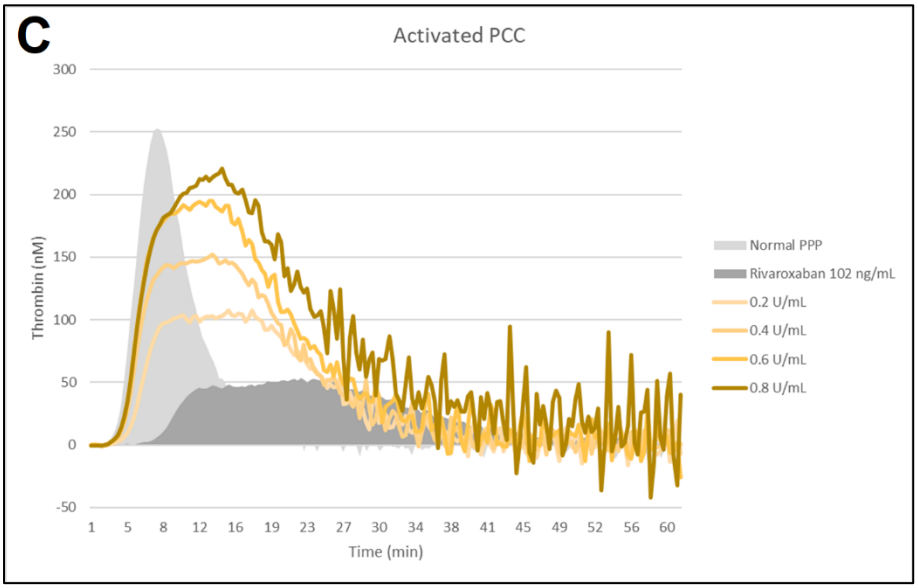
	Lag time (min)	ETP (nM*min)	Peak (nM)	Time to peak (min)
Unspiked PPP ranges	**3.41-4.92**	**1423.6-1971.4**	**134.02-316.84**	**6.31-10.18**
Rivaroxaban 102 ng/ml	8.00 (0)	1374.0 (17.0)	52.10 (1.02)	22.17 (0.17)
rVIIa				
2.5 µg/ml	3.67 (0)	1637.5 (1.5)	114.31 (0.57)	7.67 (0)
5 µg/ml	3.67 (0)	1788.5 (3.5)	140.45 (0.29)	7.67 (0)
10 µg/ml	3.67 (0)	1823.5 (0.5)	169.11 (0.50)	7.33 (0)
25 µg/ml	2.67 (0)	2299.0 (91.0)	287.73 (4.73)	5.33 (0)
50 µg/ml	1.67 (0)	3094.0 (178.0)	251.53 (4.22)	4.33 (0)
Fresh frozen plasma				
250 µl/ml	7.17 (0.17)	1382.5 (8.5)	63.01 (0.02)	19.00 (0.33)
500 µl/ml	6.67 (0)	1502.0 (27.0)	80.70 (2.97)	16.33 (0)
750 µl/ml	6.17 (0.17)	1609.5 (2.5)	125.64 (3.46)	11.33 (0)
Activated PCC				
0.2 U/ml	5.00 (0)	2061.0 (40.0)	103.91 (2.69)	15.00 (0)
0.4 U/ml	4.50 (0.17)	2722.5 (12.5)	148.77 (3.57)	13.17 (0.50)
0.6 U/ml	4.33 (0)	3507.5 (69.5)	192.65 (2.92)	12.33 (0)
0.8 U/ml	4.67 (0)	4717.0 (NC)*	223.23 (2.35)	13.50 (0.17)
1.0 U/ml	No calibrator fit			
1.2 U/ml	No calibrator fit			
4-factor PCC				
0.2 U/ml	6.67 (0)	1783.5 (0.5)	79.28 (1.30)	19.33 (0)
0.4 U/ml	6.33 (0)	2366.0 (98.0)	106.30 (4.81)	18.50 (0.17)
0.6 U/ml	6.33 (0)	3274.0 (169.0)	142.50 (0.19)	17.83 (0.17)
0.8 U/ml	6.33 (0)	4193.0 (257.0)	164.75 (1.14)	18.50 (0.50)
1.0 U/ml	No calibrator fit			
1.2 U/ml	6.17 (0.17)	Curve not finished**	254.39 (1.83)	19.83 (0.50)
3-factor PCC				
0.2 U/ml	7.17 (0.17)	1799.0 (4.0)	78.88 (1.89)	20.33 (0.33)
0.4 U/ml	6.83 (0.17)	2435.5 (16.5)	112.60 (2.44)	18.83 (0.17)
0.6 U/ml	6.33 (0)	2916.5 (38.5)	131.84 (0.60)	18.67 (0)
0.8 U/ml	6.67 (0)	4139.5 (109.5)	151.67 (0.64)	21.50 (0.17)
1.0 U/ml	No calibrator fit			
1.2 U/ml	No calibrator fit			

Table 6.7 The effect of increasing concentrations of different reversal agents on prophylactic rivaroxaban, measured using the CAT

Results are reported as mean (SD) of two measurements. * Only one curve was finished ** This curve was not finished at the end of the running time of the thrombin generation assay, which was 120 minutes for this plate.


A
Factor VIIa
Thrombin (nM)
Time (min)
Normal PPP
Rivaroxaban 102 ng/mL
2.5 µg/mL
5 µg/mL
10 µg/mL
25 µg/mL
50 µg/mL


B
Fresh Frozen Plasma
Thrombin (nM)
Time (min)
Normal PPP
Rivaroxaban 102 ng/mL
250 µL/mL
500 µL/mL
750 µL/mL


C
Activated PCC
Thrombin (nM)
Time (min)
Normal PPP
Rivaroxaban 102 ng/mL
0.2 U/mL
0.4 U/mL
0.6 U/mL
0.8 U/mL

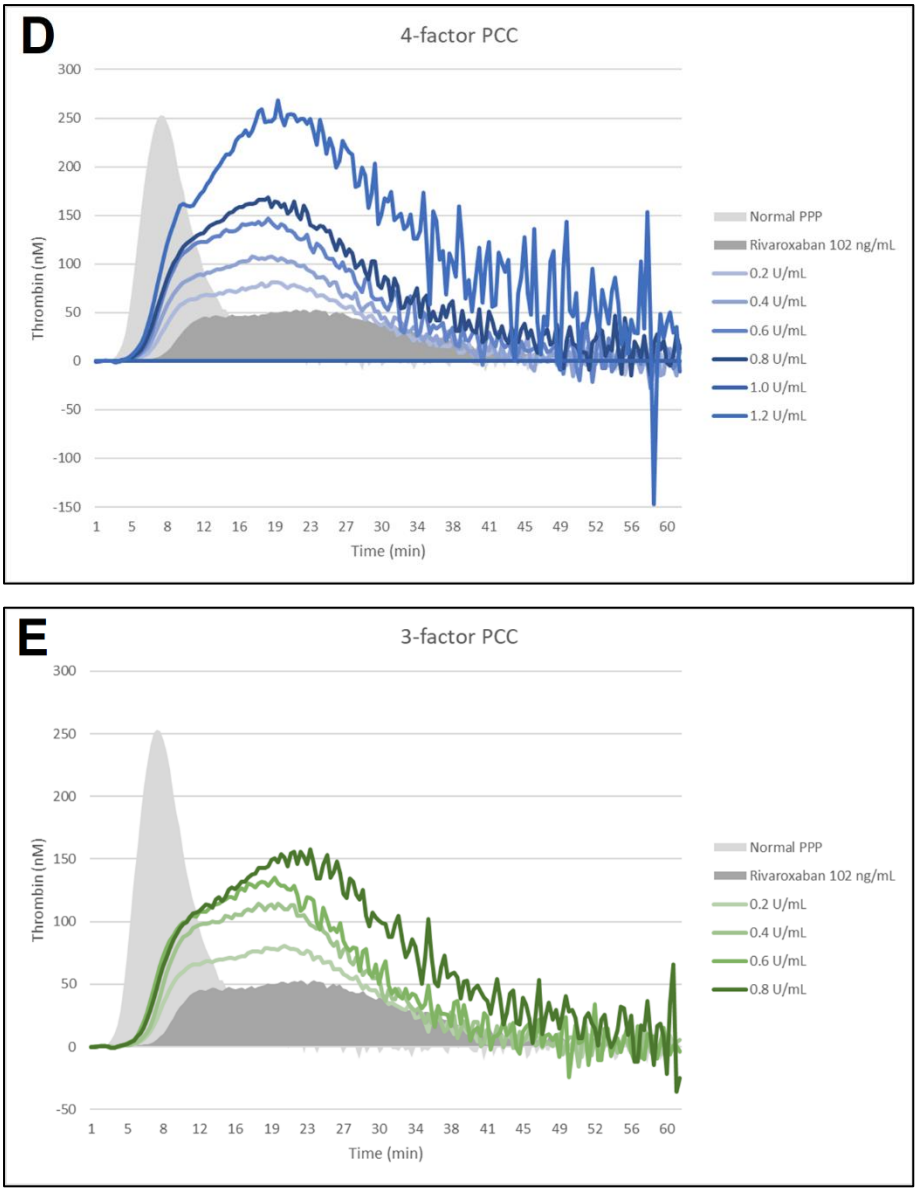


Figure 6.9 Thrombin generation curves with increasing concentrations of different reversal agents on prophylactic rivaroxaban: factor VIIa (A), fresh frozen plasma (B), activated PCC (C), 4-factor PCC (D), 3-factor PCC (E)

The curves obtained with high concentrations of activated PCC (1.2 U/ml, 1.0 U/ml) and 4-factor PCC (1.0 U/ml) and 3-factor PCC (1.2 U/ml, 1.0 U/ml) could not be plotted because there was no calibrator fit. The curve obtained with 4-factor PCC at 1.2 U/ml was not finished at 120 minutes.

6.3.3.5 ETP normalisation

The results of the different reversal agents on the DOAC, expressed as percentage of normalisation of the ETP when compared to the same unspiked plasma, are reported in Table 6.8.

For the reversal of dabigatran, rVIIa at concentration 2.5 µg/ml obtained only 52% normalisation of the ETP, while the ETP was completely normalised with concentration 50 µg/ml, which is more than 10 times higher. FFP at the highest concentration 750 µl/ml obtained 95% normalisation of the ETP. Activated PCC, 4-factor and 3-factor PCC normalised the ETP at a very small concentration 0.5 U/ml (approximately 25 U/kg), and showed hypercoagulability at higher concentrations.

For the reversal of apixaban, rVIIa at concentration 2.5 µg/ml obtained only 68% normalisation of the ETP, while the ETP was completely normalised with concentration 25 µg/ml. FFP at the highest concentration 750 µl/ml obtained only 79% normalisation of the ETP. Activated PCC normalised the ETP at concentration 0.4 U/ml, 4-factor PCC at concentration 0.6 U/ml, while the highest concentration of 3-factor PCC 1.2 U/ml (approximately 50 U/kg) failed to achieve ETP normalisation.

For the reversal of edoxaban, rVIIa at concentration 2.5 µg/ml obtained 92% normalisation of the ETP. FFP at the highest concentration 750 µl/ml obtained 89% normalisation of the ETP. Activated PCC normalised the ETP at the lowest concentration 0.2 U/ml (approximately <10 U/kg), while 4-factor and 3-factor PCC normalised the ETP at concentration 0.4 U/ml.

For the reversal of rivaroxaban, rVIIa at concentration 2.5 µg/ml obtained 95% normalisation of the ETP. FFP at the highest concentration 750 µl/ml obtained 94% normalisation of the ETP. Activated PCC, 4-factor and 3-factor PCC normalised the ETP at the lowest concentration 0.2 U/ml (approximately <10 U/kg).

		Dabigatran 230 ng/ml	Apixaban 225 ng/ml	Edoxaban 192 ng/ml	Rivaroxaban 102 ng/ml
Pre-reversal		44	49	72	80
rVIIa	2.5 µg/ml	52	68	92	95
	5 µg/ml	55	80	93	104
	10 µg/ml	60	93	98	106
	25 µg/ml	83	139	127	134
	50 µg/ml	117	214	178	180
Fresh frozen plasma	250 µl/ml	55	51	74	80
	500 µl/ml	73	66	77	87
	750 µl/ml	95	79	89	94
Activated PCC	0.2 U/ml	76	85	107	120
	0.4 U/ml	NA	108	154	158
	0.5 U/ml	140	NA	NA	NA
	0.6 U/ml	NA	151	190	204
	0.8 U/ml	252	223	251	275
	1.0 U/ml	NA	260	NC	NC
	1.2 U/ml	NC	545	NC	NC
4-factor PCC	0.2 U/ml	65	65	80	104
	0.4 U/ml	NA	86	115	138
	0.5 U/ml	124	NA	NA	NA
	0.6 U/ml	NA	118	145	191
	0.8 U/ml	253	124	218	244
	1.0 U/ml	NA	151	255	NC
	1.2 U/ml	NC	183	NC	NC
3-factor PCC	0.2 U/ml	72	65	94	105
	0.4 U/ml	NA	80	136	142
	0.5 U/ml	132	NA	NA	NA
	0.6 U/ml	NA	NC	172	170
	0.8 U/ml	226	NC	211	241
	1.0 U/ml	NA	NC	220	NC
	1.2 U/ml	NC	93	NC	NC

Table 6.8 The percentage of normalisation of the ETP with different concentrations of the reversal agents on plasma spiked with the DOACs

6.3.3.6 Effect of the reversal agents on DOAC plasma concentrations

The DOAC plasma concentrations were tested again after the addition of the reversal agents at dosages similar to or slightly higher than those recommended in clinical practice, which were rVIIa 10 µg/ml (corresponding to ~ 450 µg/kg), FFP 500 µl/ml (corresponding to ~ 20 ml/kg), different types of PCC 1.2 U/ml (corresponding to ~

50 U/kg). As shown in Table 6.9, a certain reduction of DOAC plasma concentrations was observed, which was more evident for FFP. This finding could be probably due to a dilutional effect. However, none of the reversal agents reduced the plasma concentrations below the lower limit of detection of the specific chromogenic assays.

	Dabigatran (ng/ml)	**Apixaban (ng/ml)**	**Edoxaban (ng/ml)**	**Rivaroxaban (ng/ml)**
Pre-reversal	230	225	192	102
After-reversal				
• rVIIa 10 µg/ml	200	196	172	95
• FFP 500 µl/ml	101	115	87	56
• Activated PCC 1.2 U/ml	183	152	137	77
• 4-factor PCC 1.2 U/ml	188	161	142	75
• 3-factor PCC 1.2 U/ml	188	159	135	63

Table 6.9 Effect of the reversal agents on the DOAC plasma concentrations

6.3.4 Discussion

This study analysed, through the use of the CAT, the effect of five different reversal agents on plasma spiked with the four DOACs. Several *in vitro* studies have evaluated different concentrations of the reversal agents either in samples spiked *in vitro* with the DOACs (Dinkelaar et al., 2013; Dinkelaar et al., 2014; Perzborn et al., 2014) or *ex vivo* from patients or healthy controls receiving the DOACs (Herrmann et al., 2014; Marlu et al., 2012; Schultz et al., 2017a; Schultz et al., 2017b). However, this was the first time that the four DOAC and the five reversal agents have been evaluated simultaneously.

Rivaroxaban was spiked at concentration 102 ng/ml, which corresponds to a prophylactic concentration, since the mean peak plasma concentration in patients taking 10 mg OD for orthopaedic prophylaxis was 101 ng/ml (90% prediction interval

7-273 ng/ml) (European Medicines Agency, 2018f). However, this rivaroxaban concentration can also be seen as a low therapeutic concentration, since the mean peak concentration in patients taking 20 mg OD was reported to be 215 ng/ml, but the 90% prediction interval ranged from 22 to 535 ng/ml (European Medicines Agency, 2018f). The lowest dose of rVIIa (2.5 µg/ml, ~ 90 µg/kg) obtained a good reversal with 95% ETP normalisation. FFP at the highest concentration (750 µl/ml, ~ 30 ml/kg) gave 94% ETP normalisation, which is a good result but in clinical practice there is a concern of fluid overload, since it corresponds to more than two litres in a 70 kg patient. The lowest concentration of activated PCC (0.2 U/ml, ~ 8.5 U/kg) obtained 120% normalisation of the ETP, while the lowest concentration of 4-factor and 3-factor PCC (0.2 U/ml, ~ 8.5 U/kg) obtained 104% and 105% normalisation, respectively.

Apixaban was spiked at concentration 225 ng/ml, which corresponds to a therapeutic concentration, since the median peak plasma concentration in VTE patients taking 10 mg BID was 251 ng/ml (European Medicines Agency, 2018c). In order to obtain 93% normalisation of the ETP, the rVIIa had to be administered at concentration 10 µg/ml (~ 450 µg/kg). FFP, even at the highest dose, was not enough with only 79% normalisation. Activated PCC obtained 108% normalisation at concentration 0.4 U/ml and 4-factor PCC obtained 118% at concentration 0.6 U/ml. Conversely, the 3-factor PCC obtained 93% normalisation at a concentration of 1.2 U/ml.

Edoxaban was spiked at concentration 192 ng/ml, which corresponds to a therapeutic concentration, since the median peak plasma concentration for the dose 60 mg OD has been reported to be 170 ng/ml in AF patients and 234 ng/ml in VTE patients (Douxfils et al., 2018). Compared to apixaban, edoxaban was reversed by lower concentrations of the reversal agents. The lowest dose of rVIIa obtained a good reversal with 92%

ETP normalisation, while FFP at the highest concentration gave 89% ETP normalisation. The lowest concentration of activated PCC obtained 107% normalisation of the ETP, while the lowest concentration of 4-factor and 3-factor PCC obtained 80% and 94% normalisation, respectively.

Dabigatran was spiked at concentration 230 ng/ml, which corresponds to a therapeutic concentration, since the mean peak plasma concentration in patients taking 150 mg BID was reported to be 210 ng/ml (Testa et al., 2016). In order to obtain 117% normalisation of the ETP, the rVIIa had to be administered at concentration 50 µg/ml. FFP at a concentration of 750 µl/ml obtained 95% normalisation. At concentration 0.5 U/ml (~ 20 U/kg), activated PCC obtained 140% normalisation, 4-factor PCC 124% and 3-factor PCC obtained 132%.

Taken together, these results suggest that different concentrations of reversal agents might be needed in order to normalise the coagulation profile, based on the drug administered and the plasma concentration. Therefore, the initial step in the management of a DOAC-related bleeding should be the measurement of the plasma concentration, in order to guide the optimal reversal. Different doses of PCC, based on the initial INR, are also recommended for the management of VKA-related bleeding (Hull & Garcia, 2019). These findings contradict the results of previous studies on DOAC-treated healthy volunteers which showed that PCC 25 U/kg or 37.5 U/kg were not enough to completely normalise the ETP (Barco et al., 2016; Cheung et al., 2015; Zahir et al., 2015). However, previous reversal experiments *in vitro* reported low concentrations of PCC or activated PCC to be effective, suggesting that the CAT is more sensitive to the effect of these reversal products. For instance, activated PCC and 3-factor PCC at concentration 0.5 U/ml normalised the ETP in plasma from patients receiving dabigatran 150 mg BID or rivaroxaban 10 mg OD

(Herrmann et al., 2014). In another study activated PCC or 4-factor PCC at concentration 0.25 U/ml were enough to normalise the ETP in plasma from healthy volunteers receiving one administration of rivaroxaban 20 mg or dabigatran 150 mg, with higher concentrations showing progressive hypercoagulability (Marlu et al., 2012). In DOAC-spiking studies *in vitro*, it was demonstrated that the optimal concentrations of 4-factor PCC required to normalise the ETP was dependent on the concentration of the drugs (Dinkelaar et al., 2013; Dinkelaar et al., 2014). For therapeutic dabigatran (200 ng/ml) it was 0.2 U/ml, increasing to 1.0 U/ml for supratherapeutic dabigatran (800 ng/ml) (Dinkelaar et al., 2014). For therapeutic rivaroxaban (200 ng/ml) it was 0.10 U/ml, increasing to 0.28 U/ml for supratherapeutic rivaroxaban (800 ng/ml) (Dinkelaar et al., 2013). However, for therapeutic apixaban (200 ng/ml) it was 0.7 U/ml, increasing to 2.9 U/ml for supratherapeutic apixaban (800 ng/ml) (Dinkelaar et al., 2014), suggesting that the reversal of apixaban might require higher doses of PCC.

The main limitation of this study is the fact that each DOAC has been evaluated only once and at a single concentration, although every concentration of the reversal agents has been tested in duplicate. Ideally, each DOACs should have been spiked at least at a prophylactic and a therapeutic concentration and similar concentrations should have been used for the different molecules. Furthermore, the reversal was performed *in vitro* and extrapolation to the bed side might be unsatisfactory. The main strengths of this study are the use of a pool of plasma, in order to reduce inter-patient variability, and the fact that all the reversal agents were tested on the same plate, in order to reduce inter-plate variability. However, due to the small number of tests performed, these results can be considered hypothesis generating only and should be confirmed in further studies.

6.4 The effect of DOAC Stop® on a broad range of oral and parenteral anticoagulants

6.4.1 Aim

The aim of this study was to investigate the effect of DOAC Stop® on a broad range of oral and parenteral anticoagulants and on a broad range of clotting and global coagulation assays, including the CAT and the TEG.

6.4.2 Methods

Normal PPP, warfarinised PPP and PPP spiked with the direct factor Xa inhibitors (apixaban, edoxaban, rivaroxaban), the direct thrombin inhibitors (argatroban, bivalirudin, dabigatran) and the indirect factor Xa inhibitors (enoxaparin, fondaparinux) were prepared as described in paragraph 2.3. Aliquots were stored at -80° C until the analysis. Only the highest concentrations of each anticoagulant were treated with DOAC Stop®. There were two batches of normal PPP: the first one was used to spike apixaban, edoxaban, rivaroxaban, danaparoid, enoxaparin, and bivalirudin; the second one was used to spike argatroban, dabigatran, and fondaparinux. However, all the results after DOAC Stop® were always compared to the same untreated plasma.

DOAC Stop® was purchased from Haematex Research (Australia), lot HX1A-100A. According to the manufacturer's instructions, one minitab of DOAC Stop® should be used with 0.5-1.5 ml of plasma. In order to compare the plasma before and after DOAC Stop® treatment, untreated and treated aliquots of citrated plasma were processed according to the same protocol (except for the addition of DOAC Stop®) as follows:

1. The aliquots were thawed for five minutes in the water bath at 37°C;

2. The test plasma was transferred in a plastic centrifuge tube;
3. A mini-tab of DOAC Stop® was added for every 1 ± 0.1 ml of plasma and it was gently mixed until dissolved (this step was skipped for the untreated plasma);
4. The tubes were placed on the roller mixer for five minutes for further mixing;
5. The tubes were centrifuged for five minutes at 2000g (corresponding to 3150 rpm on the Eppendorf Centrifuge 5810, using the rotor A-4-62 with a radius of 18 cm) in order to centrifuge down the particulate of DOAC Stop®;
6. The supernatant plasma was transferred in another plastic tube and used immediately after preparation for the APTT and PT/INR assays and for the native TEG. Some aliquots of plasma (both untreated and treated) were frozen at -80°C for the other tests (anti-Xa, DTT, LA, factor assays, CAT).

Therefore, all samples (both untreated and treated) underwent four cycles of freeze-thaw. The concentrations of the direct and indirect factor Xa inhibitors were measured using the anti-Xa assay with specific calibrators (paragraph 2.10), while the concentrations of the direct thrombin inhibitors were measured using the DTT with specific calibrators (paragraph 2.9).

Frozen samples were shipped to Sheffield (UK) in dry ice and the CAT was performed at TF 5pM and 1pM (paragraph 2.13), using two calibrator wells and two test wells. Native TEG was performed using both channels (paragraph 2.14). All the samples were prepared and analysed between March and June 2019. Only the CAT and the TEG were performed in duplicate, using two wells at the same time for the CAT and the two channels at the same time for the TEG. The other assays (APTT, PT/INR, LA, factor assays) were performed only once on each plasma sample.

The non-parametric Wilcoxon matched-pairs signed-ranks test was used to compare untreated and treated categories of plasma (normal PPP, warfarinised PPP, direct factor Xa inhibitors, direct thrombin inhibitors, indirect factor Xa inhibitors). Furthermore, for this study, normalisation of the CAT results or the TEG results was defined as values, after DOAC Stop® treatment, within the ranges of the same unspiked plasma sample, calculated as mean ± 1.96 * SD. To obtain these ranges, the unspiked normal PPP batch I and II were run eight times each in different plates on different days for the CAT, and they were run 10 times each in different channels on different days for the TEG. Results were also graphed as box-and-whisker plots, where the line inside the box represents the median value, and the edges of the box represent the first and third quartiles.

6.4.3 Results

6.4.3.1 Anticoagulant concentrations

The direct factor Xa inhibitors (apixaban, edoxaban and rivaroxaban) and the direct thrombin inhibitors (argatroban, bivalirudin and dabigatran) after treatment with DOAC Stop® were undetectable. No changes were observed in the concentrations of the indirect factor Xa inhibitors (enoxaparin and fondaparinux), as shown in Table 6.10.

	Untreated	Treated with DOAC Stop®
Anti-Xa assays		
Apixaban	265 ng/ml	0 ng/ml
	182 ng/ml	0 ng/ml
Edoxaban	220 ng/ml	4 ng/ml
	151 ng/ml	4 ng/ml
Rivaroxaban	339 ng/ml	0 ng/ml
	241 ng/ml	0 ng/ml
Enoxaparin	1.68 U/ml	1.66 U/ml
	0.93 U/ml	0.92 U/ml
Fondaparinux	2.16 μg/ml	2.14 μg/ml
	1.62 μg/ml	1.56 μg/ml
Diluted thrombin time assays		
Argatroban	6.16 μg/ml	0.03 μg/ml
	3.06 μg/ml	0.01 μg/ml
Bivalirudin	26.4 μg/ml	0 μg/ml
	13.3 μg/ml	0 μg/ml
Dabigatran	318 ng/ml	0 ng/ml
	203 ng/ml	0 ng/ml

Table 6.10 Changes in the anticoagulant concentrations after DOAC Stop® treatment
Note: The lower limit of detection of the assay for apixaban, dabigatran, edoxaban and rivaroxaban was 30 ng/ml. The lower limit of detection for argatroban was 0.03 μg/ml.

6.4.3.2 APTT and PT/INR assays

The DOAC Stop® treatment did not produce any effect on the normal PPP, the warfarinised PPP and the PPP spiked with the indirect factor Xa inhibitors (enoxaparin, fondaparinux), as shown in Table 6.11. A complete normalisation of the APTT and PT/INR was observed for the PPP spiked with the direct Xa inhibitors (apixaban, edoxaban, rivaroxaban). With regards to the direct thrombin inhibitors, a complete normalisation was obtained for dabigatran, while bivalirudin and argatroban reached a nearly complete normalisation, which can be explained by the high concentrations of these drugs that provided very high PT and APTT before DOAC Stop® treatment. Of note, when considering the untreated DOACs, the APTT was

more prolonged with the thrombin inhibitor dabigatran, while the direct factor Xa inhibitors had a greater effect on the PT.

	PT				APTT			
Normal ranges	**9.2-11.8 sec**		**INR 0.84-1.04**		**24.8-35.0 sec**		**Ratio 0.89-1.16**	
	U	**T**	**U**	**T**	**U**	**T**	**U**	**T**
Normal plasma								
Batch I	10.4	10.6	1.00	1.02	28.3	28.1	0.95	0.94
Batch II	10.7	10.6	1.03	1.02	27.3	27.5	0.92	0.92
Warfarinised plasma								
INR 4	43.3	43.1	4.22	4.20	42.0	42.6	1.41	1.43
INR 3	32.7	33.7	3.18	3.28	42.3	42.8	1.42	1.44
INR 2	21.8	23.0	2.11	2.23	35.1	36.5	1.18	1.22
Direct factor Xa inhibitors								
Apixaban 265 ng/ml	14.3	10.8	1.38	1.04	33.2	29.4	1.11	0.99
Apixaban 182 ng/ml	12.9	10.7	1.24	1.03	33.1	29.8	1.11	1.00
Edoxaban 220 ng/ml	14.4	10.8	1.39	1.04	36.1	28.9	1.21	0.97
Edoxaban 151 ng/ml	13.2	10.8	1.27	1.04	33.6	28.7	1.13	0.96
Rivaroxaban 339 ng/ml	20.0	10.9	1.94	1.05	36.3	28.7	1.22	0.96
Rivaroxaban 241 ng/ml	17.0	10.9	1.64	1.05	35.0	29.5	1.17	0.99
Direct thrombin inhibitors								
Argatroban 6.16 µg/ml	52.6	11.0	5.14	1.06	138.4	37.5	4.64	1.26
Argatroban 3.06 µg/ml	29.4	10.8	2.86	1.04	100.8	34.8	3.38	1.17
Bivalirudin 26.4 µg/ml	121.8	11.8	12.00	1.14	210.3	46.8	7.05	1.57
Bivalirudin 13.3 µg/ml	70.7	11.1	6.93	1.07	158.2	40.2	5.31	1.35
Dabigatran 318 ng/ml	14.2	11.1	1.37	1.07	63.2	33.1	2.12	1.11
Dabigatran 203 ng/ml	13.2	11.1	1.27	1.07	56.1	32.1	1.88	1.08
Indirect factor Xa inhibitors								
Enoxaparin 1.68 U/ml	12.6	12.9	1.21	1.24	70.5	74.6	2.37	2.50
Enoxaparin 0.93 U/ml	11.4	11.7	1.10	1.13	50.8	51.3	1.70	1.72
Fondaparinux 2.16 µg/ml	11.8	11.9	1.14	1.15	34.3	34.1	1.15	1.14
Fondaparinux 1.62 µg/ml	11.1	11.5	1.07	1.11	35.0	35.9	1.17	1.20

Table 6.11 Changes in the PT/INR and the APTT results after DOAC Stop® treatment

6.4.3.3 Lupus anticoagulant assays

Among the different anticoagulants, only the plasma spiked with the two concentrations of rivaroxaban gave false positive results on the LA assay (1.34 for both concentrations), as shown in Table 6.12. After treatment with DOAC Stop® the plasma spiked with rivaroxaban was normalised. A result could not be issued for argatroban 6.16 μg/ml and bivalirudin 26.4 μg/ml because they were outside the upper limit of the test range for the dRVVT Screen (range 16-240 sec) and the dRVVT Confirm (range 6-121 sec). Argatroban 3.06 μg/ml and bivalirudin 13.3 μg/ml were not tested for the LA due to insufficient plasma.

Of note, these results of the LA assay before treatment confirmed the different pattern of the DOAC on the dRVVT assay (Favaloro, Mohammed, et al., 2019). As previously reported, dabigatran prolongs in parallel the LA screen and the LA confirm, therefore resulting in a normal ratio, with low risk of false positive results (Favaloro, Mohammed, et al., 2019). Apixaban prolongs more the LA confirm than the LA screen, therefore resulting in a ratio <1, suggesting instead a potential for false negative results. Conversely, rivaroxaban prolongs more the LA screen than the LA confirm, with higher risk of false positive results (Favaloro, Mohammed, et al., 2019). Other studies suggested that the dRVVT, in particular the dRVVT confirm, could potentially be a screening tool for patients on DOAC (Patel, Byrne, et al., 2019); however, in this study, the increase seen with the DOAC was similar to the one observed with the VKA.

Two types of positive controls for the LA assay, prepared following the DOAC Stop® protocol, were also tested. First, three dilutions of the manufacturer's LA positive control (HemosIL®, Instrumentation Laboratory, Italy) were prepared. There was no difference after DOAC Stop® treatment in these three samples. Second, two different

anonymised plasma samples from patients with a diagnosis of LA were prepared. While the strong positive LA remained positive after DOAC Stop® treatment (from 2.41 to 2.52), the weak positive LA became negative (from 1.61 to 1.15). This incidental finding raised several questions, such as whether some of antiphospholipid antibodies might have been removed by the DOAC Stop® treatment and whether the DOAC Stop® treatment might give different results in single/double/triple positive patients with APS.

		Untreated			**Treated with DOAC Stop®**		
		dRVVTS	**dRVVTC**	**NR**	**dRVVTS**	**dRVVTC**	**NR**
Normal PPP	Batch I	0.94	0.93	1.01	0.95	0.94	1.01
	Batch II	0.96	0.92	1.03	0.95	0.92	1.03
Warfarinised PPP	INR 4.22	1.61	1.54	1.05	1.62	1.60	1.01
	INR 3.18	1.69	1.44	1.17	1.69	1.45	1.16
	INR 2.11	1.38	1.27	1.09	1.40	1.28	1.09
Apixaban	265 ng/ml	1.44	1.58	0.91	0.96	0.96	1.00
	182 ng/ml	1.26	1.46	0.86	0.98	0.95	1.03
Edoxaban	220 ng/ml	2.25	2.24	1.01	0.98	0.97	1.01
	151 ng/ml	1.93	1.91	1.01	0.97	0.95	1.02
Rivaroxaban	339 ng/ml	2.45	1.83	1.34	0.99	0.96	1.03
	241 ng/ml	2.12	1.58	1.34	0.98	0.95	1.03
Argatroban	6.16 µg/ml	4.84	failed	failed	1.26	1.31	0.96
Bivalirudin	26.4 µg/ml	failed	failed	failed	1.87	1.60	1.16
Dabigatran	318 ng/ml	2.84	2.81	1.01	1.06	1.07	0.99
	203 ng/ml	2.45	2.41	1.02	1.04	1.03	1.01
Enoxaparin	1.68 U/ml	1.50	1.45	1.03	1.55	1.48	1.05
	0.93 U/ml	1.06	1.04	1.02	1.07	1.07	1.00
Fondaparinux	2.16 µg/ml	1.19	1.20	0.99	1.21	1.18	1.03
	1.62 µg/ml	1.13	1.12	1.01	1.13	1.11	1.02
LA positive control (HemosIL®)	Dilution n.1	2.14	1.18	1.82	2.11	1.20	1.76
	Dilution n.2	2.04	1.19	1.71	2.02	1.18	1.71
	Dilution n.3	2.00	1.19	1.68	1.95	1.19	1.63
LA positive control	Sample n.1	2.46	1.53	1.61	1.15	1.00	1.15
	Sample n.2	2.29	0.95	2.41	2.28	0.91	2.52

Table 6.12 Changes in the lupus anticoagulant results after DOAC Stop® treatment
The cut-off for a positive lupus anticoagulant assay is NR >1.2.

6.4.3.4 Calibrated Automated Thrombin Generation Assay

CAT with TF 5pM

After treatment with DOAC Stop®, there were no significant differences in the CAT parameters of the normal PPP, the warfarinised PPP and the plasma spiked with the indirect factor Xa inhibitors (Figures 6.10-6.13). However, the curves for enoxaparin 1.68 U/ml could not be compared, because they were flat traces both before- and after-DOAC Stop®, suggesting no effect of treatment.

After DOAC Stop® treatment the plasma spiked with the direct factor Xa inhibitors showed a significant reduction of the lag time and the time to peak, and a significant increase of the ETP and the peak ($p=0.028$ for all comparisons). All these parameters were normalised after DOAC Stop® treatment, except the ETP of the rivaroxaban 241 ng/ml (Table 6.13).

After DOAC Stop® treatment the plasma spiked with the direct thrombin inhibitors showed a significant reduction of the lag time and the time to peak, and a significant increase of the peak ($p=0.028$ for all comparisons). There was a non-significant post-treatment increase of the ETP ($p=0.068$), which can be explained by the fact that the change in the ETP could not be calculated for the two highest concentrations of argatroban, because no tail could be identified in the curves obtained from the untreated plasma after 180 min.

Furthermore, some differences were observed in the pattern of normalisation of the different direct thrombin inhibitors (Table 6.13). After DOAC Stop® treatment the dabigatran-spiked plasma resulted in a normalisation of most of the CAT parameters, except the ETP and the peak. In the bivalirudin-spiked plasma the ETP and the peak were normalised for both concentrations, while the time to peak only for bivalirudin

13.3 µg/ml. The DOAC Stop® treatment normalised the lag time, the peak and the time to peak for the plasma spiked with argatroban at 3.06 µg/ml, and none of them for the plasma spiked with argatroban at 6.16 µg/ml.

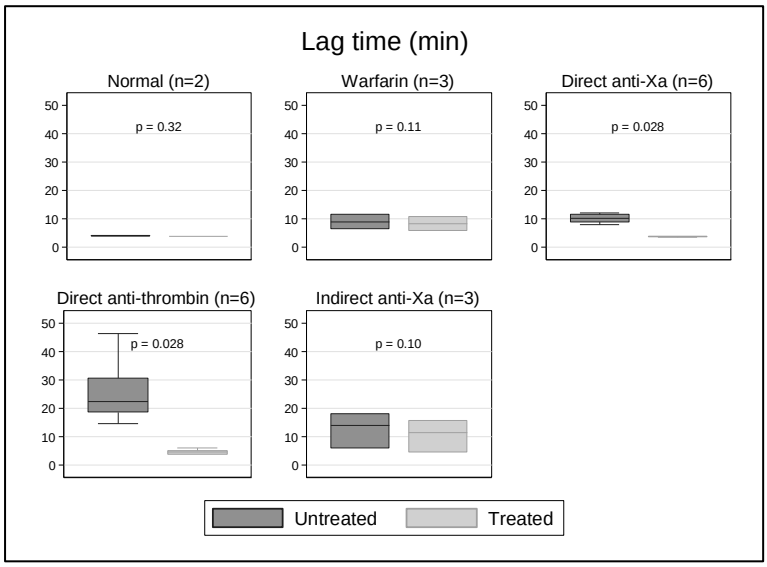


Figure 6.10 Changes in the lag time (on the CAT at TF 5pM) after DOAC Stop® treatment

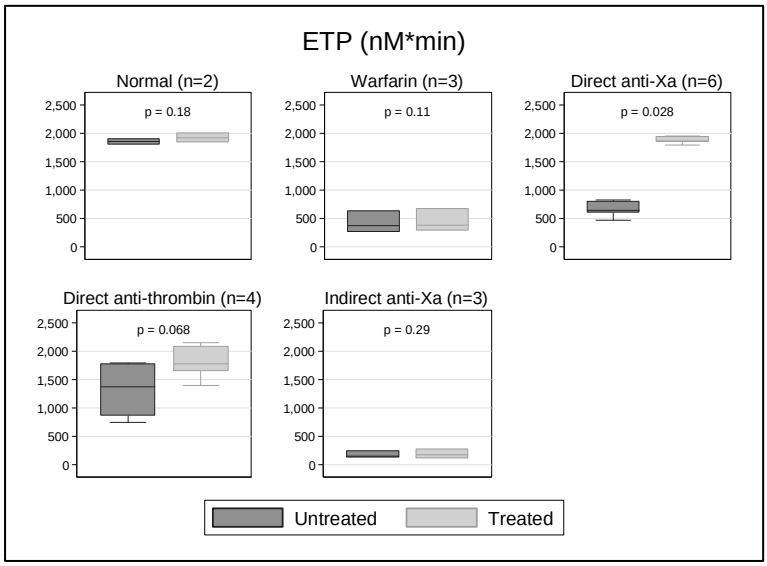


Figure 6.11 Changes in the endogenous thrombin potential (on the CAT at TF 5pM) after DOAC Stop® treatment

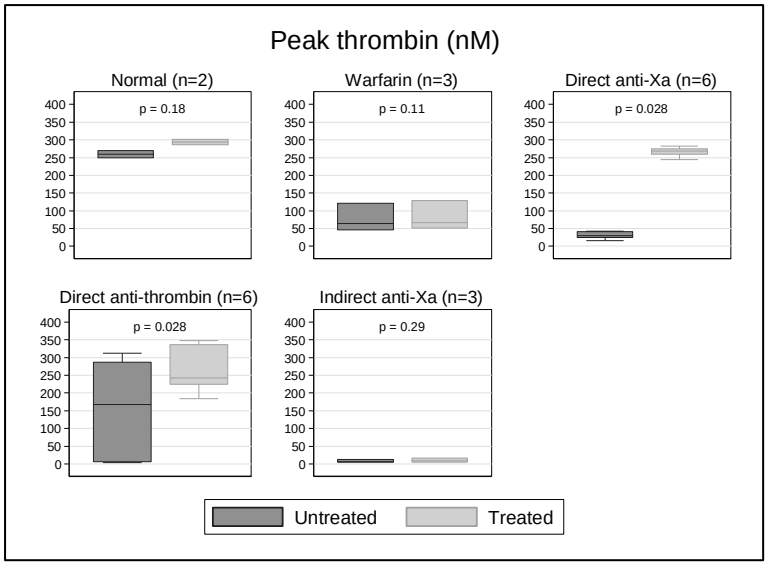


Figure 6.12 Changes in the peak thrombin (on the CAT at TF 5pM) after DOAC Stop® treatment

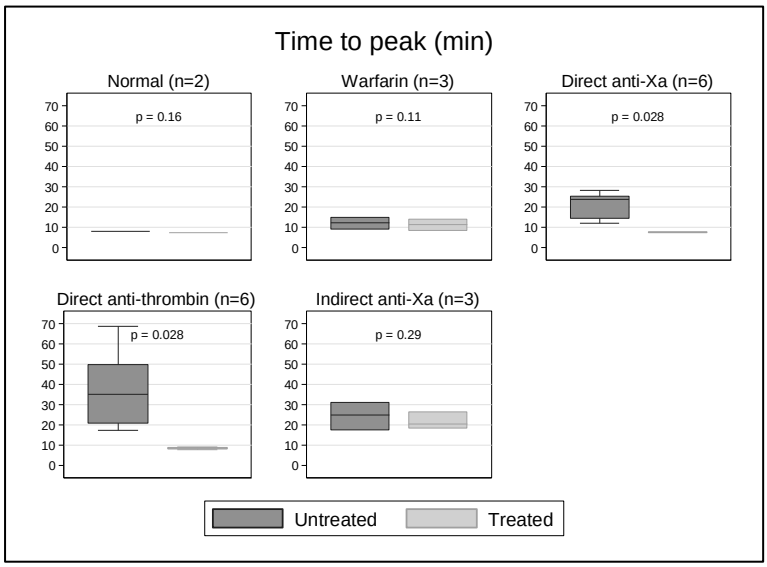


Figure 6.13 Changes in the time to peak (on the CAT at TF 5pM) after DOAC Stop® treatment

		Lag time (min)		ETP (nM*min)		Peak (nM)		Time to peak (min)	
Unspiked plasma ranges		*3.54-4.34*		*1852.7-2225.4*		*243.45-349.02*		*6.86-8.53*	
		Untreated	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**
Normal PPP	Batch I	4.17 (0.23)	3.84 (0.23)	1911.0 (52.3)	2013.0 (12.7)	270.33 (5.48)	301.69 (4.96)	8.00 (0)	7.33 (0)
	Batch II	3.84 (0.23)	3.84 (0.23)	1802.5 (64.3)	1841.5 (156.3)	249.95 (9.58)	284.96 (18.87)	8.00 (0)	7.33 (0)
Warfarinised PPP	INR 4.22	11.67 (0)	10.84 (0.23)	275.0 (12.7)	296.5 (19.1)	46.67 (1.62)	51.17 (3.17)	15.00 (0)	14.00 (0)
	INR 3.18	9.00 (0)	8.33 (0)	374.0 (19.8)	381.5 (16.3)	63.82 (2.19)	66.57 (2.34)	12.33 (0)	11.33 (0)
	INR 2.11	6.50 (0.24)	6.00 (0)	641.0 (4.2)	678.5 (44.5)	121.74 (1.68)	129.20 (6.39)	9.17 (0.23)	8.50 (0.24)
Apixaban	265 ng/ml	8.92 (0.47)	3.91 (0)	643.0 (0)	1855.5 (23.3)	27.57 (2.54)	262.72 (3.20)	14.43 (1.65)	7.58 (0)
	182 ng/ml	8.08 (0.24)	3.91 (0)	830.5 (10.6)	1857.0 (41.0)	42.94 (0.13)	272.97 (5.35)	12.09 (0.23)	7.58 (0)
Edoxaban	220 ng/ml	11.59 (0)	3.74 (0.24)	643.0 (5.7)	1945.5 (132.2)	31.25 (0.64)	282.46 (11.67)	24.78 (0.24)	7.42 (0.23)
	151 ng/ml	9.92 (0.47)	3.57 (0)	808.0 (22.6)	1887.5 (37.5)	40.86 (0.81)	275.30 (4.14)	22.95 (0.47)	7.42 (0.23)
Rivaroxaban	339 ng/ml	12.09 (0.23)	3.91 (0)	467.5 (20.5)	1956.0 (123.0)	15.66 (0.33)	259.95 (11.79)	28.28 (0.47)	7.91 (0)
	241 ng/ml	10.42 (0.23)	3.91 (0)	606.0 (11.3)	1793.5 (9.2)	23.09 (0.67)	243.70 (1.99)	25.45 (0.71)	7.91 (0)
Argatroban	6.16 µg/ml	26.17 (0.23)	4.84 (0.23)	No tail found	1396.0 (127.3)	3.25 (0)	183.82 (19.2)	68.67 (18.86)	8.84 (0.23)
	3.06 µg/ml	18.50 (0.24)	4.33 (0)	No tail found	1799.5 (7.8)	6.50 (0.12)	246.47 (4.82)	36.50 (3.07)	8.00 (0)
Bivalirudin	26.4 µg/ml	46.34 (1.89)	6.00 (0)	1795.0 (36.8)	2091.0 (113.1)	286.51 (5.74)	348.55 (18.49)	49.83 (2.12)	9.17 (0.23)
	13.3 µg/ml	30.67 (2.35)	5.00 (0)	1763.5 (16.3)	2155.0 (79.2)	312.43 (7.36)	336.71 (11.09)	33.67 (2.35)	8.33 (0)
Dabigatran	318 ng/ml	18.50 (0.71)	3.84 (0.23)	748.0 (2.8)	1652.5 (23.3)	148.06 (3.37)	224.60 (2.05)	20.83 (0.71)	7.84 (0.23)
	203 ng/ml	14.67 (0)	3.84 (0.23)	980.0 (2.8)	1753.5 (139.3)	186.41 (2.50)	238.16 (17.67)	17.33 (0)	8.00 (0)
Enoxaparin	1.68 U/ml	Flat CAT traces							
	0.93 U/ml	5.84 (0.23)	4.50 (1.65)	137.0 (0)	118.0 (2.82)	5.61 (0.18)	5.23 (0.18)	17.50 (0.71)	18.34 (0.47)
Fondaparinux	2.16 µg/ml	18.17 (0.71)	15.67 (0)	151.0 (9.90)	171.0 (9.90)	6.90 (0.32)	8.89 (0.95)	31.17 (0.23)	26.34 (0.94)
	1.62 µg/ml	14.00 (0)	11.50 (0.24)	242.5 (0.71)	274.5 (3.54)	12.90 (0.20)	16.56 (0.11)	24.84 (0.23)	20.33 (0)

Table 6.13 Changes in the thrombin generation (TF 5 pM) results after DOAC Stop® treatment
Results are reported as mean (SD) of two measurements.

CAT with TF 1pM

A similar pattern was observed when the plasma was tested with the CAT at TF 1pM (Figures 6.14-6.17). There was no significant change after DOAC Stop® treatment for the normal PPP, the warfarinised PPP and the plasma spiked with the indirect factor Xa inhibitors. Regarding the latter, only the two concentrations of fondaparinux could be compared, because the curves for enoxaparin 1.68 U/ml and 0.93 U/ml were flat traces both before- and after- DOAC Stop®, suggesting no effect of treatment.

After DOAC Stop® treatment the plasma spiked with the direct factor Xa inhibitors showed a significant reduction of the lag time and the time to peak, and a significant increase of the ETP and the peak ($p=0.028$ for all comparisons).

After DOAC Stop® treatment the plasma spiked with the direct thrombin inhibitors showed a non-significant post-treatment increase of the lag time and the time to peak ($p=0.068$ for both comparisons). Conversely to the direct thrombin inhibitors tested at 5pM, the increase of the ETP and the peak were not statistically significant ($p=0.11$ and $p=0.47$, respectively). However, the plasma spiked with argatroban 3.06 μg/ml and with bivalirudin 13.3 μg/ml could not be tested at 1pM because of insufficient plasma. Furthermore, the change in ETP could not be calculated for argatroban 6.16 μg/ml, because no tail could be identified from the untreated plasma after 180 min, probably due to the high concentration tested.

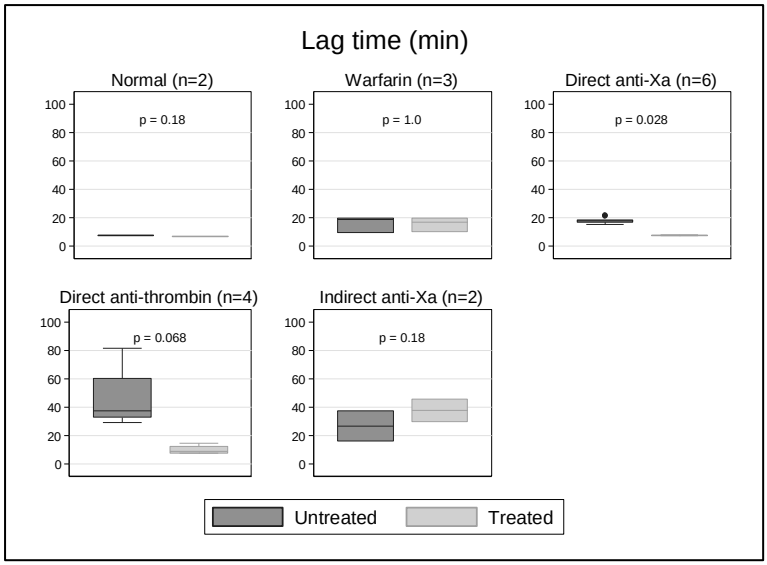


Figure 6.14 Changes in the lag time (on the CAT at TF 1pM) after DOAC Stop® treatment

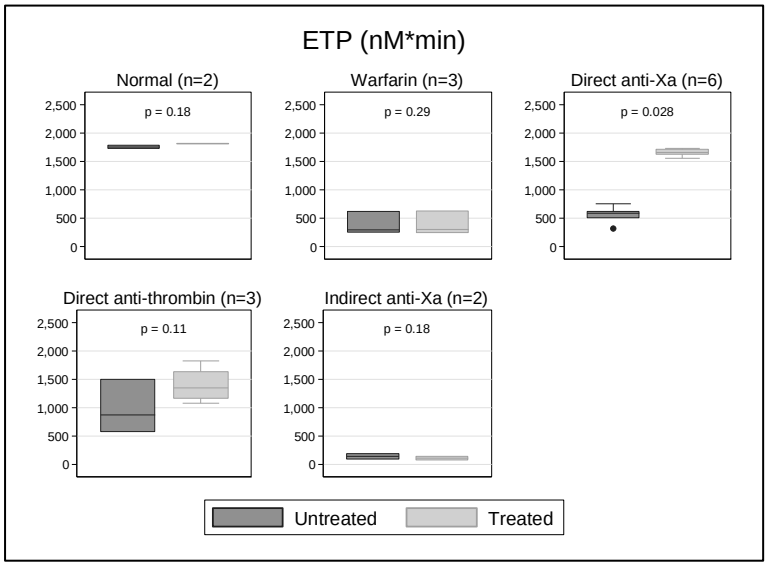


Figure 6.15 Changes in the endogenous thrombin potential (on the CAT at TF 1pM) after DOAC Stop® treatment

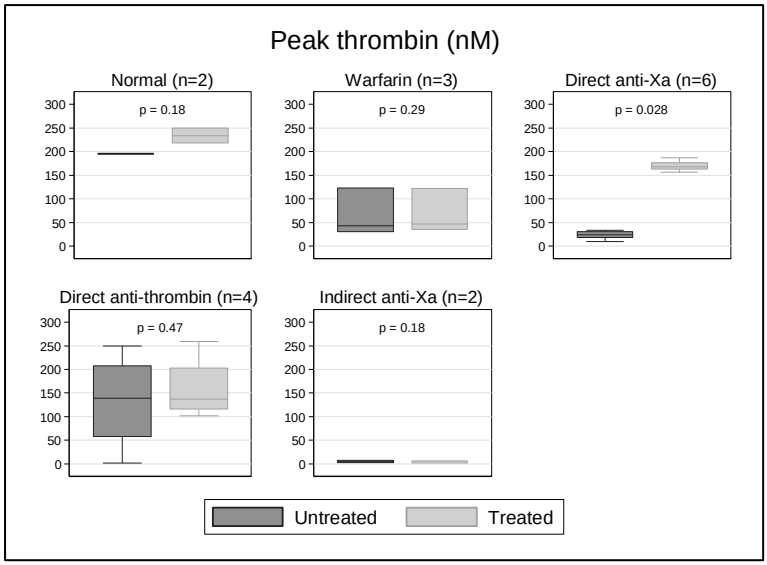


Figure 6.16 Changes in the peak thrombin (on the CAT at TF 1pM) after DOAC Stop® treatment

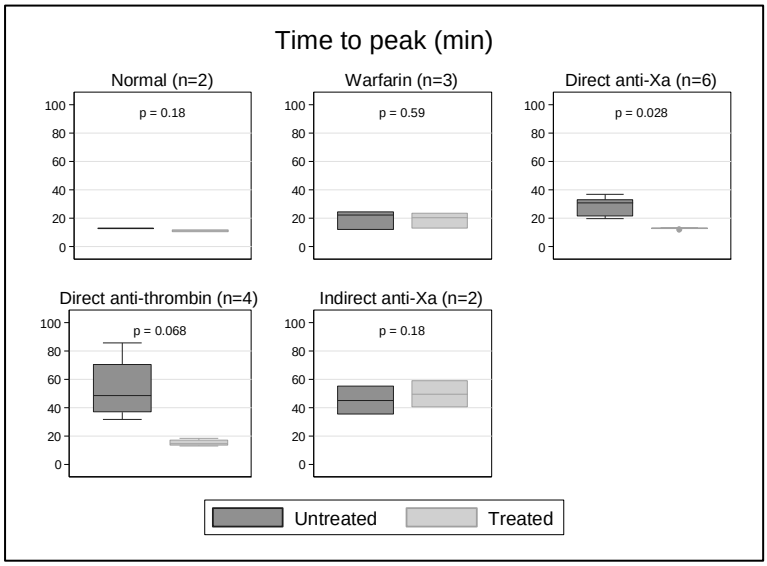


Figure 6.17 Changes in the time to peak (on the CAT at TF 1pM) after DOAC Stop® treatment

6.4.3.5 Thromboelastography

After treatment with DOAC Stop®, there were no significant differences in the TEG parameters of the normal PPP and the warfarinised PPP (Figures 6.18-6.21). The curves of the plasma spiked with the indirect factor Xa inhibitors could not be compared, because they were flat traces both before- and after-DOAC Stop®, suggesting no effect of treatment.

After DOAC Stop® treatment the plasma spiked with the direct factor Xa inhibitors showed a significant decrease of the R time ($p=0.046$) and the K time ($p=0.028$), and a significant increase of the angle and the MA ($p=0.028$ for both comparisons). Most of these parameters were normalised after DOAC Stop® treatment, except the R time for apixaban 265 ng/ml, edoxaban 220 ng/ml, rivaroxaban 339 ng/ml; the K time for apixaban 265 ng/ml; the angle for apixaban 265 ng/ml and edoxaban 220 ng/ml (Table 6.14).

After DOAC Stop® treatment the plasma spiked with the direct thrombin inhibitors showed a significant decrease of the R time and the K time ($p=0.028$ for both comparisons), and a significant increase of the angle ($p=0.028$) and the MA ($p=0.027$). Several parameters were still not normalised after DOAC Stop® treatment, such as the K time for argatroban 6.16 μg/ml and dabigatran 318 ng/ml; the angle for argatroban 6.16 μg/ml; and the R time for all anticoagulants (Table 6.14).

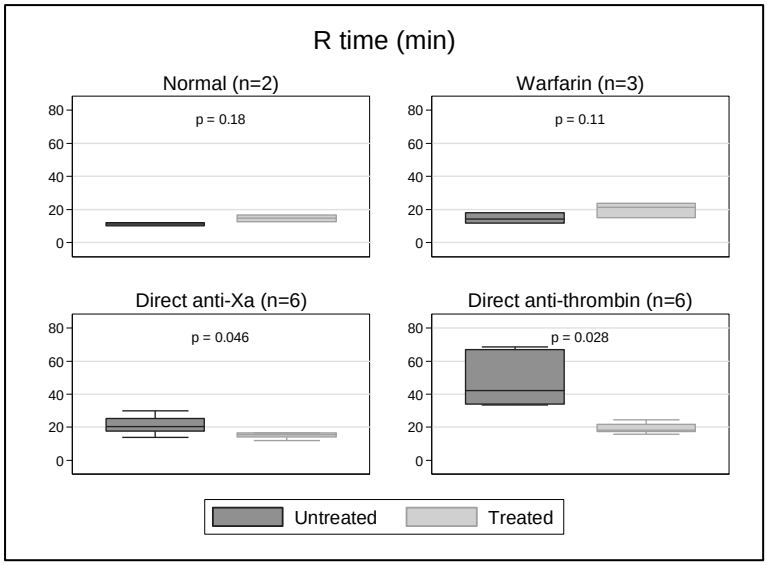


Figure 6.18 Changes in the R time (on the TEG) after DOAC Stop® treatment

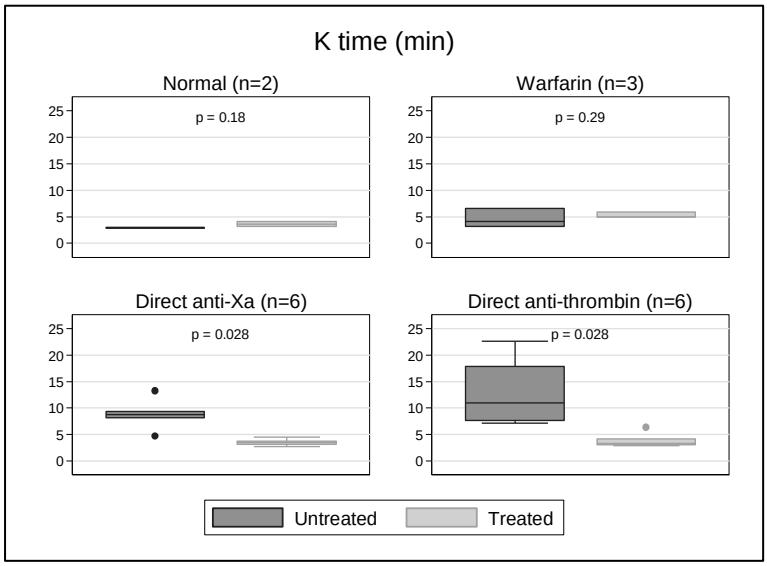


Figure 6.19 Changes in the K time (on the TEG) after DOAC Stop® treatment

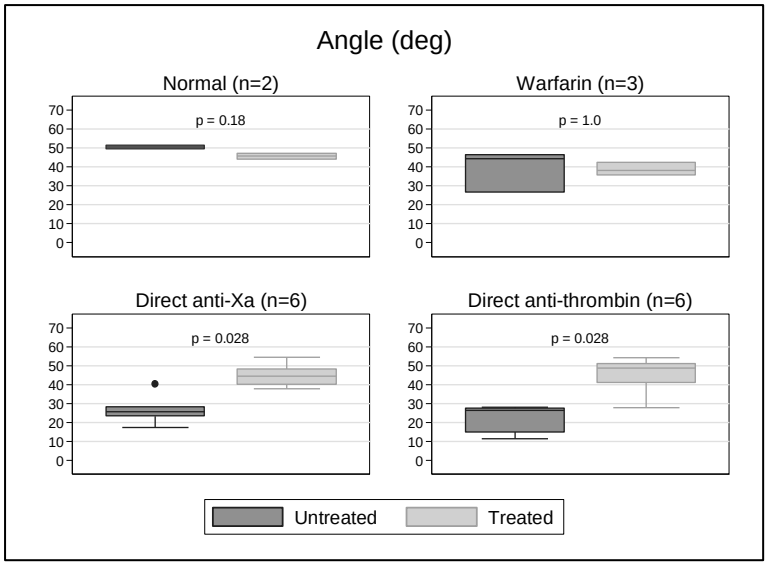


Figure 6.20 Changes in the angle (on the TEG) after DOAC Stop® treatment

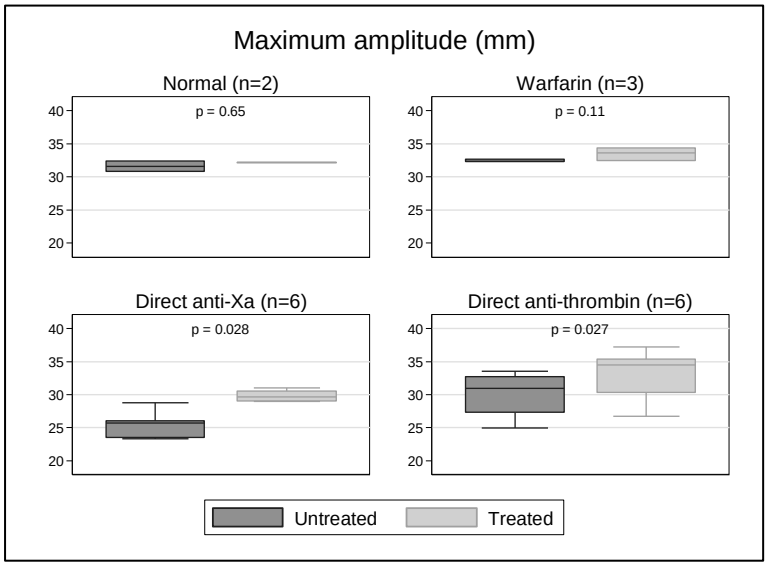


Figure 6.21 Changes in the maximum amplitude (on the TEG) after DOAC Stop® treatment

		R time (min)		K time (min)		Angle (deg)		Maximum amplitude (mm)	
Unspiked plasma ranges		***9.23-15.01***		***1.56-4.12***		***40.27-63.44***		***28.83-36.57***	
		Untreated	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**
Normal PPP	Batch I	10.05 (0.64)	12.55 (0.49)	3.05 (0.21)	3.15 (1.06)	51.45 (2.05)	47.30 (9.48)	30.80 (0.42)	32.15 (2.19)
	Batch II	12.20 (1.41)	16.80 (0.14)	2.80 (0.14)	4.10 (2.12)	49.75 (2.76)	44.20 (8.63)	32.40 (3.68)	32.20 (3.11)
Warfarinised PPP	INR 4.22	18.15 (0.21)	23.80 (1.41)	6.60 (0.57)	5.90 (0.28)	26.80 (3.25)	35.50 (2.69)	32.35 (0.49)	33.65 (0.35)
	INR 3.18	14.25 (0.07)	21.25 (4.31)	4.10 (0.14)	5.0 (0.71)	44.50 (5.94)	38.30 (7.07)	32.35 (2.47)	32.45 (0.64)
	INR 2.11	11.70 (0.42)	15.20 (1.56)	3.20 (0.57)	5.0 (3.96)	46.50 (6.08)	42.60 (21.92)	32.65 (0.07)	34.40 (0.28)
Apixaban	265 ng/ml	17.70 (0.14)	16.55 (1.91)	8.55 (0.21)	4.50 (1.84)	28.25 (2.47)	37.90 (14.42)	23.45 (0.64)	29.05 (1.77)
	182 ng/ml	13.80 (1.98)	14.95 (1.91)	4.70 (0.14)	3.40 (0)	40.55 (1.06)	48.25 (2.05)	28.75 (0.49)	29.0 (1.13)
Edoxaban	220 ng/ml	25.35 (3.18)	16.50 (0.42)	8.90 (0.14)	3.65 (0.49)	25.05 (3.89)	40.15 (5.59)	25.95 (1.63)	30.55 (2.05)
	151 ng/ml	20.60 (1.41)	13.95 (3.04)	9.35 (2.19)	3.10 (0.14)	23.35 (4.60)	45.15 (0.35)	26.05 (1.63)	29.50 (1.56)
Rivaroxaban	339 ng/ml	29.80 (2.40)	16.30 (0.14)	13.25 (0.49)	3.70 (0.42)	17.40 (0.71)	43.75 (0.21)	23.30 (1.84)	31.0 (1.27)
	241 ng/ml	19.95 (1.06)	12.00 (2.41)	8.10 (0.85)	2.75 (0.78)	26.40 (0.57)	54.60 (7.07)	25.45 (1.06)	29.80 (0.71)
Argatroban	6.16 µg/ml	66.85 (4.17)	24.60 (3.68)	17.85 (13.65)	6.35 (2.19)	14.90 (13.15)	27.95 (4.45)	30.10 (3.25)	37.25 (0.07)
	3.06 µg/ml	36.60 (19.66)	17.45 (0.07)	7.10 (1.84)	3.25 (0.78)	27.15 (8.39)	50.45 (2.76)	32.75 (7.42)	34.85 (0.07)
Bivalirudin	26.4 µg/ml	68.50 (4.81)	21.65 (4.03)	22.65 (3.04)	3.00 (0)	11.40 (3.54)	51.20 (4.95)	24.95 (1.06)	26.75 (0.64)
	13.3 µg/ml	33.40 (2.69)	15.75 (2.05)	7.65 (3.04)	3.45 (0.49)	27.70 (7.21)	47.20 (1.84)	27.30 (2.40)	30.35 (0.92)
Dabigatran	318 ng/ml	47.75 (1.34)	17.80 (1.27)	10.20 (7.35)	4.15 (0.92)	25.60 (15.27)	41.05 (6.01)	33.55 (2.62)	35.35 (0.49)
	203 ng/ml	33.85 (4.60)	18.55 (4.03)	11.80 (11.60)	2.85 (0.07)	28.05 (23.55)	54.25 (2.90)	31.75 (0.07)	34.15 (1.63)
Enoxaparin	1.68 U/ml	Flat TEG traces							
	0.93 U/ml	Flat TEG traces							
Fondaparinux	2.16 µg/ml	Flat TEG traces							
	1.62 µg/ml	Flat TEG traces							

Table 6.14 Changes in the thromboelastography results after DOAC Stop® treatment
Results are reported as mean (SD) of two measurements.

6.4.3.6 Factor assays

The following samples were tested with the factor assays: normal PPP batch I and II; warfarinised PPP INR 2.11, INR 3.18, INR 4.22; apixaban 265 ng/ml, edoxaban 220 ng/ml, rivaroxaban 339 ng/ml, dabigatran 318 ng/ml. Factor assay results of the same class of anticoagulant drugs were analysed together (e.g. normal PPP, warfarinised PPP, plasma spiked with the four DOACs).

Fibrinogen

After treatment with DOAC Stop® (Figure 6.22), there was no significant reduction of fibrinogen levels across the three categories of plasma (p=0.18 for the normal PPP; p=0.59 for the warfarinised PPP; p=0.068 for the plasma spiked with the DOACs). None of the results was below the normal ranges for fibrinogen (2-3.93 g/l).

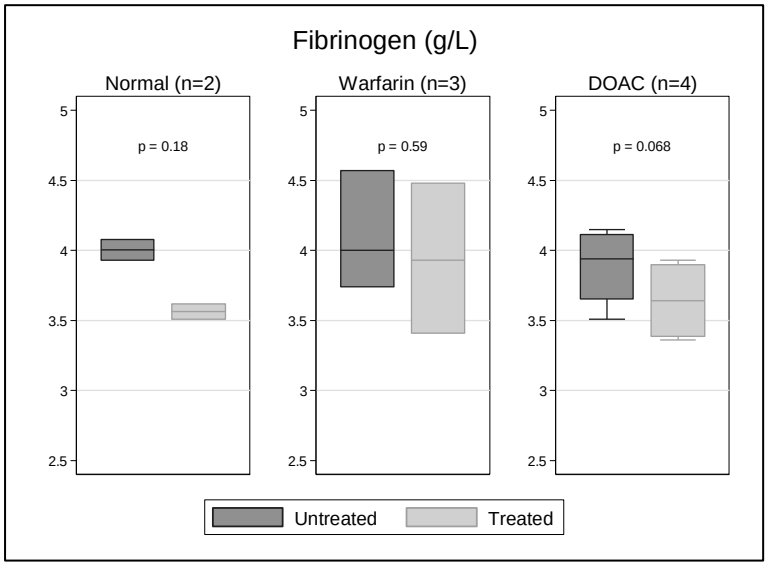


Figure 6.22 Changes in fibrinogen levels after DOAC Stop® treatment

Factor II

Low factor II levels were observed in the warfarinised plasma, due to the fact that the synthesis of factor II is vitamin K dependent and is therefore affected by the VKAs. Furthermore, a decrease of factor II levels was observed with increasing INR values (from 29.6% with INR 2.11 to 15.3% with INR 4.22). The same trend was observed with increasing concentrations of the direct factor Xa inhibitors (83.7% with edoxaban 220 ng/ml; 81.7% with apixaban 265 ng/ml; 63.1% with rivaroxaban 339 ng/ml). However, since the factor II assay is PT-dependent, it can reflect the degree of PT prolongation associated with these concentrations (Table 6.15).

After treatment with DOAC Stop® (Figure 6.23), there was no significant difference in factor II levels for the normal PPP (p=0.32) and the warfarinised PPP (p=1.0). The plasma spiked with the DOACs showed a non-significant increase of factor II levels compared to the same untreated plasma (p=0.068), which can reflect the PT normalisation associated with DOAC Stop® treatment.

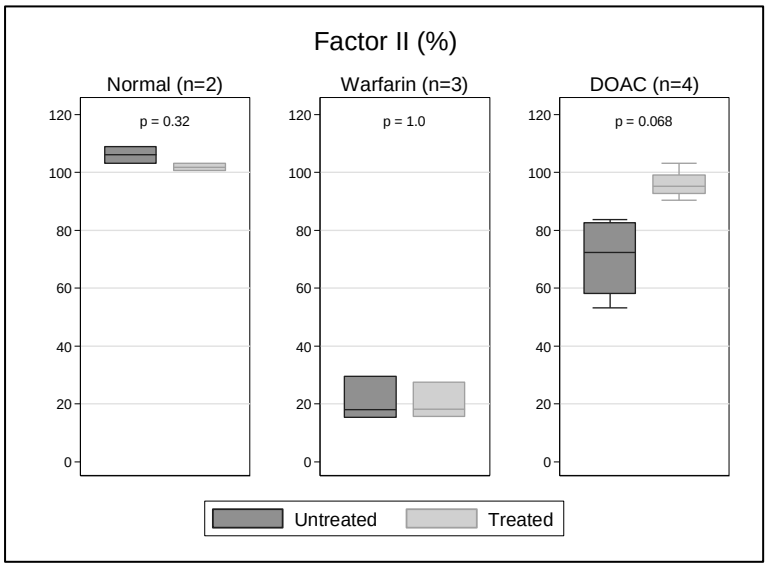


Figure 6.23 Changes in factor II levels after DOAC Stop® treatment

Factor VII

Low factor VII levels were observed in the warfarinised PPP, due to the fact that the synthesis of factor VII is also vitamin K dependent and is therefore affected by warfarin treatment. Furthermore, a decrease of factor VII levels was observed with increasing INR values (from 21.4% with INR 2.11 to 7.5% with INR 4.22). The plasma spiked with the DOACs was not tested with the factor VII assay, due to insufficient reagents.

After treatment with DOAC Stop® (Figure 6.24), there was no significant reduction of factor VII levels across the two categories of plasma.

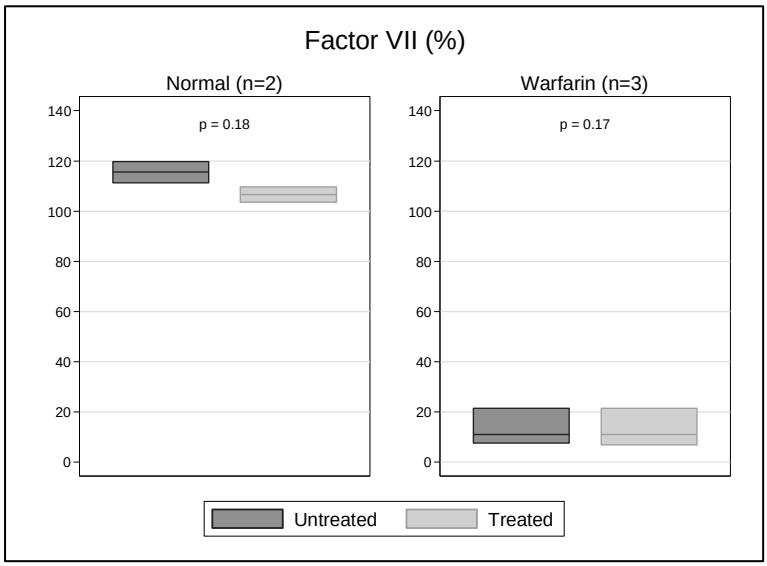


Figure 6.24 Changes in factor VII levels after DOAC Stop® treatment

Factor VIII

The warfarinised plasma showed normal levels of factor VIII. Similarly, the plasma spiked with the direct Xa inhibitors showed normal levels of factor VIII, although in the lower part of the normal ranges (between 71.6% and 90.4%). Low levels of factor VIII (34.5%) were observed in the plasma spiked with dabigatran. However, since the factor VIII assay is APTT-dependent, these results can reflect the higher degree of APTT prolongation associated with the direct thrombin inhibitor compared to the direct Xa inhibitors (Table 6.15).

After treatment with DOAC Stop® (Figure 6.25), there was a non-significant reduction of factor VIII levels for the normal PPP (p=0.18) and the warfarinised PPP (p=0.11). The plasma spiked with the DOACs showed a non-significant increase of factor VIII levels compared to the same untreated plasma (p=0.068), which can reflect the APTT normalisation associated with DOAC Stop® treatment.

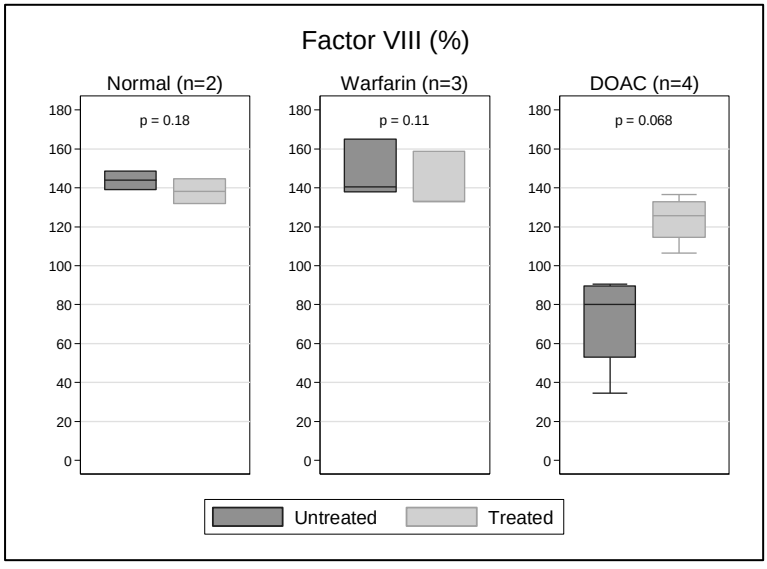


Figure 6.25 Changes in factor VIII levels after DOAC Stop® treatment

Factor IX

Low factor IX levels were observed in the warfarinised PPP, due to the fact that the synthesis of factor IX is vitamin K dependent and is therefore affected by the VKAs. Furthermore, a decrease of factor IX levels was observed with increasing INR values (from 59.2% with INR 2.11 to 36.6% with INR 4.22). The same trend was observed with increasing concentrations of the direct Xa inhibitors (from 88.2% with edoxaban 220 ng/ml to 60.6% with rivaroxaban 339 ng/ml). The highest degree of factor IX reduction in the DOAC-spiked plasma was observed with dabigatran 318 ng/ml (33.9%), which can reflect the higher degree of APTT prolongation of this thrombin inhibitor, since the factor IX assay is also APTT-dependent.

After treatment with DOAC Stop® (Figure 6.26), there was a non-significant reduction of factor IX levels for the normal PPP (p=0.18) and the warfarinised PPP (p=0.11). The plasma spiked with the DOACs showed a non-significant increase of factor IX levels compared to the same untreated plasma (p=0.068), which can reflect the APTT normalisation.

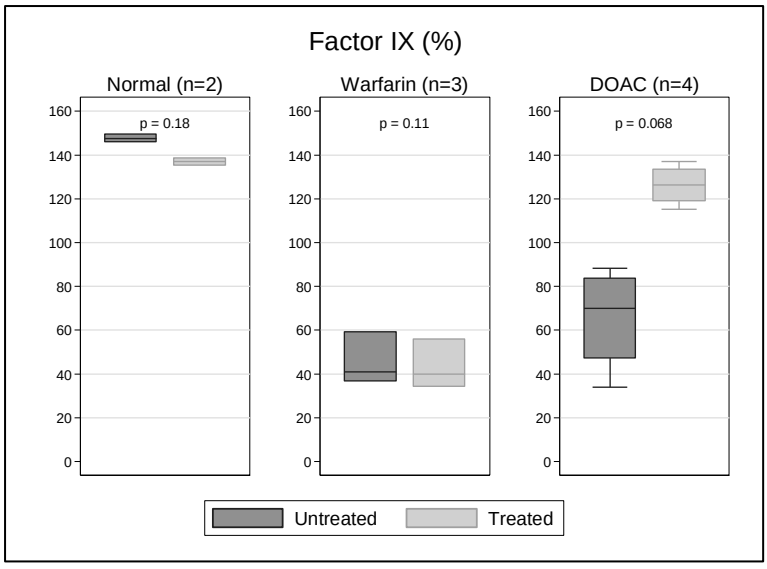


Figure 6.26 Changes in factor IX levels after DOAC Stop® treatment

Factor X

Low factor X levels were observed in the warfarinised PPP, due to the fact that the synthesis of factor X is also vitamin K dependent and is therefore affected by warfarin treatment. Furthermore, a decrease of factor X levels was observed with increasing INR values (from 15.6% with INR 2.11 to 8.6% with INR 4.22). A slight reduction was observed in the plasma spiked with dabigatran, apixaban and edoxaban (from 73.6% to 77.4%), while a more marked reduction was observed in the plasma spiked with rivaroxaban (57.7%). Since the factor X assay is PT-dependent, these results can reflect the higher degree of PT prolongation associated with rivaroxaban (Table 6.15). After treatment with DOAC Stop® (Figure 6.21), there was non-significant reduction of factor X levels for the normal PPP (p=0.18) and the warfarinised PPP (p=0.11). The plasma spiked with the DOACs showed a non-significant increase of factor X levels compared to the same untreated plasma (p=0.068), which can reflect the PT normalisation associated with DOAC Stop® treatment.

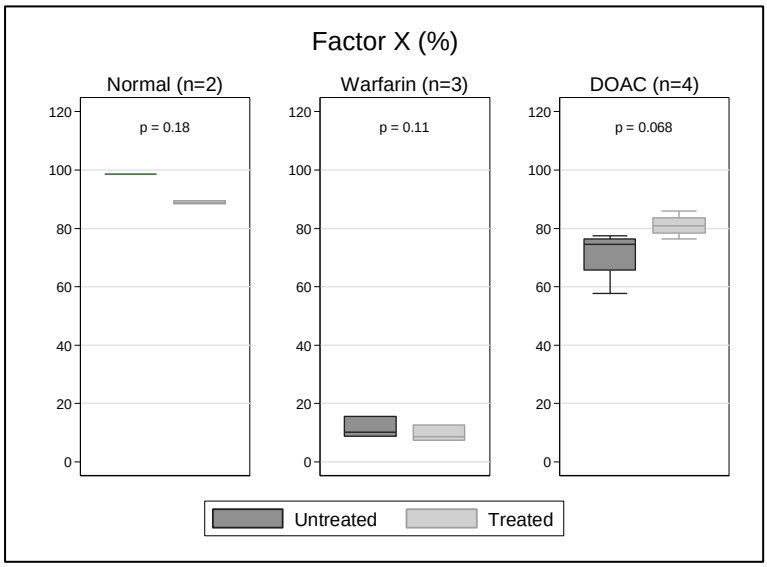


Figure 6.27 Changes in factor XI levels after DOAC Stop® treatment

Factor XI

The warfarinised plasma showed normal levels of factor XI. Similarly, the plasma spiked with apixaban and edoxaban showed normal levels of factor XI, although in the lower part of the normal ranges (68.9% and 74.7%, respectively). Low levels of factor XI were observed in the plasma spiked with rivaroxaban (57.6%), and particularly with dabigatran (32.5%). Since the factor XI assay is APTT-dependent, the higher reduction with dabigatran can reflect the degree of APTT prolongation associated with this drug.

After treatment with DOAC Stop® (Figure 6.28), there was a non-significant reduction of factor XI levels for the normal PPP (p=0.18) and the warfarinised PPP (p=0.11). The plasma spiked with the DOACs showed a non-significant increase of factor XI levels compared to the same untreated plasma (p=0.068), which can reflect the APTT normalisation associated with DOAC Stop® treatment.

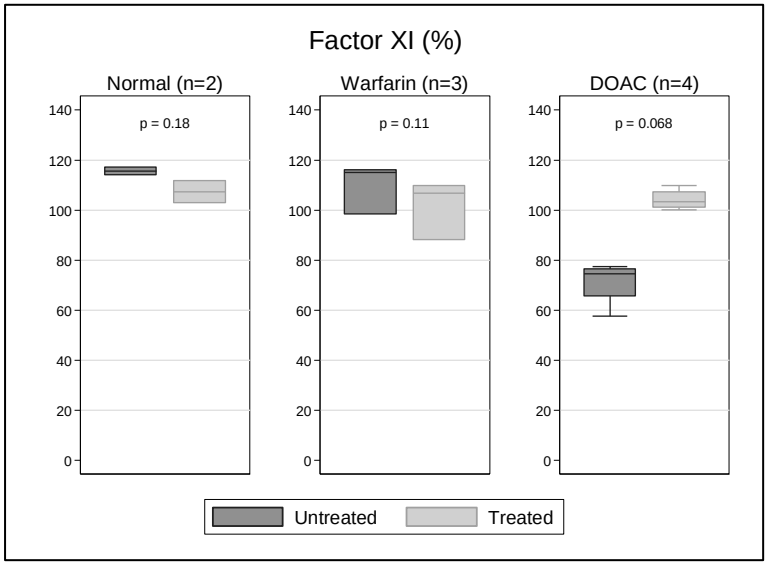


Figure 6.28 Changes in factor XI levels after DOAC Stop® treatment

Factor XII

All plasmas showed normal levels of factor XII. However, being the factor XII assay APTT-dependent, the lowest levels of factor XII were observed in the plasma spiked with dabigatran (57.4%).

After treatment with DOAC Stop® (Figure 6.29), there was a non-significant reduction of factor XII levels for the normal PPP (p=0.18) and the warfarinised PPP (p=0.11). The plasma spiked with the DOACs showed a non-significant increase of factor XII levels compared to the same untreated plasma (p=0.068), which can reflect the APTT normalisation associated with DOAC Stop® treatment.

Detailed results of factor assays are reported in Table 6.15.

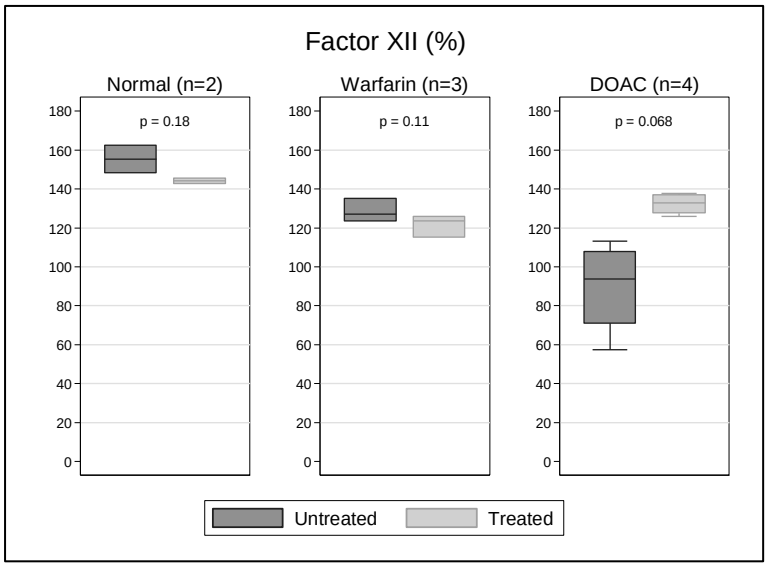


Figure 6.29 Changes in factor XI levels after DOAC Stop® treatment

	Fibrinogen (g/l)		Factor II (%)		Factor VII (%)		Factor VIII (%)		Factor IX (%)		Factor X (%)		Factor XI (%)		Factor XII (%)	
Normal ranges	***2-3.93***		***79-131***		***70-120***		***50-150***		***65-150***		***77-131***		***65-150***		***50-150***	
	U	**T**	**U**	**T**	**U**	**T**	**U**	**T**	**U**	**T**	**U**	**T**	**U**	**T**	**U**	**T**
Normal PPP batch I	4.08	3.51	109.0	100.5	111.3	103.6	148.7	144.6	149.5	135.3	98.7	89.5	117.2	102.9	162.5	145.5
Normal PPP batch II	3.93	3.62	103.2	103.2	119.8	109.7	139.2	131.7	145.8	138.7	98.7	88.3	113.9	111.8	148.2	142.9
Warfarinised PPP INR 4.22	4.57	4.48	15.3	15.5	7.5	6.9	165.0	158.9	36.6	34.3	8.6	7.2	115.0	106.8	127.0	123.6
Warfarinised PPP INR 3.18	4.00	3.41	18.0	18.2	11.0	10.9	140.6	132.9	40.9	40.0	10.2	8.6	116.1	109.8	135.3	125.9
Warfarinised PPP INR 2.11	3.74	3.93	29.6	27.5	21.4	21.4	137.9	132.9	59.2	55.9	15.6	12.6	98.3	88.0	123.6	115.1
Apixaban 265 ng/ml	3.80	3.41	81.7	95.2	NA	NA	88.8	122.2	79.2	122.5	75.5	80.4	68.9	101.9	102.6	129.3
Edoxaban 220 ng/ml	4.08	3.87	83.7	95.2	NA	NA	90.4	136.7	88.2	130.3	77.4	85.9	74.7	109.8	113.1	137.8
Rivaroxaban 339 ng/ml	4.15	3.93	63.1	103.2	NA	NA	71.6	129.2	60.6	137.0	57.7	81.5	57.6	104.8	84.7	136.5
Dabigatran 318 ng/ml	3.51	3.36	53.2	90.4	NA	NA	34.5	106.5	33.9	115.2	73.6	76.4	32.5	100.1	57.4	125.9

Table 6.15 Changes in the factor assay levels after DOAC Stop® treatment

6.4.4 Discussion

This study evaluated the effect of the treatment with DOAC Stop®, a new binding agent, on a broad range of oral and parenteral anticoagulants. It was found that the concentrations of the DOAC (apixaban, edoxaban, rivaroxaban and dabigatran) were below the lower limit of detection of the corresponding assays (30 ng/ml), anti-Xa or DTT as appropriate. Although a small residual DOAC activity might be present below this level, this is the safe cut-off usually considered for the pre-operative management of the DOAC (Erdoes et al., 2018; Levy et al., 2016).

When analysing the plasma spiked with the DOAC, the DOAC Stop® resulted in a complete normalisation of APTT, PT/INR, and lupus anticoagulant. There was also a normalisation of most of the CAT results, the only exceptions being the ETP of rivaroxaban 241 ng/ml and the ETP and the peak of the dabigatran-spiked plasma. The degree of normalisation assessed by the TEG was more variable, with the R time, the K time and the angle often not completely normalised. These results on the DOAC-spiked plasma partly confirmed the recently published literature on this topic, showing a normalisation of several coagulation assays (Exner et al., 2018; Favaloro, Gilmore, et al., 2019; Favresse et al., 2018; Jacquemin et al., 2018; Platton & Hunt, 2019). There is only one published study that evaluated the thrombin generation assay (Kopatz et al., 2018) and reported a complete reversal of the DOAC-spiked plasma after DOAC Stop® treatment. However, so far, the DOAC Stop® has never been tested on the TEG before.

The effect of DOAC Stop® was also observed on the parenteral direct thrombin inhibitors argatroban and bivalirudin. Argatroban plasma concentrations were normalised, as well as PT/INR and LA. Conversely, APTT, CAT and TEG parameters were only partly normalised, which can be explained by the high concentrations tested,

the ones recommended for patients with HIT undergoing PCI. No effect of DOAC Stop® was observed on the indirect factor Xa inhibitors. Although the direct thrombin inhibitor bivalirudin and the indirect factor Xa inhibitor fondaparinux have never been tested before, both the DOAC Stop® binding to the direct thrombin inhibitors (such as argatroban and lepirudin) and the lack of interference with the indirect factor Xa inhibitors (such as enoxaparin and danaparoid) have been previously reported (Exner, Ahuja, et al., 2019). Therefore, these results can be seen as a valuable confirmation of these DOAC Stop® properties.

Nevertheless, when untreated and treated normal PPP and warfarinised PPP were analysed with the global coagulation assays, several new findings emerged. First, the normal PPP showed a small increase of the peak of thrombin generated after DOAC Stop® treatment, suggesting hypercoagulability. The only previous study that evaluated the DOAC Stop® on the thrombin generation assay similarly reported that plasma spiked with the DOACs after DOAC Stop® treatment was slightly more procoagulant than the control plasma (Kopatz et al., 2018). The authors also found that the procoagulant effect was more pronounced when low volumes of plasma were treated with DOAC Stop® and that, among the natural anticoagulants (AT, protein S, and TFPI), there was a small but significant reduction of the free TFPI which may explain the increase in thrombin generation (Kopatz et al., 2018).

Second, the warfarinised PPP showed contrasting findings: a reduction of the lag time and the time to peak on the CAT (suggesting a partial normalisation of the results), and a prolongation of the R time on the TEG (suggesting more hypocoagulability). In order to explain these findings, several factor assays were performed. Although the results were not statistically significant, a consistent trend towards a reduction of the plasma levels of all the tested coagulation factors (I, II, VII, VIII, IX, X, XI, XII) was

found after DOAC Stop® treatment in all tested normal PPP and warfarinised PPP. The lack of statistical significance can be explained by the low number of samples tested. However, the aim of the factor assays was to perform an exploratory analysis to explain the findings on the global coagulation assays. Nonetheless these findings raise the question on the possible effects of DOAC Stop® on patient plasma with more pronounced factor deficiencies.

A first hypothesis was that these results could be partly explained by the molecular weight of the different coagulation factors: fibrinogen 338 kDa (McDonagh et al., 1972), prothrombin 70 kDa (Davie & Kulman, 2006), factor VII 50 kDa (Kazama et al., 1993), factor VIII 293 kDa (Mazurkiewicz-Pisarek et al., 2016), factor IX 57 kDa (Vysotchin et al., 1993), factor X 59 kDa (Venkateswarlu et al., 2002), factor XI 160 kDa (Emsley et al., 2010), factor XII 80 kDa (Dementiev et al., 2018). It was hypothesised that those factors with high molecular weight were more likely to remain entrapped in the DOAC Stop®, which is made of activated charcoal, during processing. However, it was reported that "the pore capacity of the adsorbing agent in DOAC Stop® […] is approximately 5000" (Exner et al., 2018, p.122), thus it is not supposed to bind the coagulation factors and the natural anticoagulants which are all above this cut-off.

Therefore, the different results between the CAT and the TEG could be probably explained by the different execution and different sensitivity of these two assays. A native TEG, performed without the addition of kaolin or TF, is more sensitive to the contact activation pathway (Chitlur et al., 2014), whereas the thrombin generation assay, performed with the addition of TF, is more sensitive to the extrinsic pathway (van Veen et al., 2008). Furthermore, there are some reports on animal blood showing that the TEG is particularly sensitive to factor XII deficiency, with normalisation of

the trace when the TEG was activated with TF (Blois et al., 2015), whilst on the CAT a significant amount of thrombin is generated even with very low factors concentrations (with the exception of prothrombin) (van Veen et al., 2008).

The plasma spiked with the DOAC showed an opposite profile with increasing concentrations of factors II, VIII, IX, XI and XII after DOAC Stop® treatment. This finding can be explained by the interference of the DOAC with the basic coagulation assays, PT and APTT, considering that the factor assays for II, VII and X are PT-based, while the factor assays for VIII, IX, XI and XII are APTT-based. Therefore, the effect of DOAC Stop® treatment was more visible as a normalisation of these results. The only exception was fibrinogen, which showed a small reduction also in these samples. Fibrinogen is the largest among the tested coagulation factors and fibrinogen, using the Clauss method, is insensitive to the DOAC (Favaloro & Lippi, 2017).

The main strengths of this study include the large number of different anticoagulants tested at the same time and the rigorous protocol of samples preparation, in which untreated and treated plasma underwent the same centrifugation process and the same number of freeze-thaw cycles to make the results more comparable. The main limitations of this study include the low number of samples tested, the fact that some coagulation assays have not been performed in duplicate (APTT, PT/INR, dRVVT, factor assays) and the fact that other less common tests (such as kininogen or prekallikrein) could have provided a better insight into the effect of DOAC Stop® on normal and warfarinised PPP. However, these results can contribute to the knowledge of the effects of this new product and could provide the background for further research studies, in order to better assess the safety of using this binding agent in clinical practice.

6.5 Conclusion

This chapter evaluated the effect of different agents used for the reversal of the anticoagulant drugs. The first study analysed *ex vivo* the effect of FFP for VKA reversal and found that, while its administration resulted in a significant reduction of the INR and all the CAT parameters, a complete normalisation of the haemostatic balance was not obtained in any patient.

The second study analysed different reversal agents for DOAC reversal *in vitro* and showed that different concentrations of the reversal agents might be needed in order to normalise the coagulation profile, based on the plasma concentrations. For prophylactic concentrations, a small dose of rVIIa, activated PCC, 3-factor or 4-factor PCC might be enough, while for therapeutic concentrations higher doses are probably needed. The reversal effect of FFP is limited by the high volume required.

The third study analysed the effect of the binding agent DOAC Stop® *in vitro* and found that while basic coagulation assays (APTT, PT/INR) are normalised, there might be a potential for false negative results in patients with lupus anticoagulant. Furthermore, the factor assays showed a non-significant reduction of the plasma levels of several coagulation factors, suggesting a certain level of DOAC Stop® binding.

These results can form the basis for future research studies on this topic and contribute to better management of the anticoagulated patients. The local reversal strategy with FFP for VKA-treated patients might need to be revised, with the use of higher doses of FFP or other more efficient reversal products (i.e. PCC). In DOAC-treated patients, activated PCC, PCC and rVIIa at appropriate dosages could be a reasonable approach, when specific antidotes are not available. Finally, caution should be used when interpreting the results obtained with the DOAC Stop® in clinical practice, until there is more evidence in the literature on its binding effect.

Chapter 7 :

Maltese Translations of the DASS and the PACT-Q

7.1 Introduction

Anticoagulation is a chronic treatment for most clinical indications, such as recurrent VTE, AF, or mechanical heart valve replacement (Ageno et al., 2012). It can therefore affect patients' health related QoL and satisfaction (Borg Xuereb et al., 2016; Casais et al., 2005; Wild et al., 2008). For instance, VKAs have a narrow therapeutic index and several food/drug interactions, therefore requiring periodic monitoring of the INR and dose adjustment. All these elements can contribute to the burden of VKA (Borg Xuereb et al., 2012).

Since patients' negative beliefs can result in non-adherence to medications and, therefore, reduced effectiveness (Phatak & Thomas, 2006; Waterman et al., 2004), the health related QoL should be always evaluated in the decision-making process. Understanding the degree of satisfaction can allow for specific interventions, with the aim to increase the adherence to medications and to reduce the adverse clinical outcomes.

Generic scales are available, such as the SF-36 (Ware & Sherbourne, 1992) and the SF-12 (Ware et al., 1996), which can be applied to patients with different conditions, such as rheumatological, orthopaedic and haematological diseases (Arian et al., 2019; Kanazawa et al., 2019; Matcham et al., 2014). Furthermore, there are specific questionnaires, which assess the satisfaction associated with the anticoagulant treatment. They include the PACT-Q (Prins, Marrel, et al., 2009), the DASS (Samsa et al., 2004), the ACTS (Cano et al., 2012), the Deep Venous Thrombosis Quality of Life questionnaire (DVTQOL) (Hedner et al., 2004), and the Pulmonary Embolism Quality of Life Questionnaire (PEmb-QoL) (Cohn et al., 2009). However, there was no specific scale assessing the degree of satisfaction of anticoagulated patients available in the Maltese language.

For the purpose of this study, it was chosen to translate the DASS and the PACT-Q, because they were the most comprehensive and the most validated questionnaires. They were already translated in several languages and applied to patients with a broad range of clinical indications to anticoagulation (Agnelli et al., 2015; De Caterina et al., 2018; Gafou et al., 2007; Hasan et al., 2015; Matchar et al., 2010; Mohamed et al., 2015; Pelegrino et al., 2012; Prins, Guillemin, et al., 2009; Samsa et al., 2004).

7.2 Aims

The aim of this study was to validate the Maltese translations of the DASS and the PACT-Q questionnaires. The validation was performed by assessing their psychometric properties (reliability and validity) in comparison with the published literature and the results of original English version in this Maltese cohort.

7.3 Methods

7.3.1 Study population

Between July 2017 and February 2018, the DASS and the PACT-Q questionnaires were administered to patients on warfarin treatment attending different Anticoagulation Clinics in Malta for INR monitoring, specifically five Health Centres (Cospicua, Floriana, Mosta, Qormi, Rabat) and Mater Dei Hospital (Msida). Patients with cognitive impairment, dementia or major psychiatric disorders (such as schizophrenia) were excluded. INR monitoring at the Health Centres was performed using POC coagulometers, whilst at Mater Dei Hospital was performed using the standard laboratory INR.

Overall, 174 patients completed the Maltese version of the DASS and the PACT-Q questionnaires and 157 patients completed the original English versions. The choice of the language of the questionnaires was left at the discretion of each patient. The enrolled patients were also asked to complete a form of sociodemographic data (including age, sex, living situation, level of education, working status, self-reported history of any bleeding). Further information was collected from a review of the clinical notes (such as clinical indication to VKA, duration of VKA treatment, INR at enrolment, INR results in the previous year, any hospitalisation in the previous year). Questionnaires and sociodemographic forms were identified using a code, in order to ensure anonymity and confidentiality. In case of missing answers, the list with the correspondence between the codes and patients' personal details was accessed and patients were contacted by phone. This step was done in order to avoid missing answers which would have hampered the possibility to calculate the total scores of the questionnaires.

A random sample of 40 patients underwent a retest after 1-2 weeks: half of them was retested in the same language (to calculate the intra-language correlation) and the other half was retested in the other language (to calculate the cross-language correlation). Therefore, the following types of test-retest were performed: Maltese-Maltese (n=10), English-English (n=10), Maltese-English (n=10), English-Maltese (n=10).

This study was approved by the University of Malta Research and Ethics Committee (protocol 07/2016, Appendix B). After explaining the rationale and the design of this study, eligible patients received an information sheet and, if they agreed to take part in this study, they were asked to sign a consent form. Both English and Maltese versions of the information sheet, consent form, and questionnaires were available for patients (Appendix C2).

7.3.2 The DASS questionnaire

The DASS questionnaire has 25 questions which can be answered on a 7-point scale (not at all, a little, somewhat, moderately, quite a bit, a lot, very much) (Samsa et al., 2004). The DASS questionnaire explores three dimensions: limitations (9 items, sections A and B); hassles and burdens (8 items, section C); psychological impact (8 items, section D), which can be further divided into positive psychological impact (5 items, including 4a, 4b, 4f, 4h, 4j) and negative psychological impact (3 items, including 4d, 4g, 4i). Six items (3h, 4a, 4b, 4f, 4h, 4j) require reverse-coding prior to analysis. The DASS result can be expressed as a total score, ranging from 25 to 175, with lower scores representing higher satisfaction (Samsa et al., 2004).

7.3.3 The PACT-Q questionnaire

The PACT-Q questionnaire is divided into two parts: the PACT-Q1 measures the expectations associated with the anticoagulant treatment and should be administered before initiation, while the PACT-Q2 measures the convenience and the satisfaction associated with the anticoagulant treatment and should be administered during treatment (Prins, Guillemin, et al., 2009; Prins, Marrel, et al., 2009). Since patients who were already receiving the VKA were enrolled, only the PACT-Q2 could be administered.

The PACT-Q2 questionnaire has 20 questions which can be answered on a 5-point Likert scale (not at all, a little, moderate, a lot, extremely). The PACT-Q2 explores two dimensions: convenience (13 items, sections B and C); and anticoagulant treatment satisfaction (7 items, section D). During the analysis, the items of the convenience dimension are reversed, summed, and rescaled on a 0-100 scale; the items

of the anticoagulant treatment satisfaction dimension are summed and rescaled on a 0-100 scale. The results of the two dimensions are reported separately, with higher scores corresponding to higher convenience/satisfaction.

7.3.4 Translation process

Permission to translate and use the questionnaires was obtained from the corresponding author of the original publication (for the DASS questionnaire) (Samsa et al., 2004) and from Sanofi Aventis/Mapi Research Trust (for the PACT-Q questionnaire). Published guidelines for the linguistic validation process were followed (Beaton et al., 2000; Sousa & Rojjanasrirat, 2011). Different people, all bilingual in English and Maltese, were involved in the translation process (a speech and language pathologist, Dr. E. Azzopardi; a professional translator, Dr. G. Farrugia; and a health psychologist, Dr. C. Borg Xuereb). Two forward translations were performed from English to Maltese and a backward translation from Maltese to English. A pilot testing of the Maltese DASS and PACT-Q was performed by completing and discussing the questionnaire with five patients on long-term warfarin (not included in the analysis).

7.3.5 Statistical analysis

Continuous variables were reported as mean (SD), and compared using the Student's independent samples t-test. Categorical variables were reported as counts and percentages, and compared using the Chi-square or the Fisher's exact tests, as appropriate.

The psychometric properties of the DASS and the PACT-Q questionnaires were evaluated, which involve reliability and validity. Reliability was evaluated through

internal consistency and reproducibility (Webb et al., 2006). Validity was evaluated through floor/ceiling effect, convergent/discriminant validity, construct validity, and known-group validity.

The internal consistency is defined as the correlation between different items on the same scale or subscale. It was assessed using the Cronbach's alpha coefficient, with values ≥ 0.70 indicating high internal consistency (Cronbach, 1951).

The reproducibility was evaluated in the subgroup of 40 patients who participated in the test-retest. The intra-language test-retest correlation was evaluated in those patients who were rested in the same language (Maltese-Maltese test-retest or English-English test-retest). The intraclass correlation coefficient (ICC) was calculated, with values between 0.60 and 0.74 considered acceptable (Cicchetti, 1994). The cross-language test-retest correlation was evaluated in those patients who were retested in the other language, by pooling together the Maltese–English and English–Maltese test-retest, as previously done (McCrae et al., 1998). The raw cross-language correlation and the cross-language correlation adjusted for score unreliability (by dividing the raw cross-language test-retest correlation by the square-root of the product of the Maltese-Maltese and English-English test-retest intra-language correlation) were calculated (McCrae et al., 1998; Wood et al., 2018).

Floor and ceiling effects occur when a significant proportion of the respondents (> 15%) achieves the lowest (floor effect) or the highest (ceiling effect) possible score. It means that the questionnaire is not able to capture the variance in the responses (limited content validity) (Terwee et al., 2007).

Convergent and discriminant validity were evaluated through factor analysis. An exploratory factor analysis with varimax rotation was performed to examine the structure of the DASS and the PACT-Q. The convergent validity criterion was

considered met when the correlation between each item and its dimension was ≥ 0.40. The discriminant validity criterion was considered met when each item showed higher correlation with its dimension than the others (Campbell & Fiske, 1959). A confirmatory factor analysis was subsequently performed and the following fit parameters were calculated (McDonald & Ho, 2002):

- Root mean square error of approximation (RMSEA): good fit for values ≤ 0.05, acceptable fit for values ≤ 0.08;
- Standardized root mean squared residual (SRMR): good fit for values ≤ 0.05, acceptable fit for values ≤ 0.10;
- Goodness-of fit index (GFI): acceptable fit for values ≥ 0.90;
- Adjusted goodness-of-fit index (AGFI): acceptable fit for values ≥ 0.90;
- Comparative fit index (CFI): acceptable fit for values ≥ 0.90.

Construct validity was examined by evaluating the Pearson's correlation between different subscales or between each subscale and the overall questionnaire (scale-subscale validity) (Streiner et al., 2015).

Known-group validity was examined by evaluating the correlation (Pearson's or point-biserial correlation as appropriate) between the total score (overall DASS score or each of the two PACT-Q2 subscales) and the following variables, which have been previously reported to correlate with patients' satisfaction (Gafou et al., 2007; Pelegrino et al., 2012; Radaideh & Matalqah, 2018; Samsa et al., 2004; Yildiz & Dayapoglu, 2017): increasing age; male sex; living alone; level of education primary school only; full-time or part-time paid employment; AF as indication to VKA treatment; VKA treatment duration >5 years; INR within the therapeutic range at enrolment; TTR ≥ 70% in the previous year, calculated according to the Rosendaal

method (Rosendaal et al., 1993); any hospitalisation in the previous year; history of any bleeding during VKA treatment (self-reported).

A sample size of at least 150 patients per each group was planned, considering that international recommendations suggest to test at least 50 patients (Terwee et al., 2007) and that previous validation studies enrolled around 100 patients (Frey et al., 2015; Rochat et al., 2014). For the statistical analysis reported in this chapter the following softwares were used: STATA SE v.12 (StataCorp LP, College Station, TX, USA) and SAS v. 9.4 (SAS Institute Inc, Cary, NC, USA).

7.4 Results

7.4.1 Study population

Overall, 174 patients completed the Maltese versions of the DASS and PACT-Q2 and 157 patients completed the original English versions. The baseline characteristics of the population are reported in Table 7.1. The comparison between patients who completed the Maltese and the English questionnaires showed some differences in the level of education and in the employment status ($p<0.001$ for both). Female sex was more common among those subjects who chose to complete the Maltese version ($p=0.01$), while heart valve replacement was more common among those subjects who chose the English version ($p=0.02$).

	Patients who completed the Maltese questionnaire (n=174)	Patients who completed the English questionnaire (n=157)	p value
Age (years), mean (SD)	70 (10.1)	69.8 (10.2)	0.87
Females, n (%)	92 (52.9%)	61 (38.9%)	0.01
Living situation, n (%) • Living with family members • Living alone • Other	 138 (79.3%) 31 (17.8%) 5 (2.9%)	 124 (79.0%) 30 (19.1%) 3 (1.9%)	0.82
Level of Education, n (%) • Primary school • Secondary school • College or above	 108 (62.1%) 48 (27.6%) 18 (10.3%)	 36 (22.9%) 67 (42.7%) 54 (34.4%)	< 0.001
Employment status, n (%) • Full-time or part-time paid employment • Retired/pension • Other (homemaker/housewife, unemployed)	 18 (10.3%) 103 (59.2%) 53 (30.5%)	 25 (15.9%) 114 (72.6%) 18 (11.5%)	< 0.001
Clinical indications to warfarin*, n (%)			
• Atrial fibrillation	122 (70.1%)	97 (61.8%)	0.11
• Venous thromboembolism	30 (17.2%)	23 (14.7%)	0.52
• Heart valve replacement	25 (14.4%)	38 (24.2%)	0.02
• Other	6 (3.5%)	9 (5.7%)	0.32
Warfarin treatment duration, n (%) • ≤ 5 years • > 5 years	 98 (56.3%) 76 (43.7%)	 76 (48.4%) 81 (51.6%)	0.15
INR at enrolment, n (%) • In range • Other (above or below range)	 99 (56.9%) 75 (43.1%)	 98 (62.4%) 59 (37.6%)	0.31
High TTR (≥ 70%) in the previous year **, n (%)	96 (56.5%)	89 (59.7%)	0.56
Any hospitalisation in the previous year **, n (%)	86 (50.6%)	77 (51.7%)	0.85
Self-reported history of any bleeding, n (%)	63 (36.2%)	50 (31.9%)	0.40
Site of enrolment, n (%) • Health Centres • Mater Dei Hospital	 86 (49.4%) 88 (50.6%)	 78 (49.7%) 79 (50.3%)	0.96

Table 7.1 Baseline characteristics of the study population (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)

* more than one option is possible ** data available only in 170 patients who completed the Maltese version and 149 patients who completed the English version

The analysis of the questionnaires results showed a non-significant lower satisfaction among those patients who completed the Maltese version (p=0.18 for the overall DASS, p=0.28 for the convenience subscale and p=0.09 for the anticoagulant treatment satisfaction subscale of the PACT-Q2), as reported in Table 7.2.

	Patients who completed the Maltese questionnaire (n=174)	Patients who completed the English questionnaire (n=157)	p value
DASS results, mean (SD)			
• Overall score	56.7 (18.5)	53.6 (16.9)	0.18
• Limitations subscale	17.1 (9.4)	17.0 (8.0)	0.92
• Hassles/burdens subscale	16.2 (8.0)	15.3 (6.7)	0.27
• Psychological impact subscale	23.4 (6.2)	21.3 (7.4)	0.006
PACT-Q2 results, mean (SD)			
• Convenience subscale	82.2 (16.1)	84.0 (13.7)	0.28
• Anticoagulant treatment satisfaction subscale	65.2 (11.5)	67.6 (14.6)	0.09

Table 7.2 Results of the two questionnaires in the Maltese and English languages

7.4.2 The DASS questionnaire

7.4.2.1 Internal consistency

The internal consistency of the Maltese DASS was good. The Cronbach's alpha was 0.87 for the overall DASS score (25 items). The coefficients for the subscales were 0.86 for limitations (9 items); 0.84 for hassles/burdens (8 items); 0.57 for the overall psychological impact (8 items), while it was 0.65 for the positive components (5 items) and 0.64 for the negative components (3 items).

The internal consistency of the original English DASS in this study cohort was also good. The Cronbach's alpha was 0.85 for the overall DASS score (25 items). The coefficients for the subscales were 0.82 for limitations (9 items); 0.79 for hassles/burdens (8 items); 0.71 for the overall psychological impact (8 items), while it was 0.79 for the positive components (5 items) and 0.56 for the negative components (3 items).

Detailed results for the internal consistency are reported in Table 7.3. No significant increase or decrease of the Cronbach's alpha coefficients was observed when each item, in turn, was deleted.

DASS item	Maltese version			English version		
	Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted	Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted
DASS score (overall)	0.87			0.85		
Limitations	0.86			0.82		
1a		0.60	0.85		0.54	0.79
1b		0.63	0.85		0.59	0.79
1c		0.72	0.84		0.48	0.80
1d		0.70	0.84		0.63	0.79
1e		0.72	0.84		0.66	0.78
2a		0.46	0.86		0.46	0.80
2b		0.31	0.88		0.43	0.81
2c		0.60	0.85		0.47	0.81
2d		0.70	0.84		0.56	0.79
Hassles / burdens	0.84			0.79		
3a		0.59	0.82		0.51	0.76
3b		0.73	0.80		0.61	0.74
3c		0.70	0.81		0.57	0.75
3d		0.48	0.83		0.66	0.74
3e		0.80	0.79		0.70	0.73
3f		0.56	0.82		0.38	0.78
3g		0.78	0.79		0.74	0.73
3h		0.004	0.88		0.12	0.86
Psychological impact (positive)	0.65			0.79		
4a		0.34	0.63		0.60	0.74
4b		0.54	0.55		0.69	0.72
4f		0.38	0.65		0.49	0.79
4h		0.48	0.59		0.60	0.75
4j		0.42	0.59		0.55	0.76
Psychological impact (negative)	0.64			0.56		
4d		0.41	0.62		0.39	0.45
4g		0.55	0.40		0.45	0.33
4i		0.41	0.59		0.30	0.56

Table 7.3 Internal consistency of the Maltese and English versions of the DASS (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)

7.4.2.2 Reproducibility

The intra-language test-retest correlation was very good, with ICC 0.73 for the Maltese-Maltese test-retest and 0.85 for the English-English test-retest. The ICCs for each subscale are reported in Table 7.4.

The cross-language test-retest correlation, pooling together Maltese-English and English-Maltese test-retests, gave unsatisfactory results. The raw ICC for the DASS total score was 0.31 and the adjusted ICC was 0.39. When analysed separately, the ICC for the English-Maltese test-retest was 0.59, while the ICC for the Maltese-English test-retest was 0. This finding suggested that those patients who initially chose the Maltese language were probably less proficient in English.

	Score difference			ICC
	Mean (SD)	Min	Max	
Maltese-Maltese test-retest				
• DASS total score	3.5 (8.9)	-6	24	0.73
• DASS limitations subscale	1.5 (3.5)	-3	9	0.69
• DASS hassles/burdens subscale	1 (3.6)	-4	8	0.87
• DASS psychological impact subscale	1 (3.8)	-3	10	0.77
English-English test-retest				
• DASS total score	4.7 (9.9)	-10	19	0.85
• DASS limitations subscale	2.8 (6.1)	-3	15	0.80
• DASS hassles/burdens subscale	2.3 (2.5)	0	6	0.80
• DASS psychological impact subscale	-0.4 (4.7)	-10	6	0.71

Table 7.4 Results of the intra-language test-retest correlation for the DASS (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)
Score difference is calculated as time 2 (retest) minus time 1 (test).

7.4.2.3 Floor and ceiling effect

The response distribution for each item of the DASS was analysed. In the Maltese DASS, a significant floor effect was identified in most of the items and a significant ceiling effect only for item 4f (Table 7.5). However, similar findings emerged from the original English DASS administered to this study population (Table 7.6) and a certain degree of floor effect was also reported in the original publication of the DASS questionnaire (Samsa et al., 2004). Therefore, it is more likely to be an intrinsic characteristic of the questionnaire, rather than a weakness of the Maltese translation.

DASS item	Response category (%)							Mean (SD) in this study (Maltese version)	Mean (SD) in this study (English version)	Mean (SD) in the study by Samsa et al. (2004)
	1	2	3	4	5	6	7			
1a	**74.1**	13.2	1.7	3.5	4.0	2.9	0.6	1.61 (1.31)	1.59 (1.28)	1.84 (1.37)
1b	**75.9**	12.1	3.5	2.9	1.7	3.5	0.6	1.55 (1.25)	1.50 (1.12)	1.36 (0.99)
1c	**60.3**	16.1	6.3	2.9	4.6	6.3	3.5	2.08 (1.76)	2.04 (1.51)	1.69 (1.36)
1d	**76.4**	12.6	2.3	2.3	1.7	3.5	1.2	1.55 (1.30)	1.46 (1.14)	1.84 (1.78)
1e	**72.4**	12.6	3.5	5.8	1.2	3.5	1.2	1.66 (1.35)	1.62 (1.19)	1.88 (1.31)
2a	**39.1**	24.7	13.2	9.8	8.1	4.6	0.6	2.39 (1.54)	2.10 (1.35)	2.60 (1.66)
2b	**70.1**	13.2	2.3	4.6	2.3	4.6	2.9	1.81 (1.60)	2.14 (1.59)	1.97 (1.89)
2c	**46.6**	19.5	10.9	4.0	9.2	5.8	4.0	2.43 (1.82)	2.71 (1.96)	3.02 (2.12)
2d	**55.2**	20.1	8.1	8.1	2.3	4.0	2.3	2.03 (1.55)	1.86 (1.21)	2.20 (1.43)
3a	**63.2**	17.8	5.8	5.2	2.3	4.0	1.7	1.84 (1.47)	1.80 (1.19)	1.78 (1.22)
3b	**46.6**	19.0	9.8	11.5	4.6	5.8	2.9	2.37 (1.72)	2.15 (1.49)	2.09 (1.25)
3c	**62.1**	20.1	5.2	6.3	3.5	2.9	0.0	1.78 (1.29)	1.66 (1.16)	1.65 (1.09)
3d	**74.1**	10.3	5.2	2.9	4.0	3.5	0.0	1.63 (1.30)	1.78 (1.08)	1.76 (0.97)
3e	**57.5**	16.1	7.5	6.3	5.2	6.3	1.2	2.09 (1.64)	1.85 (1.35)	1.76 (1.24)
3f	**69.0**	14.4	7.5	3.5	4.6	0.6	0.6	1.64 (1.20)	1.33 (0.90)	1.37 (0.90)
3g	**52.3**	23.0	5.8	7.5	5.2	5.2	1.2	2.10 (1.57)	1.94 (1.19)	1.81 (1.17)
3h	7.5	52.3	23.0	5.2	4.0	4.6	3.5	2.74 (1.39)	2.77 (1.98)	2.90 (2.19)
4a	4.0	31.6	30.5	16.7	4.6	10.3	2.3	3.26 (1.44)	2.54 (1.50)	2.32 (1.67)
4b	4.0	40.2	28.2	17.2	4.0	4.6	1.7	2.98 (1.26)	2.64 (1.46)	2.78 (1.66)
4d	**20.7**	15.5	10.9	18.4	14.9	14.9	4.6	3.55 (1.89)	2.87 (1.77)	2.55 (1.64)
4f	7.5	19.0	12.1	10.3	8.6	14.9	**27.6**	4.49 (2.12)	4.01 (2.01)	4.15 (2.08)
4g	**58.7**	14.9	6.9	9.2	4.6	5.2	0.6	2.04 (1.56)	2.24 (1.48)	2.00 (1.34)
4h	11.5	59.2	18.4	6.9	1.2	1.2	1.7	2.37 (1.08)	2.41 (1.35)	2.55 (1.60)
4i	**55.8**	20.1	6.3	10.9	3.5	2.9	0.6	1.97 (1.41)	1.68 (1.22)	1.75 (1.23)
4j	10.9	45.4	24.1	11.5	2.3	2.9	2.9	2.69 (1.32)	2.91 (1.93)	2.42 (1.73)

Table 7.5 Response distribution for each item of the Maltese DASS and summary statistics (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)
Numbers in bold in the response category section indicate significant floor or ceiling effect.

DASS item	Response category (%)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1a	**76.4**	8.3	3.8	5.7	3.2	1.9	0.6
1b	**74.5**	15.3	3.2	3.2	1.9	0.6	1.3
1c	**56.7**	15.9	9.6	7.0	7.6	1.9	1.3
1d	**78.3**	12.7	1.3	3.8	0.6	2.6	0.6
1e	**67.5**	19.8	4.5	3.2	2.6	1.9	0.6
2a	**42.7**	33.1	7.0	10.2	3.8	2.6	0.6
2b	**49.0**	26.1	7.6	5.1	5.7	3.8	2.6
2c	**38.2**	24.2	8.9	4.5	12.7	3.8	7.6
2d	**53.5**	26.1	7.6	7.6	3.8	1.3	0.0
3a	**54.1**	29.3	7.0	4.5	3.2	1.3	0.6
3b	**46.5**	25.5	9.6	8.9	5.7	1.9	1.9
3c	**65.0**	20.4	5.7	3.2	5.1	0.0	0.6
3d	**52.2**	31.2	6.4	7.0	2.6	0.6	0.0
3e	**58.0**	23.6	5.1	6.4	2.6	4.5	0.0
3f	**80.3**	15.3	0.0	1.9	1.3	0.6	0.6
3g	**46.5**	31.2	10.8	6.4	3.2	1.9	0.0
3h	**33.1**	28.7	10.2	10.2	1.9	5.7	10.2
4a	**29.3**	28.7	19.1	12.7	4.5	3.2	2.6
4b	**24.2**	31.9	18.5	13.4	6.4	5.1	0.6
4d	**27.4**	29.3	6.4	15.9	12.1	4.5	4.5
4f	11.5	17.8	16.6	13.4	10.2	14.7	**15.9**
4g	**41.4**	28.0	9.6	13.4	3.8	1.3	2.6
4h	**28.0**	33.8	20.4	10.8	3.2	1.9	1.9
4i	**65.0**	21.0	2.6	6.4	3.8	0.0	1.3
4j	**28.7**	26.8	13.4	10.8	5.7	5.7	8.9

Table 7.6 Response distribution for each item of the English DASS (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)
Numbers in bold in the response category section indicate significant floor or ceiling effect.

However, when the results of the overall DASS score and each subscale were analysed, floor effect was minimal. For the Maltese DASS, floor effect was 0% for the overall score, 15.5% for the limitations subscale, 3.5% for the hassles and burdens subscale, 0% for the psychological impact overall, 1.2% for the positive psychological impact and 12.1% for the negative psychological impact. Ceiling effect was 0% for all subscales.

For the English DASS, floor effect was 0% for the overall score, 12.1% for the limitations subscale, 14.7% for the hassles and burdens subscale, 1.9% for the

psychological impact overall, 6.4% for the positive psychological impact and 14.0% for the negative psychological impact. Ceiling effect was 0% for all subscales.

7.4.2.4 Factor analysis

Results of the confirmatory factor analysis were unsatisfactory, both for the Maltese and the English DASS (Table 7.7). RMSEA and SRMR were slightly above the acceptable cut-offs. GFI, AGFI and CFI were all significantly below the adequate fit level. However, since the fit parameters were not reported in previous studies (Pelegrino et al., 2012; Samsa et al., 2004), these results could not be directly compared.

Fit parameters	Reference values (McDonald & Ho, 2002)	Maltese version of the DASS	English version of the DASS
RMSEA	≤ 0.08	0.13	0.10
SRMR	≤ 0.10	0.13	0.14
GFI	≥ 0.90	0.64	0.66
AGFI	≥ 0.90	0.57	0.65
CFI	≥ 0.90	0.67	0.63

Table 7.7 Results of the confirmatory factor analysis of the DASS questionnaire

Therefore, an additional exploratory factor analysis was performed, whose rotated factor pattern is reported in Table 7.8. For the Maltese DASS, the convergent validity criterion was met by all items except 2a, 2b, 2c (for the limitations subscale), 3h (for the hassles/burdens subscale) and 4d, 4g, 4h, 4i (for the psychological impact subscale). The discriminant validity criterion was met by all items except 2a, 2b, 2c, 2d (for the limitations subscale), 3h (for the hassles/burdens subscale) and 4d, 4g, 4i

(for the psychological impact subscale). For the English DASS, the convergent validity criterion was met by all items except 2a, 2b, 2c, 2d (for the limitations subscale), 3h (for the hassles/burdens subscale) and 4d, 4g, 4i (for the psychological impact subscale). The discriminant validity criterion was met by all items except 2a, 2b, 2c, 2d (for the limitations subscale), 3h (for the hassles/burdens subscale) and 4d, 4g, 4i (for the psychological impact subscale). However, the factor load on the three DASS subscales was similar to previously published studies (Pelegrino et al., 2012; Samsa et al., 2004). This finding suggested that it is probably a limitation of the DASS questionnaire itself, and not a problem of the Maltese translation.

	This study (Maltese version)			This study (English version)			Pelegrino et al. (2012) (Brazilian-Portugues version)			Samsa et al. (2004) (English version)		
Item	**Limitations**	**Hassles / burdens**	**Psychological impact**	**Limitations**	**Hassles / burdens**	**Psychological impact**	**Limitations**	**Hassles / burdens**	**Psychological impact**	**Limitations**	**Hassles / burdens**	**Psychological impact**
1a	**0.81**	0.23	0.11	**0.70**	0.14	0.05	**0.76**	0.12	-0.09	**0.68**	0.34	-0.07
1b	**0.75**	0.14	-0.05	**0.77**	0.23	0.07	**0.45**	0.18	0.16	**0.67**	0.30	-0.04
1c	**0.59**	0.36	-0.29	**0.43**	0.21	-0.05	**0.41**	0.17	-0.38	**0.50**	0.09	-0.04
1d	**0.86**	0.13	-0.06	**0.83**	0.10	-0.07	**0.56**	0.13	-0.19	**0.77**	0.13	-0.12
1e	**0.84**	0.31	-0.04	**0.84**	0.24	-0.03	**0.67**	0.11	-0.04	**0.81**	0.31	-0.06
2a	0.19	0.35	**-0.44**	0.19	**0.42**	-0.05	0.24	0.13	**-0.31**	**0.56**	0.32	-0.16
2b	0.16	0.07	**-0.43**	0.18	**0.38**	-0.004	0.07	**0.37**	-0.19	**0.43**	-0.06	0.06
2c	0.28	0.41	**-0.51**	0.26	**0.35**	-0.14	0.18	**0.38**	-0.27	**0.48**	0.22	-0.04
2d	0.45	**0.68**	-0.19	0.36	**0.59**	0.02	**0.47**	0.39	0.03	**0.75**	0.41	-0.02
3a	0.33	**0.61**	-0.14	0.34	**0.52**	0.09	0.30	**0.46**	0.23	0.51	**0.60**	-0.01
3b	0.09	**0.77**	0.10	0.08	**0.70**	0.08	0.15	**0.55**	0.05	0.34	**0.65**	0.06
3c	0.32	**0.73**	-0.10	0.24	**0.61**	0.17	0.21	**0.59**	0.22	0.19	**0.64**	0.08
3d	-0.22	**0.58**	0.34	-0.03	**0.72**	0.11	0.05	**0.60**	-0.06	0.26	**0.71**	-0.02
3e	0.14	**0.85**	0.16	0.04	**0.83**	0.17	0.05	**0.71**	0.15	0.12	**0.81**	0.00
3f	0.25	**0.55**	0.18	0.11	**0.41**	-0.03	0.10	**0.63**	0.07	0.27	**0.44**	-0.05
3g	0.10	**0.84**	0.09	0.04	**0.86**	0.06	0.04	**0.74**	0.08	0.20	**0.77**	0.06
3h	0.05	-0.04	**0.39**	-0.08	0.04	**0.52**	0.03	-0.07	**0.43**	0.04	-0.17	**0.51**
4a	0.10	0.15	**0.40**	0.02	0.05	**0.72**	-0.03	0.27	**0.55**	0.06	-0.06	**0.76**
4b	0.14	0.06	**0.54**	0.08	0.09	**0.82**	-0.11	0.16	**0.62**	0.01	0.11	**0.83**
4d	**0.39**	0.36	-0.005	0.31	**0.35**	0.06	0.41	0.17	**-0.53**	**0.58**	0.38	0.07
4f	-0.14	-0.05	**0.43**	-0.05	-0.02	**0.58**	0.01	0.20	**0.42**	-0.25	-0.07	**0.57**
4g	0.26	**0.50**	-0.11	0.26	**0.45**	0.07	**0.67**	0.02	0.14	0.29	**0.64**	0.03
4h	-0.05	0.14	**0.34**	0.06	0.30	**0.63**	0.09	0.20	**0.72**	-0.02	0.38	**0.74**
4i	0.23	**0.57**	-0.06	0.14	**0.32**	0.16	0.19	**0.48**	-0.07	0.07	**0.68**	0.06
4j	-0.004	0.06	**0.49**	0.01	-0.005	**0.61**	0.22	-0.24	**0.70**	-0.15	0.20	**0.70**

Table 7.8 Results of the 3-factor analysis of the DASS questionnaire, in comparison to previously published studies (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)

Numbers in bold indicate the highest loading of each factor, which is therefore likely to explore that dimension.

7.4.2.5 Correlation scale-subscales

There was a significant positive correlation between the DASS total score and its subscales, which was expected because the DASS total score is the sum of its three subscales. However, there was also a significant correlation between the different subscales (Table 7.9), thus confirming the correctness of summing them into an overall total score.

	Limitations (9 items)	**Hassles / burdens (8 items)**	**Psychological impact (8 items)**	**Positive psychological impact (5 items)**	**Negative psychological impact (3 items)**
Maltese version					
Limitations	1.00				
Hassles/burdens	0.48 *	1.00			
Psychological impact	0.24 *	0.51 *	1.00		
Positive psychological impact	-0.13	0.20 #	0.80 *	1.00	
Negative psychological impact	0.57 *	0.59 *	0.63 *	0.03	1.00
DASS total score	0.80 *	0.85 *	0.68 *	0.29 *	0.75 *
English version					
Limitations	1.00				
Hassles/burdens	0.43 *	1.00			
Psychological impact	0.22 #	0.50 *	1.00		
Positive psychological impact	0.03	0.31 *	0.90 *	1.00	
Negative psychological impact	0.43 *	0.53 *	0.57 *	0.14	1.00
DASS total score	0.74 *	0.82 *	0.74 *	0.53 *	0.66 *

Table 7.9 Correlation between the DASS total score and its subscales (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)

* $p \leq 0.001$ # $p < 0.05$

7.4.2.6 Known-group validity

The Maltese DASS showed a significant positive correlation with previous hospitalisation (p=0.03) and previous bleeding (p=0.01), and a significant negative correlation with longer anticoagulant treatment duration (p=0.02), as reported in Table 7.10. Since lower DASS total scores represent greater satisfaction, a negative correlation means greater satisfaction. Therefore, these findings suggested that patients with previous hospitalisation, previous bleeding and shorter anticoagulant treatment duration are less satisfied.

The English DASS showed a significant positive correlation with paid-employment (p=0.04) and previous bleeding (p=0.01), and a significant negative correlation with increasing age (p=0.002) and male sex (p=0.004). These findings suggested that patients of female sex, young age or actively working, and with previous bleeding were less satisfied. It can be noted that, although sometimes lacking statistical significance, the correlation between the main sociodemographic and clinical characteristics was in the same direction for the Maltese DASS and the English DASS.

Variable	Correlation coefficient for the Maltese version (p value)	Correlation coefficient the English version (p value)
Increasing age	-0.12 (0.12)	-0.25 (0.002)
Male sex	-0.15 (0.05)	-0.22 (0.004)
Living situation: living alone	-0.02 (0.72)	0.06 (0.45)
Level of education: primary school only	-0.05 (0.54)	0.01 (0.86)
Employment status: full- or part-time paid employment	0.09 (0.37)	0.17 (0.04)
Clinical indication to anticoagulation: AF	-0.09 (0.23)	-0.08 (0.30)
Anticoagulant treatment duration: >5 years	-0.18 (0.02)	-0.05 (0.55)
INR in range at enrolment	-0.06 (0.41)	-0.01 (0.94)
High TTR (≥ 70%) in the previous year	-0.01 (0.86)	-0.05 (0.53)
Any hospitalisation in the previous year	0.17 (0.03)	0.14 (0.09)
History of any bleeding on warfarin	0.20 (0.01)	0.20 (0.01)

Table 7.10 Correlation between the DASS total score and patients characteristics (Riva, Borg Xuereb, Ageno, et al., 2019) (Reproduced with permission)

7.4.3 The PACT-Q2 questionnaire

7.4.3.1 Internal consistency

The convenience subscale (13 items) of the Maltese PACT-Q2 showed good internal consistency (Cronbach's alpha coefficient = 0.86). The anticoagulant treatment satisfaction subscale (7 items) showed a Cronbach's alpha coefficient of 0.62, which was slightly below the acceptable cut-off of 0.70. When the single items were analysed, the question D2 had low item-total correlation (~ 0.3) and the Cronbach's alpha showed an increase to 0.66 when this item was deleted (Table 7.11). Item D2 corresponds to the question "Do you feel that your anticoagulant treatment has decreased your symptoms?", which could have a negative answer also in satisfied patients, especially when anticoagulation is used for stroke prevention.

The English PACT-Q2 showed good internal consistency for both the convenience (Cronbach's alpha = 0.86) and the satisfaction (Cronbach's alpha = 0.75) subscales.

PACT-Q2 item	Maltese version			English version		
	Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted	Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted
Convenience	0.86			0.86		
B1		0.63	0.84		0.56	0.84
B2		0.72	0.83		0.68	0.84
B3		0.60	0.84		0.65	0.84
B4		0.65	0.84		0.53	0.85
B5		0.49	0.85		0.51	0.85
B6		0.57	0.84		0.54	0.84
B7		0.58	0.84		0.53	0.84
B8		0.54	0.84		0.61	0.84
B9		0.65	0.84		0.62	0.84
B10		0.32	0.86		0.23	0.86
B11		0.34	0.86		0.39	0.86
C1		0.42	0.85		0.52	0.84
C2		0.36	0.85		0.55	0.84
Satisfaction	0.62			0.75		
D1		0.40	0.56		0.41	0.73
D2		0.31	0.66		0.28	0.79
D3		0.31	0.59		0.36	0.74
D4		0.33	0.59		0.53	0.71
D5		0.35	0.58		0.61	0.69
D6		0.48	0.57		0.72	0.68
D7		0.56	0.56		0.69	0.68

Table 7.11 Internal consistency of the Maltese and English versions of the PACT-Q2 (Riva, Borg Xuereb, Makris, et al., 2019) (Reproduced with permission)

7.4.3.2 Reproducibility

The intra-language correlation is reported in Table 7.12. For the Maltese-Maltese test-retest, the result was very good for the convenience subscale (ICC = 0.87), but low for the satisfaction subscale (ICC = 0.40). For the English-English test-retest, the result was very good for the convenience subscale (ICC = 0.87), but at the lower acceptable limit for the satisfaction subscale (ICC = 0.60).

The cross-language test-retest correlation, pooling together Maltese-English and English-Maltese test-retests, gave slightly lower results. The raw ICC for the convenience subscale was 0.51 while for the satisfaction subscale was 0.52. The

adjusted ICC were 0.59 for the convenience subscale and 1.06 for the satisfaction subscale. When analysed separately, the ICC for the English-Maltese test-retest was 0.76 for the convenience subscale and 0.68 for the satisfaction subscale. The ICC for the Maltese-English test-retest was 0.43 for the convenience subscale and 0.41 for the satisfaction subscale.

	Score difference			ICC
	Mean (SD)	Min	Max	
Maltese-Maltese test-retest				
• PACT-Q2 convenience	-0.1 (3.2)	-6	6	0.87
• PACT-Q2 satisfaction	0.1 (4.1)	-7	8	0.40
English-English test-retest				
• PACT-Q2 convenience	0.9 (3.0)	-3	7	0.87
• PACT-Q2 satisfaction	2.3 (3.6)	-1	+10	0.60

Table 7.12 Results of the intra-language test-retest correlation for the PACT-Q2 (Riva, Borg Xuereb, Makris, et al., 2019) (Reproduced with permission)
For both subscales the original scores were considered (not rescaled). Items in the convenience subscale were reversed.

7.4.3.3 Floor and ceiling effect

The response distribution for each item of the PACT-Q2, after reversing the items of the convenience subscale, was analysed. In the Maltese PACT-Q2, a significant ceiling effect was identified in most of the items, while a significant floor effect was found only for item D2 (Table 7.13). Similar findings emerged for the original English PACT-Q2 administered to this study cohort.

PACT-Q2 item	Maltese version						English version					
	Response category (%)					Mean (SD)	Response category (%)					Mean (SD)
	1	2	3	4	5		1	2	3	4	5	
B1 *	1.2	5.2	4.0	12.1	**77.6**	4.6 (0.9)	0.6	1.3	5.7	14.0	**78.3**	4.7 (0.7)
B2 *	2.9	6.3	8.6	20.1	**62.1**	4.3 (1.1)	0.0	5.1	6.4	16.6	**72.0**	4.6 (0.8)
B3 *	4.0	5.8	6.3	24.1	**59.8**	4.3 (1.1)	0.0	2.6	9.6	26.1	**61.8**	4.5 (0.8)
B4 *	5.2	12.6	8.6	24.1	**49.4**	4.0 (1.2)	2.6	11.5	19.1	26.8	**40.1**	3.9 (1.1)
B5 *	2.9	10.3	15.5	21.8	**49.4**	4.0 (1.2)	1.3	8.3	19.8	29.3	**41.4**	4.0 (1.0)
B6 *	2.9	8.1	4.0	10.3	**74.7**	4.5 (1.1)	0.6	2.6	7.6	21.0	**68.2**	4.5 (0.8)
B7 *	1.7	4.6	9.8	18.4	**65.5**	4.4 (1.0)	1.9	3.8	8.3	26.1	**59.9**	4.4 (0.9)
B8 *	1.2	4.0	7.5	14.4	**73.0**	4.5 (0.9)	1.3	6.4	8.9	27.4	**56.1**	4.3 (1.0)
B9 *	0.6	4.0	4.6	6.9	**83.9**	4.7 (0.8)	0.6	1.9	2.6	17.2	**77.7**	4.7 (0.7)
B10 *	5.2	8.1	5.2	17.8	**63.8**	4.3 (1.2)	1.3	3.2	3.8	13.4	**78.3**	4.6 (0.8)
B11 *	11.5	17.8	29.5	24.1	**27.0**	3.4 (1.4)	8.3	17.8	19.1	26.1	**28.7**	3.5 (1.3)
C1 *	2.3	4.0	6.9	10.9	**75.9**	4.5 (1.0)	0.0	4.5	10.8	14.0	**70.7**	4.5 (0.9)
C2 *	2.3	8.6	10.9	25.3	**52.9**	4.2 (1.1)	0.6	2.6	7.0	28.0	**61.8**	4.5 (0.8)
D1	2.3	2.9	27.0	57.5	10.3	3.7 (0.8)	6.4	8.3	14.6	39.5	**31.2**	3.8 (1.2)
D2	**51.2**	14.9	6.9	16.7	10.3	2.2 (1.5)	**42.7**	16.6	19.1	14.0	7.6	2.3 (1.3)
D3	4.0	5.8	50.6	28.7	10.9	3.4 (0.9)	0.6	3.2	54.1	21.7	**20.4**	3.6 (0.9)
D4	0.6	2.9	12.6	72.4	11.5	3.9 (0.6)	0.6	1.9	11.5	64.3	**21.7**	4.0 (0.7)
D5	0.0	5.8	9.8	71.3	13.2	3.9 (0.7)	1.3	5.1	12.7	53.5	**27.4**	4.0 (0.9)
D6	0.0	0.6	6.3	81.6	11.5	4.0 (0.4)	1.3	0.6	7.0	68.8	**22.3**	4.1 (0.7)
D7	0.0	0.0	5.8	78.7	**15.5**	4.1 (0.5)	1.3	1.3	7.6	64.3	**25.5**	4.1 (0.7)

Table 7.13 Response distribution for each item of the PACT-Q2 and summary statistics (Riva, Borg Xuereb, Makris, et al., 2019) (Reproduced with permission)
Numbers in bold in the response category section indicate significant floor or ceiling effect.
* Items of the convenience subscale (B1 to C2) are reversed

However, when the results of each subscale of the PACT-Q2 were analysed, ceiling effect was minimal. For the Maltese PACT-Q2, ceiling effect was 6.3% for the

convenience subscale and 1.2% for the satisfaction subscale. Floor effect was 0% for both subscales. For the English PACT-Q2, ceiling effect was 9.6% for the convenience subscale and 1.3 % for the satisfaction subscale. Floor effect was 0% for both subscales.

7.4.3.4 Factor analysis

Results of the confirmatory factor analysis were acceptable (Table 7.14). For the Maltese PACT-Q2, SRMS was acceptable and RMSEA was slightly above the reference cut-off. GFI, AGFI and CFI were slightly below the reference values. For the English PACT-Q2, both RMSEA and SRMR were acceptable. GFI, AGFI and CFI were slightly below the reference values.

An exploratory factor analysis was also performed, whose rotated factor pattern is reported in Table 7.15. For the Maltese PACT-Q2, the convergent validity criterion was met by all items except B10, B11, C2 (for the convenience subscale) and D2, D3 (for the satisfaction subscale). All items met the discriminant validity criterion. For the English PACT-Q2, the convergent validity criterion was met by all items except B10, B11 (for the convenience subscale) and D1, D2, D3 (for the satisfaction subscale). All items met the discriminant validity criterion.

Fit parameters	Reference values (McDonald & Ho, 2002)	Maltese version of the PACT-Q2	English version of the PACT-Q2
RMSEA	≤ 0.08	0.09	0.07
SRMR	≤ 0.10	0.10	0.08
GFI	≥ 0.90	0.82	0.80
AGFI	≥ 0.90	0.78	0.84
CFI	≥ 0.90	0.79	0.88

Table 7.14 Results of the confirmatory factor analysis of the PACT-Q2 questionnaire (Riva, Borg Xuereb, Makris, et al., 2019) (Reproduced with permission)

	Maltese version		English version	
Item	**Convenience**	**Satisfaction**	**Convenience**	**Satisfaction**
B1 *	**0.69**	0.09	**0.63**	0.06
B2 *	**0.78**	0.26	**0.69**	0.34
B3 *	**0.64**	0.07	**0.69**	0.06
B4 *	**0.70**	-0.03	**0.57**	-0.03
B5 *	**0.53**	0.04	**0.53**	0.10
B6 *	**0.60**	-0.08	**0.60**	0.16
B7 *	**0.62**	0.32	**0.56**	0.18
B8 *	**0.63**	0.13	**0.63**	0.25
B9 *	**0.70**	0.07	**0.72**	0.05
B10 *	**0.34**	0.09	**0.22**	0.15
B11 *	**0.36**	0.07	**0.38**	0.11
C1 *	**0.46**	-0.08	**0.55**	0.08
C2 *	**0.38**	0.03	**0.58**	0.11
D1	-0.06	**0.42**	0.08	**0.37**
D2	-0.36	**0.29**	-0.07	**0.25**
D3	0.10	**0.27**	0.15	**0.39**
D4	0.20	**0.49**	0.38	**0.55**
D5	0.16	**0.58**	0.14	**0.75**
D6	0.03	**0.75**	0.17	**0.89**
D7	-0.10	**0.83**	0.18	**0.91**

Table 7.15 Results of the 2-factor analysis of the PACT-Q2 questionnaire (Riva, Borg Xuereb, Makris, et al., 2019) (Reproduced with permission)
Numbers in bold indicate the highest loading of each factor, which is therefore likely to explore that dimension
* Items of the convenience subscale (B1 to C2) are reversed

7.4.3.5 Correlation scale-subscales

In the Maltese PACT-Q2, there was no correlation between the convenience subscale and the satisfaction subscale ($r = 0.01$, $p=0.83$). In the English PACT-Q2, there was a weak positive correlation ($r = 0.33$, $p<0.001$). These findings confirmed the fact that the two subscales cover different dimension and should be scored separately.

7.4.3.6 Known-group validity

The Maltese PACT-Q2 showed a negative correlation with previous bleeding ($p=0.08$ for the convenience subscale, $p=0.01$ for the satisfaction subscale). Since higher

PACT-Q2 scores represent greater satisfaction, a negative correlation means lower satisfaction.

The convenience subscale of the English PACT-Q2 showed a significant positive correlation with increasing age (p<0.0001), male sex (p=0.001), and a significant negative correlation with paid employment (p=0.006), previous hospitalisation (p=0.03) and previous bleeding (p=0.03). The satisfaction subscale of the English PACT-Q2 showed a significant positive correlation with male sex (p=0.02). Taken together, these findings suggested that patients of female sex, young age or actively working, and with previous hospitalisation or bleeding had lower convenience/satisfaction.

Variable	Correlation coefficient for the Maltese version (p value)		Correlation coefficient for the English version (p value)	
	Convenience subscale	Satisfaction subscale	Convenience subscale	Satisfaction subscale
Increasing age	0.05 (0.53)	0.02 (0.81)	0.34 (<0.0001)	0.14 (0.08)
Male sex	0.09 (0.26)	0.03 (0.68)	0.27 (0.001)	0.19 (0.02)
Living situation: living alone	0.05 (0.54)	-0.11 (0.14)	-0.07 (0.37)	0.05 (0.55)
Level of education: primary school only	-0.01 (0.95)	-0.07 (0.36)	-0.02 (0.84)	-0.11 (0.16)
Employment status: full- or part-time paid employment	-0.09 (0.24)	0.04 (0.56)	-0.22 (0.006)	-0.15 (0.07)
Clinical indication to anticoagulation: AF	-0.01 (0.94)	0.04 (0.58)	0.09 (0.27)	0.01 (0.91)
Anticoagulant treatment duration: >5 years	0.03 (0.73)	0.07 (0.36)	0.05 (0.57)	0.01 (0.92)
INR in range at enrolment	0.05 (0.49)	0.05 (0.43)	0.07 (0.36)	-0.15 (0.07)
High TTR (≥ 70%) in the previous year	0.06 (0.43)	0.12 (0.12)	0.04 (0.64)	-0.03 (0.71)
Any hospitalisation in the previous year	-0.10 (0.21)	-0.03 (0.70)	-0.18 (0.03)	0.05 (0.58)
History of any bleeding on warfarin	-0.16 (0.08)	-0.21 (0.01)	-0.17 (0.03)	-0.12 (0.11)

Table 7.16 Correlation between the PACT-Q2 subscales and patients characteristics (Riva, Borg Xuereb, Makris, et al., 2019) (Reproduced with permission)

7.5 Discussion

In this study, for the first time, the DASS and the PACT-Q2 were translated into the Maltese language and validated. They were administered to 174 patients on warfarin treatment for different clinical indications (AF, VTE, heart valve replacement). In addition, a group of 157 patients, enrolled during the same time frame from the same Anticoagulation Clinics, completed the original English versions of the DASS and the PACT-Q2. This study design was possible because Malta is a bilingual country where both Maltese and English are official languages (National Statistics Office, 2014; Vella, 2013). It has been estimated that more than two thirds of the population is bilingual (National Statistics Office, 2014), although the Maltese language predominates in the spoken language, while the English language predominates in written communications (Vella, 2013). Therefore, the psychometric properties (reliability and validity) of the Maltese translations could be evaluated in comparison to the published literature on these two questionnaires, but also in comparison to the psychometric properties of the original English versions completed in this study cohort.

7.5.1 Reliability and validity of the Maltese DASS

The Maltese translation of the DASS showed good reliability. The internal consistency was very good, with Cronbach's alpha coefficients above 0.80 for the overall DASS score and the subscales limitations and hassles/burdens. The Cronbach's alpha coefficients were slightly below the acceptable cut-off (≥ 0.70) for the positive and negative psychological impact subscales (0.65 and 0.64, respectively). This finding can be explained by the lower number of items included in these subscales (five and three items, respectively). In fact, lower Cronbach's alpha were reported also in

another study evaluating the Brazilian-Portuguese version of the DASS (0.67 and 0.38, respectively) (Pelegrino et al., 2012) and in the original English DASS in the current study cohort (0.79 and 0.56, respectively).

The reproducibility was also very good, with ICC for the intra-language correlation being 0.73 for the Maltese-Maltese test-retest and 0.85 for the English-English test-retest. These results were above the acceptable cut-off (≥ 0.60) and similar to values reported in previous studies (Radaideh & Matalqah, 2018; Samsa et al., 2004). Given the peculiarity of the Maltese population with high prevalence of bilingual subjects (National Statistics Office, 2014), the cross-language correlation could also be assessed. However, despite the fact that a rigorous process of translation was followed, the ICC for the cross-language correlation was significantly lower. A discrepancy between the ICC for the intra-language correlation and the ICC for the cross-language correlation has already been reported, together with several possible solutions (Chung et al., 2006; Wood et al., 2018). Wood et al. (2018) hypothesised that it could be due to score unreliability and suggested to adjust the cross-language correlation by the intra-language correlation. A better ICC was found after applying the suggested adjustment (raw ICC for the cross-language correlation 0.31, adjusted ICC 0.39); however, the result was still unsatisfactory. Chung et al. (2006) hypothesised that it could be due to poor bilingual proficiency and performed a sub-analysis of subjects with higher level of education. Unfortunately, the number of patients included in the test-retest with high level of education was too low to perform a specific sub-analysis. However, when the English-Maltese test-retest and the Maltese-English test-retest were analysed separately, poor results were observed only in the latter (ICC 0.59 and 0, respectively). This result suggested that those patients who initially chose to

complete the Maltese version of the questionnaire were probably less confident in English, confirming the hypothesis by Chung et al. (2006).

Validity of the Maltese DASS was acceptable. A significant floor effect was identified in the analysis of the single items, but it was already present in the original English DASS (Samsa et al., 2004), therefore being an intrinsic characteristic of the questionnaire itself. At factory analysis, most of the fit parameters were significantly below the desirable values; however, the factors load on the three DASS subscale was similar to previous studies (Pelegrino et al., 2012; Samsa et al., 2004). Construct validity was good, with significant positive correlations between the overall DASS score and its subscales and between the different subscales. Finally, the analysis of the known-group validity showed that patients with previous hospitalisation, previous bleeding and shorter anticoagulant treatment duration were less satisfied (in the analysis of the Maltese DASS), while patients of female sex, young age or actively working, and with previous bleeding were less satisfied (in the analysis of the English DASS). Differences in the study population between patients who completed the Maltese version of the DASS and patients who completed the English version of the DASS might have contributed to these slightly different results. However, these patient's characteristics have already been reported to correlate with the degree of satisfaction in previous studies: younger age (Pelegrino et al., 2012; Samsa et al., 2004); working status (Gafou et al., 2007; Radaideh & Matalqah, 2018); hospitalisation for bleeding (Radaideh & Matalqah, 2018; Samsa et al., 2004). Although the present study used two different definitions for hospitalisation and previous bleeding, they were both correlated with the DASS total score.

7.5.2 Reliability and validity of the Maltese PACT-Q2

The Maltese translation of the PACT-Q2 showed good reliability. The reliability was very good for the convenience subscale (Cronbach's alpha 0.86, ICC 0.87), while it was lower for the satisfaction subscale (Cronbach's alpha 0.62, ICC 0.40). However, this finding appeared to be a weakness of this subscale, rather than a limitation of the Maltese translation. In fact, low reliability of the satisfaction subscale was reported also in the English PACT-Q2 in this study (Cronbach's alpha 0.86, ICC 0.87 for the convenience subscale vs. Cronbach's alpha 0.75, ICC 0.60 for the satisfaction subscale) and in the original publication of the PACT-Q (Cronbach's alpha 0.84 for the convenience subscale vs. 0.76 for the satisfaction subscale) (Prins, Guillemin, et al., 2009). This finding can be explained by the low number of items, a response bias, and a change in the status between the test and the retest. The Cronbach's alpha is known to be dependent on the number of items in each subscale (Terwee et al., 2007) and, while the convenience subscale has 13 items, the satisfaction subscale has seven items only. Response bias occurs when participants are influenced by their belief of which answers are socially acceptable or which answers are expected by the researchers (Mazor et al., 2002). In the satisfaction subscale there are several questions (D4-D7) asking directly the level of satisfaction with different aspects of the anticoagulant treatment (such as the level of independence, the appointments, the anticoagulant drug and the overall satisfaction). Therefore, the satisfaction subscale might have been particularly susceptible to the response bias, because the participants might have felt obliged to show that they were satisfied with the service. They might have also feared reprisal on their treatment, even though the consent forms specified the confidentiality of the answers. Finally, although the retest was performed within two weeks, there might have been changes in the level of satisfaction due to

intercurrent clinical events or different experience of service provision during medical appointments.

Validity of the Maltese PACT-Q2 was good. A significant ceiling effect was identified in the analysis of the single items. However, ceiling effect was 6.3% for the convenience subscale and 1.2% for the satisfaction subscale, which was even better than the original report of the PACT-Q2 (22.1% for the convenience subscale and 3.3% for the satisfaction subscale) (Prins, Guillemin, et al., 2009). The factor analysis showed that fit parameters were very close to the acceptable reference values. Furthermore, all the items met the discriminant validity criterion, while the only exceptions to the convergent validity criterion were B10, B11, C2, D2, D3. These results confirmed the findings of the original publication of the PACT-Q2, where items B10-B11 and D2-D3 did not meet the convergent validity criterion (Prins, Guillemin, et al., 2009). Correlation between the two subscales (convenience and satisfaction) was weak, confirming the fact that they cover different dimensions and they should be scored separately (Prins, Guillemin, et al., 2009). Finally, the analysis of the known-group validity found that patients with history of bleeding had lower satisfaction. Although this group was not specifically evaluated in previous validation studies of the PACT-Q (Mohamed et al., 2015; Prins, Guillemin, et al., 2009), a prospective study of 807 AF patients on VKA reported lower scores in the convenience dimension after bleeding complications (Kooistra et al., 2016). Furthermore, studies validating the DASS questionnaire also reported lower satisfaction in patients with previous bleeding (Radaideh & Matalqah, 2018; Samsa et al., 2004).

7.5.3 The importance of patients reported outcomes

Anticoagulant therapy has a fundamental role for the prevention and treatment of VTE and for stroke prevention in patients with AF or mechanical heart valves. It has been estimated that around 1% of the Maltese population (~ 4,000 people) is anticoagulated (Zammit et al., 2011). However, there was no specific questionnaire available in the Maltese language to evaluate the QoL of anticoagulated patients. It has been shown that low satisfaction correlates with poor medication adherence and poor INR control (Balkhi et al., 2018; Bartoli-Abdou et al., 2018; Davis et al., 2005; Thomson Mangnall et al., 2016; Weernink et al., 2018) and that, in turn, a low TTR correlates with an increased risk of thromboembolic and bleeding complications (Pokorney et al., 2015). Assessing the QoL is one of the steps to improve the quality of the care for anticoagulated patients. Healthcare professionals can promote specific educational interventions to reduce the burden associated with VKA treatment. In addition, they can also reinforce the positive aspects which are relevant for each patient. For instance, this study highlighted that the burden of anticoagulant therapy and INR monitoring is greater for young working people, compared to older retirees.

It was decided to translate the DASS and the PACT-Q, because they are both psychometric questionnaires specifically developed for anticoagulated patients. The DASS explores three dimensions (limitations, hassles/burdens, psychological impact), includes 25 questions (to be answered on a 7-point scale), and can provide one overall final score. Conversely, the PACT-Q2 explores two dimensions (convenience, anticoagulant treatment satisfaction), it is shorter (including 20 questions to be answered on a 5-point scale), however it does not provide one overall final score, because the items of the two subscales should be scored separately.

7.5.4 Strengths and limitations

The main strengths of this study are the completeness of data, without any missing answers, and the rigorous process of translation and analysis, in order to validate the Maltese DASS and the Maltese PACT-Q2. Furthermore, the patients were enrolled from different locations around the Maltese island, therefore this sample is likely to be generalizable to the overall anticoagulated Maltese population. Finally, a peculiarity of this study was the group of patients, enrolled from the same setting, who completed the original English versions of the questionnaires, therefore allowing a comparison between the psychometric properties in the two languages.

However, this study has also some limitations which need to be acknowledged. First, only patients on VKA treatment were included, therefore these results might not be generalizable to patients treated with the DOAC or with parenteral anticoagulants. However, at the time of enrolment the majority of anticoagulated patients in Malta were on warfarin, since the DOAC were not centrally funded. Second, since the patients were already receiving the VKA treatment, only the PACT-Q2 could be evaluated, because the PACT-Q1 has to be administered before treatment initiation. Third, the number of patients included in the test-retest was smaller than what is generally recommended for the calculation of the ICC (Terwee et al., 2007).

7.6 Conclusion

The results of this study showed that the Maltese translation of the DASS had a good reliability and validity. The Maltese translation of the PACT-Q2 showed an acceptable level of reliability and good validity. These findings were comparable to the original English versions of the DASS and the PACT-Q2. Therefore, the Maltese DASS and the Maltese PACT-Q2 are valid and reliable instruments to assess the level of

satisfaction of Maltese-speaking anticoagulated patients. They can be used by healthcare professionals in the setting of anticoagulation clinics or in future research projects assessing patients' satisfaction or barriers to anticoagulant treatment.

Chapter 8 :

Patients' Satisfaction with the Point-of-care INR

8.1 Introduction

Anticoagulation is a long-term treatment for most clinical indications and it can therefore affect the patients' perception of the QoL. It can be associated with positive aspects, such as the reassurance provided by the treatment itself or the contact with supportive healthcare professionals (Borg Xuereb et al., 2016), but also negative aspects, such as the need for lifestyle changes or regular blood tests, or concerns about possible bleeding complications (Borg Xuereb et al., 2016; Prins, Marrel, et al., 2009; Samsa et al., 2004). Health-related QoL should always be considered when dealing with chronic treatment, since patient dissatisfaction can lead to decreased adherence (Balkhi et al., 2018; A. T. Hirsh et al., 2005; Ware & Davies, 1983), poor anticoagulation control and worse clinical outcomes (Ho et al., 2009; Samsa et al., 2004). It is important to identify those patients with low QoL or who are not satisfied, in order to promote specific interventions (Lane et al., 2006). For this purpose, specific psychometric questionnaires addressing anticoagulated patients have been developed, such as the DASS (Samsa et al., 2004) and the PACT-Q (Prins, Guillemin, et al., 2009; Prins, Marrel, et al., 2009).

VKA treatment is monitored using the INR which can be performed through venepuncture and laboratory coagulometers (laboratory INR) or through finger-prick and POC coagulometers (POC INR), the latter usually performed by patients themselves as self-testing. Several studies reported patients' preference for the POC INR monitoring, by using generic QoL scales (Jowett et al., 2006; Khan et al., 2004) or simple questions (Kong et al., 2008; Oral Anticoagulation Monitoring Study Group, 2001; Shiach et al., 2002). The PACT-Q has never been evaluated, so far, in patients monitored with the POC. The DASS has been evaluated in patients switched from anticoagulation clinic management to POC self-testing with online remote monitoring

(Meyer et al., 2013) and in patients randomized to POC self-testing vs. clinic-testing (Matchar et al., 2010). However, it is still unclear whether the same degree of satisfaction is associated with the use of POC coagulometers by healthcare professionals in the anticoagulation clinics. A small study used the DASS to compare vein-testing vs. POC-testing, but did not find any statistically significant difference between the two groups (Gafou et al., 2007).

8.2 Aim

The aim of this study was to compare patients' satisfaction associated with warfarin treatment in two different settings characterized by diverse ways of monitoring (anticoagulation clinics with standard laboratory INR monitoring vs. anticoagulation clinics with POC INR monitoring).

8.3 Methods

8.3.1 Study population

The inclusion criteria were patients on long-term warfarin treatment for different clinical indications (e.g. AF, VTE, heart valve replacement, and others) enrolled from two different settings.

The first cohort consisted of patients monitored with the classical venepuncture and the standard laboratory INR, enrolled from the Anticoagulation Clinic at Mater Dei Hospital (Msida). In this setting blood collection was performed early in the morning, samples were analysed in the Coagulation Laboratory and warfarin dose was prescribed after the INR results become available, i.e. in the early afternoon. The INR was measured using the automated coagulation analyser ACL TOP 500

(Instrumentation Laboratory, Italy) and the HemosIL® RecombiPlasTin 2G reagent (Instrumentation Laboratory, Italy). The Anticoagulation Clinic at Mater Dei Hospital has a walk-in policy and assists hundreds of patients every day. Patients with less than 12 months experience with the standard laboratory INR for VKA monitoring were excluded.

The second cohort consisted of patients monitored with a finger-prick and the use of POC coagulometers. In Malta, since 2014 the POC devices were used by healthcare professionals at several Health Centres spread around the island. In this setting patients were allocated a specific appointment and a time slot for INR testing, which was immediately followed by warfarin dose adjustment by the attending physicians. Patients were enrolled from the Anticoagulation Clinics at five Health Centres (Cospicua, Floriana, Mosta, Qormi, Rabat), which represent the different areas of Malta. The POC coagulometer CoaguChek XS Plus (Roche Diagnostics International Ltd, Germany) was used for the determination of the INR. Patients with less than 12 months experience with the POC INR for VKA monitoring were excluded.

All the patients monitored with the POC INR had a previous experience with the standard laboratory INR, since in the local context they were usually switched to the POC INR after some time. At the beginning of the POC system, the local protocol for switching required some strict criteria (target INR ≤ 3.0; at least three consecutive INR values within the therapeutic range; absence of APS, liver disease, severe renal failure, active cancer, or dual antiplatelet therapy), but afterwards it was left at the discretion of the attending physicians.

8.3.2 Study design

A cross-sectional study was performed. Consecutive adult patients attending the above-mentioned Anticoagulation Clinics on random days between July 2017 and February 2018, were invited to participate. After explaining the rationale and the design of this study, eligible patients received an information sheet and, if they agreed to take part in this study, they were asked to sign a consent form. Both English and Maltese versions of the information sheets, consent forms, and questionnaires were available for patients (Appendix C2). This study was approved by the University of Malta Research and Ethics Committee (protocol 07/2016, Appendix B).

Patients' satisfaction associated with the anticoagulant treatment was evaluated through the administration of two specific psychometric questionnaires at the time of enrolment: the DASS (Samsa et al., 2004) and the PACT-Q2 (Prins, Marrel, et al., 2009). The PACT-Q1 was not administered because it measures the expectations associated with the anticoagulant treatment and should be administered before treatment initiation (Prins, Marrel, et al., 2009), while these cohorts consisted of patients on long-term warfarin. The choice of whether to complete the Maltese or the English versions of the questionnaires was left at the discretion of each patient. The Maltese translations of the DASS and PACT-Q2 questionnaires were previously validated (Chapter 7). The patients were offered the option to fill the questionnaires in the waiting area at the Anticoagulation Clinics or to fill the questionnaire at home and send it by post with a pre-paid self-addressed envelope. In this latter case, they received a maximum of two reminders, if needed. Questionnaires were identified with a numeric code, in order to ensure anonymity. The list with the correspondence between the code and patients' details was used to contact the patients only in case of missing answers.

Through a demographic form completed by the patients and a review of medical notes, the following information were collected: baseline characteristics of the population (age, sex, nationality, spoken languages, living situation, level of education, employment status), details of the warfarin treatment (indication for anticoagulant treatment, starting date, prescribed duration, INR target range, INR results in the 12 months before inclusion). Experience of unsuitable blood specimens was defined as any previous coagulation blood samples that was either haemolysed, lipemic, insufficient or filled in excess (from 2012 up to the day of enrolment).

8.3.3 Statistical analysis

Continuous variables were expressed as mean (SD) or median (IQR); categorical variables were expressed as counts and percentages. Normality was evaluated using the Wilk-Shapiro test. Continuous variables were compared using the Student's t-test for normally distributed variables or the non-parametric Mann-Whitney U test for not-normally distributed variables; categorical variables were compared using the Chi square or Fisher's exact tests, as appropriate.

The DASS was expressed as total score (ranging from 25 to 175), and as score of each subscale (limitations, hassles/burdens, psychological impact). Six items (3h, 4a, 4b, 4f, 4h, 4j) were reversed prior to analysis, according to Samsa et al. (2014). The PACT-Q2 score was reported separately for the two subscales (convenience, anticoagulant treatment satisfaction). During the analysis, the items of the convenience dimension were reversed, summed, and rescaled on a 0-100 scale; the items of the anticoagulant treatment satisfaction dimension were summed and rescaled on a 0-100 scale (Prins, Guillemin, et al., 2009). The DASS and PACT-Q2 results were compared between the two groups (standard laboratory INR vs. POC INR). For the DASS lower

scores correspond to higher satisfaction, while for the PACT-Q2 higher scores correspond to higher satisfaction.

The TTR was calculated according to the Rosendaal method (Rosendaal et al., 1993), from the outpatients INR values of the 12 months prior to enrolment. High TTR was defined ≥ 70%, according to the latest ESC guidelines for the antithrombotic therapy in AF patients (Lip et al., 2018).

To assess the role of the POC monitoring on patients' satisfaction, a multiple regression analysis was performed, adjusting for several potential confounding variables. Three models were created, using the questionnaires scores (DASS total score, PACT-Q2 convenience, or PACT-Q2 satisfaction) as dependent variable. The independent variables were age, male sex, living alone, level of education, paid employment, warfarin treatment duration, AF as clinical indication, INR in range at enrolment, high TTR, hospitalisation in the previous year, previous bleeding (self-reported), indirect experience of warfarin side effects (self-reported), experience of unsuitable blood specimens, choice of the Maltese language of the questionnaire, use of the POC for INR monitoring.

A sensitivity analysis was performed by including only those patients in the two groups who fulfilled the initial criteria for switching to the POC INR monitoring (lack of severe diseases; target INR ≤ 3.0; stable INR, defined as at least three consecutive INR values within the therapeutic range 12 months ± 1 month prior to enrolment). These same criteria were followed because it is known that unstable INR or severe comorbidities can influence patients' perception and satisfaction with chronic treatments (Kneeland & Fang, 2010) and therefore could potentially create an imbalance between the two groups. Another sensitivity analysis was performed considering separately the Maltese and the English versions of the PACT-Q2, since

the Maltese translation of the anticoagulant treatment satisfaction subscale of the PACT-Q2 showed lower reliability than the original English version (Chapter 7).

Sample size calculation was based on the following hypothesis. In The Home INR Study (THINRS), patients who performed INR self-testing at home obtained a mean (SD) overall DASS score of 46.8 (16.3) points, while patients who underwent INR testing in clinic obtained a mean (SD) overall DASS score of 49.2 (18.0) points (Matchar et al., 2010). It was hypothesised to detect a difference between the two groups of 5 points, with a SD of 15 units. In order to achieve a power of 80% and a significance level of 0.05, the necessary sample size was 142 patients per group. Therefore, the planned sample size was at least 150 patients per group. For the analysis reported in this chapter, the statistical programs STATA/SE v.12 (StataCorp LP, College Station, TX, USA) and SPSS v.21 (SPSS Inc., Chicago, Illinois, USA) were used.

8.4 Results

8.4.1 Study population

For the POC INR cohort, 174 patients accepted to participate, but only 164 (94.3%) questionnaires were returned. Furthermore, five questionnaires were not included in the analysis because these patients had less than 12 months experience with the POC INR. Therefore, 159 questionnaires were analysed for the POC INR group.

For the laboratory INR cohort, 249 patients accepted to participate, but only 167 (67.1%) questionnaires were returned. Furthermore, 13 questionnaires were not included in the analysis because five patients had less than 12 months experience with the laboratory INR, six patients were actually on POC INR monitoring and they

attended the Anticoagulation Clinic at Mater Dei Hospital only once because of the need of other blood tests, and two patients had a mixed POC and laboratory INR monitoring in the previous 12 months. Therefore, 154 questionnaires were analysed for the laboratory INR group. The flow diagram with details on the number of enrolled patients and analysed questionnaires is reported in Figure 8.1.

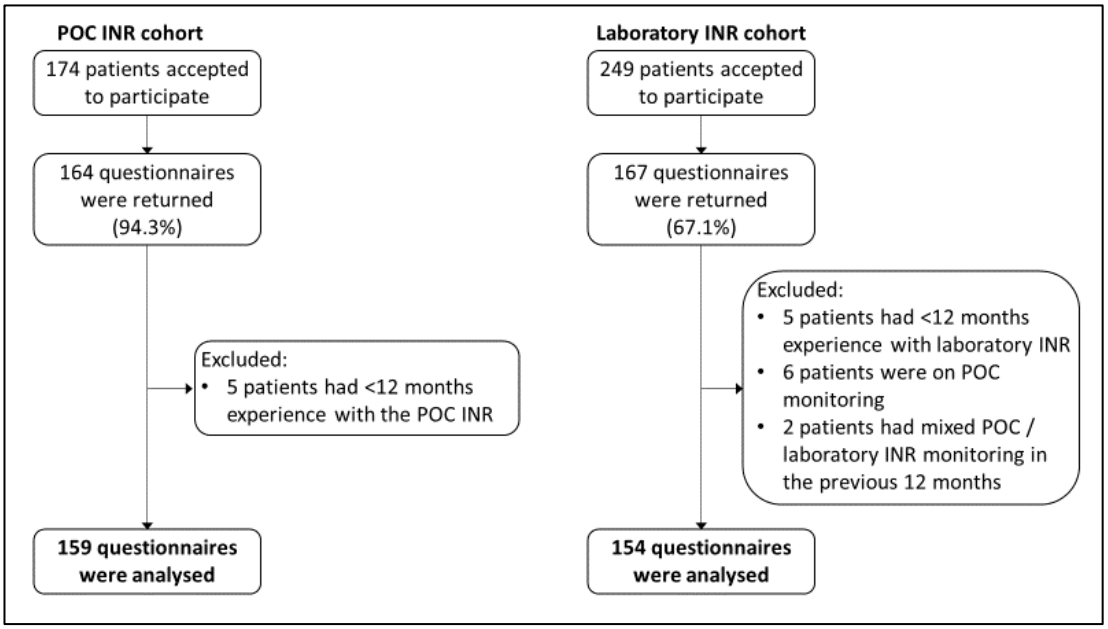


Figure 8.1 Flow chart of the study population selection

The comparison between the two cohorts showed that patients in the POC INR cohort had a predominance of male sex and INR in range on the day of enrolment, longer warfarin treatment duration, higher TTR in the previous year, and more indirect experience of the side effects of the anticoagulant treatment. Patients in the laboratory INR cohort had more hospitalisations in the year prior to enrolment and higher prevalence of English as spoken language, even though there was no difference in the proportion of patients who filled the Maltese versions of the questionnaires in the two

settings. Differences among the two cohorts emerged also in the level of education (Table 8.1).

	POC INR cohort (n=159)	**Laboratory INR cohort (n=154)**	**P value**
Age (years), median (IQR)	72 (65-78)	70.5 (63-76)	0.066
Sex, n (%) • Males • Females	 94 (59.1%) 65 (40.9%)	 71 (46.1%) 83 (53.9%)	0.021
Nationality, n (%) • British • Maltese • Other	 12 (7.6%) 141 (88.7%) 6 (3.8%)	 10 (6.5%) 136 (88.3%) 8 (5.2%)	0.79
Spoken languages*, n (%)			
• English	105 (66.0%)	118 (76.6%)	0.039
• Maltese	146 (91.8%)	139 (90.3%)	0.63
• Other	38 (23.9%)	35 (22.7%)	0.81
Living situation, n (%) • Living alone • Living with family members • Other	 23 (14.5%) 131 (82.4%) 5 (3.1%)	 37 (24.0%) 114 (74.0%) 3 (2.0%)	0.087
Level of Education, n (%) • Primary school • Secondary school • College or vocational school • Graduate or professional school • University degree • Other	 76 (47.8%) 51 (32.1%) 12 (7.6%) 4 (2.5%) 15 (9.4%) 1 (0.6%)	 57 (37.0%) 59 (38.3%) 25 (16.2%) 5 (3.3%) 7 (4.6%) 1 (0.7%)	0.038
Employment status, n (%) • Full-time paid employment • Part-time paid employment • Homemaker/housewife • Retired/pension • Unemployed • Not working due to present health status • Other	 10 (6.3%) 3 (1.9%) 29 (18.2%) 110 (69.2%) 1 (0.6%) 2 (1.3%) 4 (2.5%)	 22 (14.3%) 8 (5.2%) 24 (15.6%) 93 (60.4%) 1 (0.7%) 4 (2.6%) 2 (1.3%)	0.10
Warfarin treatment duration, n (%) • 1 year • >1 year to ≤ 2years • >2 years to ≤ 3years • >3 years to ≤ 4years • >4 years to ≤ 5 years • >5 years	 1 (0.6%) 8 (5.0%) 13 (8.2%) 20 (12.6%) 15 (9.4%) 102 (64.2%)	 12 (7.8%) 23 (14.9%) 23 (14.9%) 32 (20.8%) 14 (9.1%) 50 (32.5%)	<0.001
POC duration, n (%) • 1 year • >1 year to ≤ 2years • >2 years to ≤ 3years • >3 years to ≤ 4years	 3 (1.9%) 40 (25.2%) 93 (58.5%) 23 (14.5%)	 not applicable	

	POC INR cohort (n=159)	Laboratory INR cohort (n=154)	P value
Clinical indication for warfarin*, n (%)			
• AF	111 (69.8%)	93 (60.4%)	0.080
• VTE	22 (13.8%)	28 (18.2%)	0.29
• Heart valve replacement	28 (17.6%)	32 (20.8%)	0.48
• Other	9 (5.7%)	6 (3.9%)	0.60
INR target range, n (%) • 2.0 to 3.0 • 2.5 to 3.5 • Other	 144 (90.6%) 8 (5.0%) 7 (4.4%)	 140 (90.9%) 13 (8.4%) 1 (0.7%)	0.060
INR at enrolment, n (%) • In range • Below range • Above range	 103 (64.8%) 24 (15.1%) 32 (20.1%)	 84 (54.6%) 47 (30.5%) 23 (14.9%)	0.005
TTR in the last 12 months (%)**, median (IQR)	74.3 (62.7-87.5)	71.4 (60.2-81.8)	0.040
High TTR (≥ 70%) in the last 12 months**, n/N (%)	94 (59.5%)	83 (55.7%)	0.50
Hospitalisation in the last 12 months, n (%) • Yes • No • Unknown	 60 (37.7%) 98 (61.6%) 1 (0.6%)	 96 (62.3%) 53 (34.4%) 5 (3.3%)	<0.001
Warfarin prescribed duration (self-reported), n (%) • Limited period of time • Lifelong • I don't know yet	 2 (1.3%) 143 (89.9%) 14 (8.8%)	 1 (0.7%) 131 (85.1%) 22 (14.3%)	0.35
Previous bleeding on warfarin (self-reported), n (%) *"Have you ever had any bruise or bleeding while you were taking warfarin?"* • Yes • No	 50 (31.5%) 109 (68.6%)	 55 (35.7%) 99 (64.3%)	0.42
Indirect experience of warfarin side effects (self-reported), n (%) *"Do you know someone who has had side effects from blood-thinning medications?"* • Yes • No	 17 (10.7%) 142 (89.3%)	 6 (3.9%) 148 (96.1%)	0.021
Experience of unsuitable blood specimens, n (%) • Yes • No	 60 (37.7%) 99 (62.3%)	 65 (42.2%) 89 (57.8%)	0.42
Language of the questionnaires, n (%) • English • Maltese	 76 (47.8%) 83 (52.2%)	 74 (48.1%) 80 (52.0%)	0.96

Table 8.1 Baseline characteristics of the study population

*more than one option was possible ** data available only in 158 patients in the POC INR group and 149 patients in the laboratory INR group

8.4.2 Patients' satisfaction in the two cohorts

Patients in the POC INR cohort were more satisfied than patients in the laboratory INR cohort, as shown by a statically significant lower score in the overall DASS and in the subscales hassles/burdens and psychological impact ($p<0.001$ for all comparisons). They also had statistically significant higher scores in the PACT-Q2 subscales convenience ($p<0.001$) and anticoagulant treatment satisfaction ($p=0.039$). Since the results were not normally distributed, they were compared using the Mann-Whitney U test (Table 8.2); however, mean (SD) values were also calculated, in order to allow the comparison with previous cohort studies that reported only mean values.

	POC INR cohort (n=159)		**Laboratory INR cohort (n=154)**		**Mann-Whitney U test: p value**
	Mean (SD)	**Median (IQR)**	**Mean (SD)**	**Median (IQR)**	
DASS results					
• Sections 1 and 2: limitations	16.4 (8.0)	13 (11-19)	17.7 (9.6)	14 (11-21)	0.47
• Section 3: hassles and burdens	13.6 (6.7)	11 (9-16)	17.9 (7.9)	16 (12-23)	<0.001
• Section 4: psychological impact	20.7 (6.7)	20 (16-24)	23.8 (6.7)	24 (19-28)	<0.001
• Overall DASS	50.8 (17.1)	47 (39-60)	59.4 (18.0)	56 (47-66)	<0.001
PACT-Q2 results					
• Sections B and C: convenience	85.9 (13.8)	88.5 (80.8-96.2)	79.8 (16.2)	82.7 (73.1-90.4)	<0.001
• Section D: anticoagulant treatment satisfaction	67.6 (11.8)	64.3 (60.7-75.0)	65.2 (14.5)	64.3 (57.1-75.0)	0.039

Table 8.2 Results of the DASS and PACT-Q2 questionnaires in the two groups of patients

8.4.3 Detailed results of the DASS questionnaire

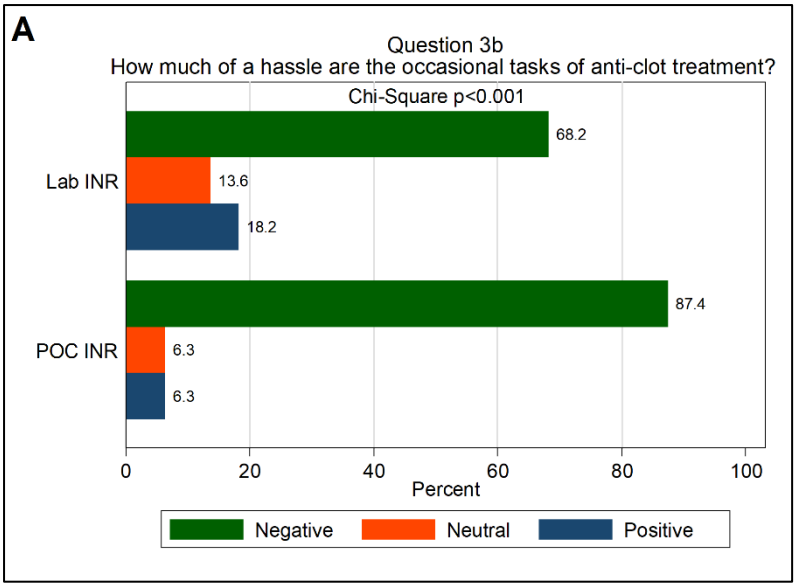
The comparison between the two study cohorts showed significant differences in the median scores obtained in the following questions of the DASS questionnaire (Table 8.3):

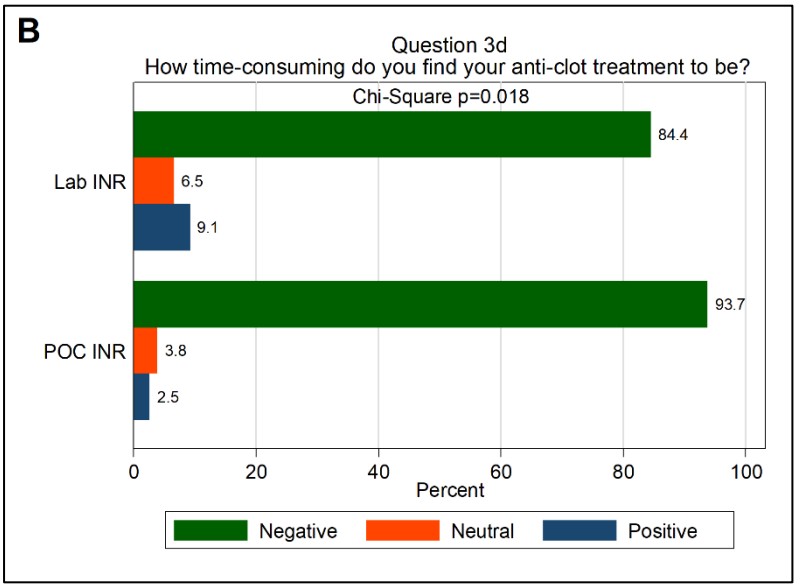
- 2d "Overall, how much does anti-clot treatment affect your daily life?" ($p=0.014$);
- 3a "How much of a hassle (inconvenience) are the daily tasks of anti-clot treatment?" ($p=0.005$);
- 3b "How much of a hassle (inconvenience) are the occasional tasks of anti-clot treatment?" ($p<0.001$);
- 3d "How time-consuming do you find your anti-clot treatment to be?" ($p<0.001$);
- 3e "How frustrating do you find your anti-clot treatment to be?" ($p<0.001$);
- 3f "How painful do you find your anti-clot treatment to be?" ($p<0.001$);
- 3g "Overall, how much of a burden do you find your anti-clot treatment to be?" ($p<0.001$);
- 3h "Overall, how confident are you about handling your anti-clot treatment?" ($p=0.014$);
- 4a "How well do you feel that you understand the medical reason for your anti-clot treatment?" ($p=0.003$);
- 4b "How much do you feel reassured because of your anti-clot treatment?" ($p<0.001$);
- 4d "How much do you worry about bleeding and bruising?" ($p=0.003$);
- 4f "Overall, how much has anti-clot treatment had a positive impact on your life?" ($p=0.004$);
- 4i "Compared with other treatments you have had, how difficult is your anti-clot treatment to manage?" ($p=0.005$).

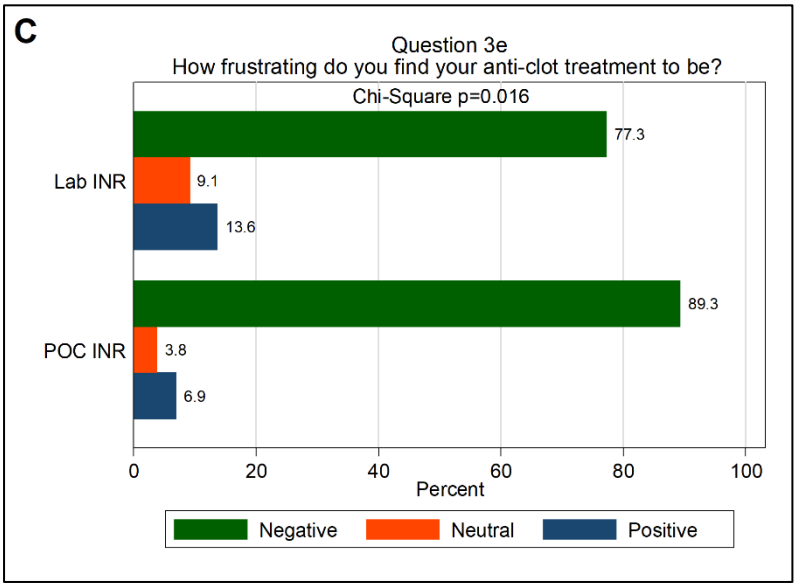
DASS item	POC INR cohort (n=159)		Laboratory INR cohort (n=154)		Mann-Whitney U test: p value
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Limitations subscale					
1a	1.6 (1.3)	1 (1-1)	1.7 (1.4)	1 (1-2)	0.19
1b	1.5 (1.1)	1 (1-1)	1.6 (1.3)	1 (1-2)	0.19
1c	2.0 (1.5)	1 (1-3)	2.0 (1.6)	1 (1-2)	0.63
1d	1.5 (1.1)	1 (1-1)	1.5 (1.3)	1 (1-1)	0.85
1e	1.6 (1.2)	1 (1-2)	1.7 (1.4)	1 (1-2)	0.63
2a	2.3 (1.5)	2 (1-3)	2.3 (1.5)	2 (1-3)	0.71
2b	1.8 (1.4)	1 (1-2)	2.1 (1.7)	1 (1-2)	0.21
2c	2.5 (1.8)	2 (1-3)	2.7 (2.0)	2 (1-4)	0.46
2d	1.8 (1.3)	1 (1-2)	2.1 (1.5)	2 (1-3)	0.014
Hassles and burdens subscale					
3a	1.6 (1.1)	1 (1-2)	2.1 (1.6)	1 (1-2)	0.005
3b	1.8 (1.3)	1 (1-2)	2.8 (1.8)	2 (1-4)	<0.001
3c	1.6 (1.2)	1 (1-2)	1.9 (1.3)	1 (1-2)	0.065
3d	1.4 (1.0)	1 (1-1)	2.0 (1.4)	1 (1-2)	<0.001
3e	1.7 (1.3)	1 (1-2)	2.3 (1.6)	2 (1-3)	<0.001
3f	1.3 (0.9)	1 (1-1)	1.7 (1.2)	1 (1-2)	<0.001
3g	1.7 (1.3)	1 (1-2)	2.4 (1.5)	2 (1-3)	<0.001
3h *	2.6 (1.7)	2 (1-3)	2.8 (1.6)	2 (2-3)	0.014
Psychological impact subscale					
4a *	2.7 (1.5)	2 (2-3)	3.1 (1.5)	3 (2-4)	0.003
4b *	2.5 (1.2)	2 (2-3)	3.1 (1.5)	3 (2-4)	<0.001
4d	2.9 (1.9)	2 (1-5)	3.5 (1.8)	4 (2-5)	0.003
4f *	3.9 (2.0)	4 (2-6)	4.5 (2.1)	5 (3-7)	0.004
4g	2.1 (1.5)	1 (1-3)	2.2 (1.6)	2 (1-3)	0.55
4h *	2.3 (1.2)	2 (2-3)	2.5 (1.2)	2 (2-3)	0.064
4i	1.6 (1.1)	1 (1-2)	2.1 (1.5)	1 (1-3)	0.005
4j *	2.7 (1.7)	2 (2-3)	2.8 (1.5)	2 (2-4)	0.28

Table 8.3 Results of each DASS item in the two cohorts
For each questions the answers can range from 1 to 7. * Items were reverse coded prior to the analysis

In a further analysis, results were divided into three categories: negative (if the answers were “not at all”, “a little”, “somewhat”), neutral (if the answer was “moderately”), positive (if the answers were “quite a bit”, “a lot”, “very much”). For this analysis the original answers, not reverse coded, were considered. Results that were statistically significant are reported in the Figures 8.2-8.3.


A
Question 3b
How much of a hassle are the occasional tasks of anti-clot treatment?
Chi-Square p<0.001
Lab INR
POC INR
68.2
13.6
18.2
87.4
6.3
6.3
0
20
40
60
80
100
Percent
Negative
Neutral
Positive


B
Question 3d
How time-consuming do you find your anti-clot treatment to be?
Chi-Square p=0.018
Lab INR
POC INR
84.4
6.5
9.1
93.7
3.8
2.5
0
20
40
60
80
100
Percent
Negative
Neutral
Positive


C
Question 3e
How frustrating do you find your anti-clot treatment to be?
Chi-Square p=0.016
Lab INR
POC INR
77.3
9.1
13.6
89.3
3.8
6.9
0
20
40
60
80
100
Percent
Negative
Neutral
Positive

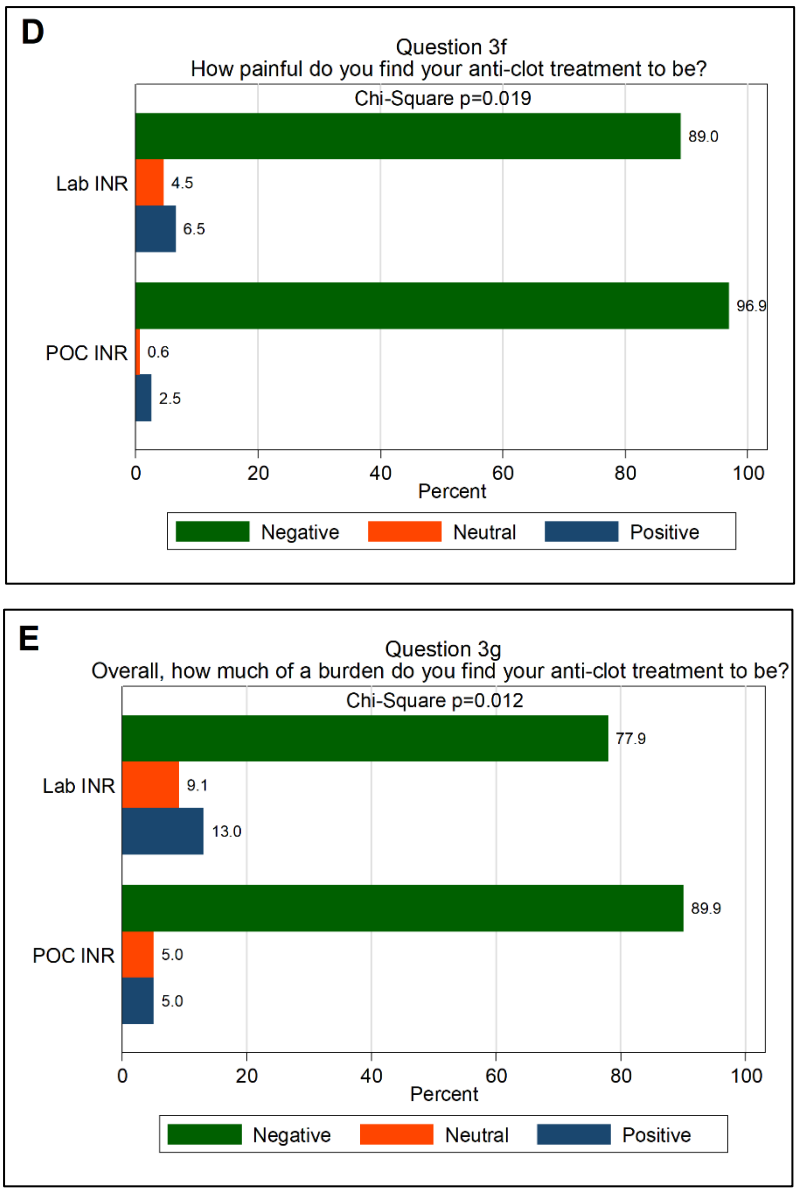
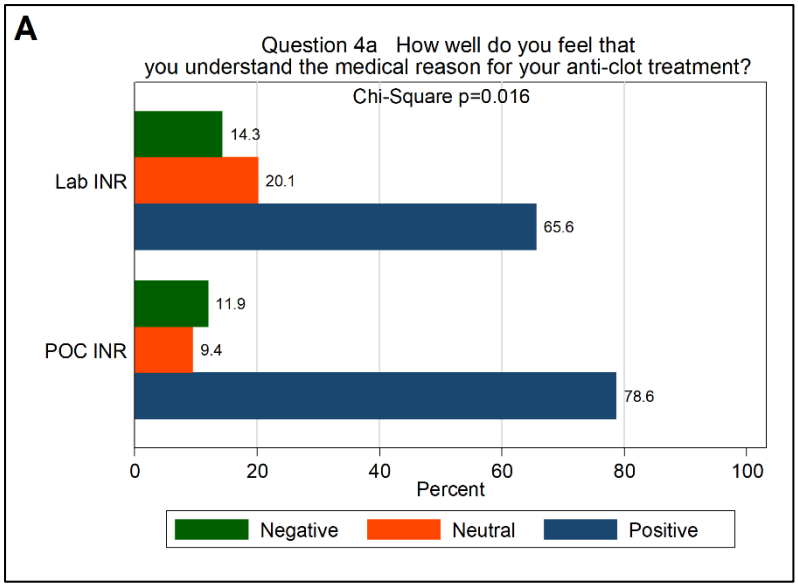
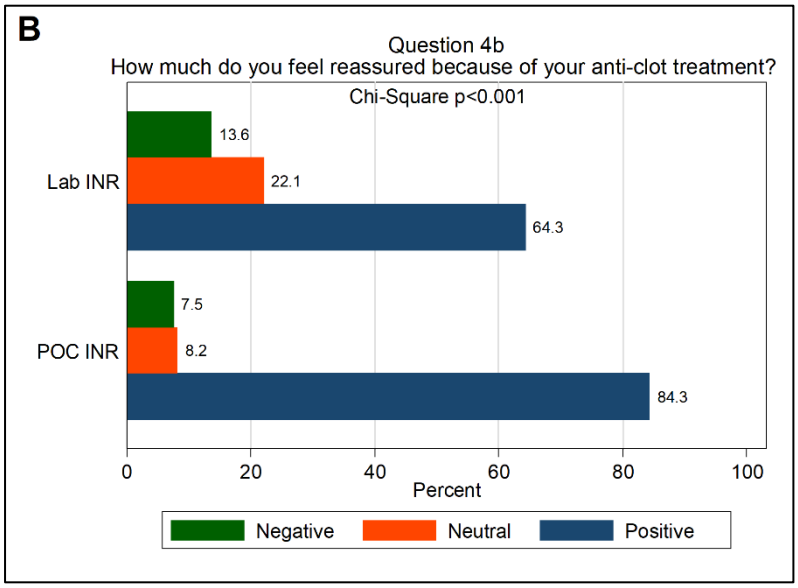
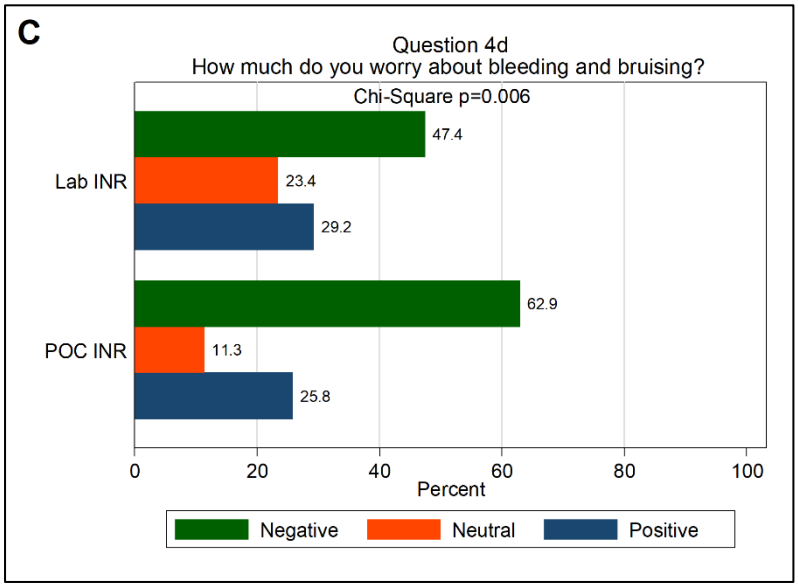


Figure 8.2 Categorical answers to the DASS items of the hassles and burdens subscale


A
Question 4a How well do you feel that
you understand the medical reason for your anti-clot treatment?
Chi-Square p=0.016
Lab INR
POC INR
14.3
20.1
65.6
11.9
9.4
78.6
0
20
40
60
80
100
Percent
Negative
Neutral
Positive


B
Question 4b
How much do you feel reassured because of your anti-clot treatment?
Chi-Square p<0.001
Lab INR
POC INR
13.6
22.1
64.3
7.5
8.2
84.3
0
20
40
60
80
100
Percent
Negative
Neutral
Positive


C
Question 4d
How much do you worry about bleeding and bruising?
Chi-Square p=0.006
Lab INR
POC INR
47.4
23.4
29.2
62.9
11.3
25.8
0
20
40
60
80
100
Percent
Negative
Neutral
Positive

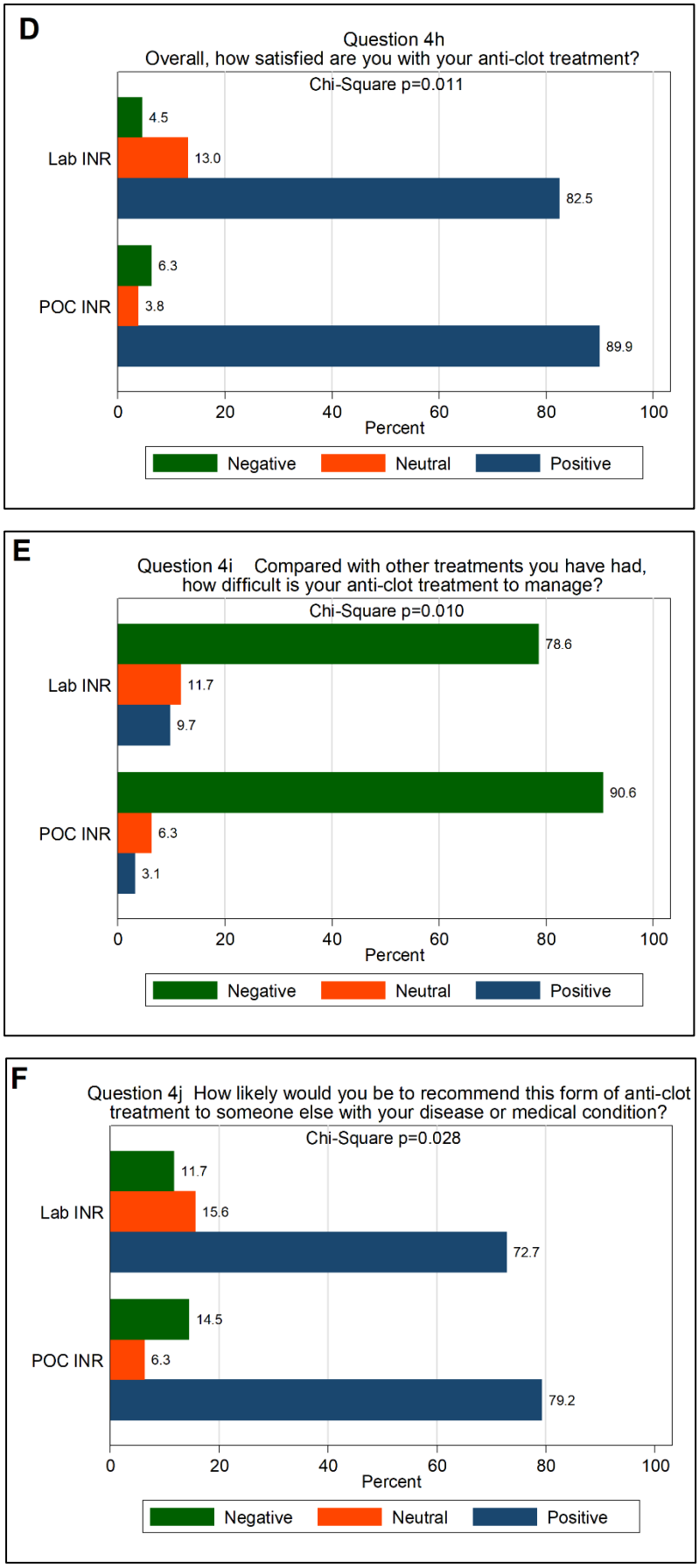


Figure 8.3 Categorical answers to the DASS items of the psychological impact subscale

8.4.4 Detailed results of the PACT-Q2 questionnaire

The comparison between the two study cohorts showed significant differences in the median scores obtained in the following questions of the PACT-Q2 questionnaire (Table 8.4):

- B2 “How bothered are you by taking your anticoagulant treatment?” (p=0.001);
- B4 “Certain medications cannot be taken with anticoagulant treatments; how difficult is this for you?” (p=0.023);
- B5 “It is recommended that certain foods be avoided while taking an anticoagulant treatment; how difficult is this for you?” (p=0.007);
- B6 “How difficult is it for you to take your anticoagulant treatment when you are away from home?” (p=0.019);
- B7 “How difficult is it for you to plan your time around your anticoagulant treatment (i.e., appointments with nurses, doctors or labs …)?” (p<0.001);
- B8 “How bothered are you by the medical follow-up required with your anticoagulant treatment?” (p=0.001);
- B9 “How difficult is it for you to take your anticoagulant treatment as directed on a regular basis?” (p=0.038);
- B11 “How worried are you about having to interrupt or stop your anticoagulant treatment?” (p<0.001);
- D1 “How reassured do you feel by your anticoagulant treatment?” (p<0.001);
- D2 “Do you feel that your anticoagulant treatment has decreased your symptoms (i.e., leg pain or swelling, palpitations, shortness of breath, or chest pain…)?” (p=0.003);

- D5 “How satisfied are you with the methods (i.e., appointments with nurses, doctors, labs…) used to ensure the follow-up of your disease and anticoagulant treatment?” ($p<0.001$);
- D7 “Overall, how satisfied are you with your anticoagulant treatment?” ($p=0.006$).

PACT-Q2 item	POC INR cohort (n=159)		Laboratory INR cohort (n=154)		Mann-Whitney U test: p value
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	
Convenience subscale					
B1 *	4.7 (0.7)	5 (5-5)	4.5 (0.9)	5 (4-5)	0.10
B2 *	4.6 (0.8)	5 (5-5)	4.3 (1.1)	5 (4-5)	0.001
B3 *	4.4 (0.9)	5 (4-5)	4.3 (1.0)	5 (4-5)	0.16
B4 *	4.1 (1.2)	5 (3-5)	3.8 (1.2)	4 (3-5)	0.023
B5 *	4.2 (1.1)	5 (3-5)	3.9 (1.1)	4 (3-5)	0.007
B6 *	4.6 (0.8)	5 (5-5)	4.4 (1.1)	5 (4-5)	0.019
B7 *	4.6 (0.8)	5 (4-5)	4.2 (1.1)	5 (4-5)	<0.001
B8 *	4.6 (0.8)	5 (4-5)	4.3 (1.0)	5 (4-5)	0.001
B9 *	4.8 (0.7)	5 (5-5)	4.6 (0.8)	5 (5-5)	0.038
B10 *	4.5 (1.0)	5 (4-5)	4.4 (1.1)	5 (4-5)	0.34
B11 *	3.7 (1.4)	4 (2-5)	3.1 (1.2)	3 (2-4)	<0.001
C1 *	4.6 (0.8)	5 (4-5)	4.5 (1.0)	5 (4-5)	0.51
C2 *	4.4 (1.0)	5 (4-5)	4.3 (0.9)	5 (4-5)	0.071
Anticoagulant treatment satisfaction subscale					
D1	3.9 (0.9)	4 (4-4)	3.6 (1.0)	4 (3-4)	<0.001
D2	2.0 (1.3)	1 (1-3)	2.5 (1.5)	2 (1-4)	0.003
D3	3.5 (0.9)	3 (3-4)	3.4 (0.9)	3 (3-4)	0.39
D4	4.0 (0.7)	4 (4-4)	3.9 (0.7)	4 (4-4)	0.13
D5	4.1 (0.7)	4 (4-5)	3.8 (0.8)	4 (3-4)	<0.001
D6	4.1 (0.5)	4 (4-4)	4.0 (0.6)	4 (4-4)	0.16
D7	4.2 (0.5)	4 (4-4)	4.0 (0.6)	4 (4-4)	0.006

Table 8.4 Results of each PACT-Q2 item in the two cohorts
For each questions the answers can range from 1 to 5. * Items were reverse coded prior to the analysis

In a further analysis, results were also divided into three categories: negative (if the answers were “not at all”, “a little”), neutral (if the answer was “moderately”), positive (if the answers were “a lot”, “extremely”). For this analysis the original answers, not reverse coded, were considered. Results that were statistically significant are reported in the Figures 8.4-8.5.

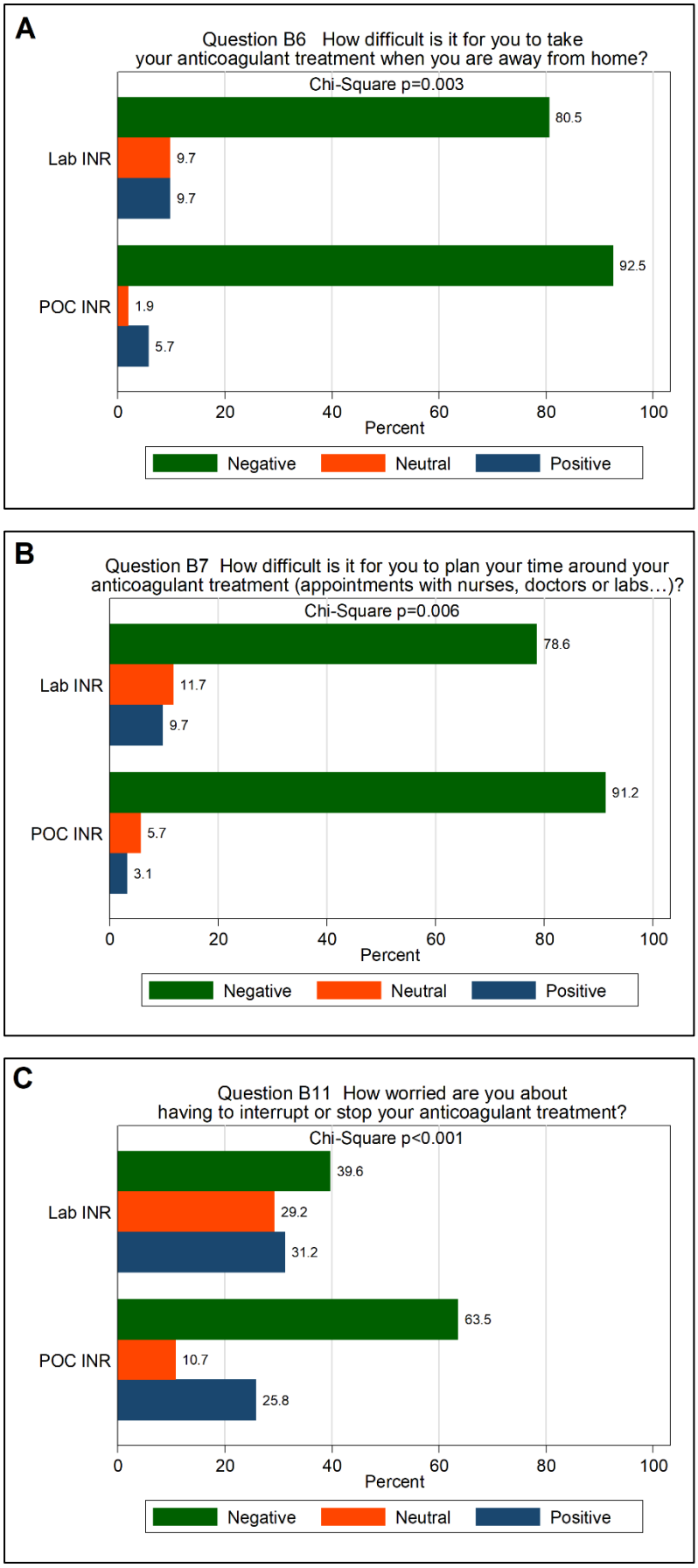


Figure 8.4 Categorical answers to the PACT-Q2 items of the convenience subscale

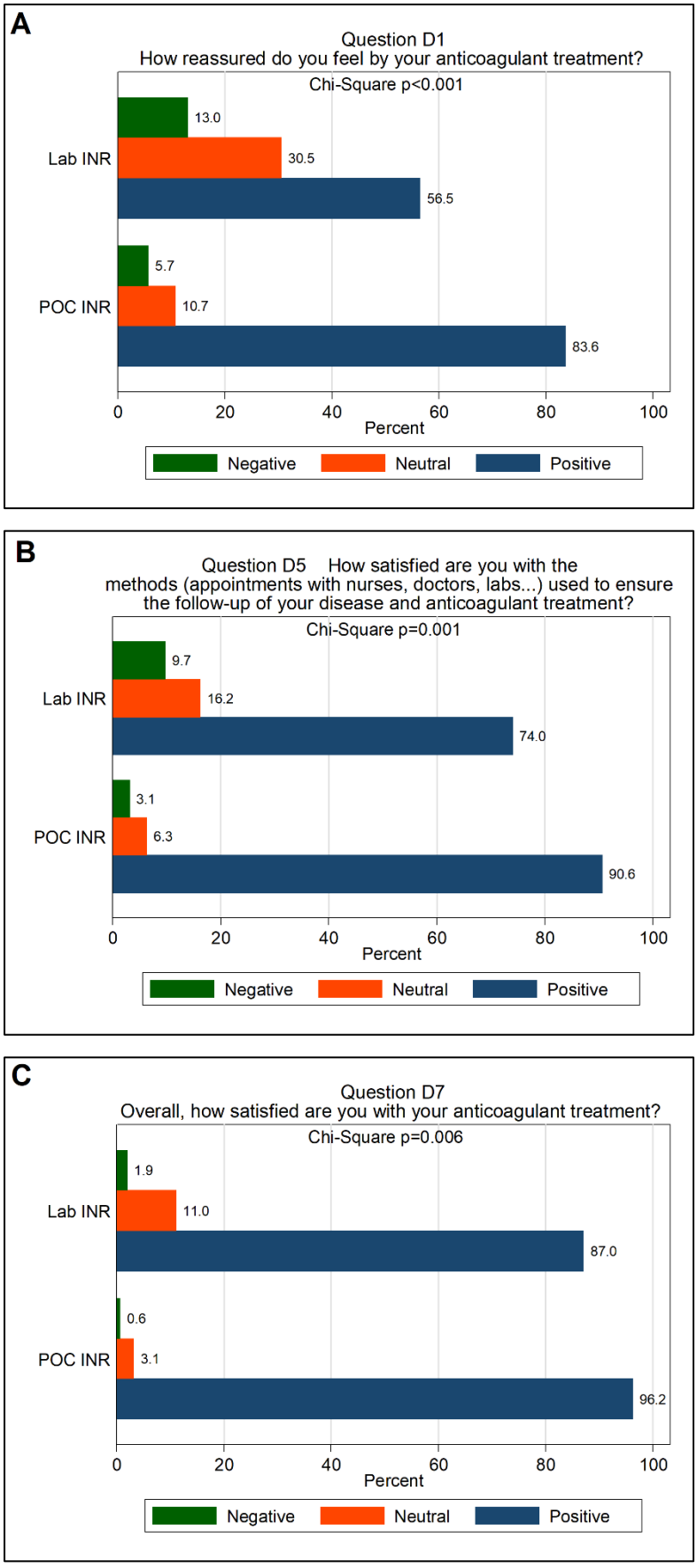


Figure 8.5 Categorical answers to the PACT-Q2 items of the anticoagulant treatment satisfaction subscale

8.4.5 Contribution of the POC monitoring to patients' satisfaction

Three different multiple regression models were created to confirm whether the POC monitoring significantly contributed to patients' satisfaction (Table 8.5). After adjusting for potential confounding variables, the use of the POC devices remained significantly associated with the DASS total score (beta coefficient = -0.156, p=0.013) and the PACT-Q2 convenience score (beta coefficient = 0.161, p=0.012), thus confirming the beneficial effect of the POC in reducing the hassles and burdens associated with warfarin treatment.

	DASS total score: beta coefficient (p value)	**PACT-Q2 convenience score: beta coefficient (p value)**	**PACT-Q2 satisfaction score: beta coefficient (p value)**
Age	-0.130 (0.055)	0.164 (0.019)	0.081 (0.24)
Male sex	-0.128 (0.033)	0.119 (0.052)	0.041 (0.51)
Living alone	0.005 (0.93)	0.005 (0.93)	-0.023 (0.69)
Level of education	-0.072 (0.28)	0.089 (0.19)	0.224 (0.001)
Paid employment (full-time or part-time)	0.071 (0.28)	-0.102 (0.13)	-0.099 (0.14)
Warfarin treatment duration	-0.051 (0.39)	-0.062 (0.31)	0.061 (0.32)
AF as clinical indication for warfarin	0.018 (0.77)	-0.085 (0.18)	-0.038 (0.55)
INR in range at enrolment	0.025 (0.65)	0.016 (0.78)	-0.089 (0.13)
High TTR (≥ 70%) in the previous year	0.032 (0.58)	-0.001 (0.99)	0.012 (0.84)
Hospitalisation in the previous year	0.065 (0.26)	-0.060 (0.31)	0.065 (0.27)
Previous bleeding on warfarin (self-reported)	0.186 (0.002)	-0.127 (0.033)	-0.239 (<0.001)
Indirect experience of warfarin side effects (self-reported)	0.003 (0.96)	-0.006 (0.92)	0.056 (0.33)
Experience of unsuitable blood specimens	0.119 (0.031)	-0.034 (0.55)	-0.046 (0.41)
Maltese language of the questionnaire	0.028 (0.63)	-0.009 (0.88)	0.004 (0.95)
Use of the POC for INR monitoring	-0.156 (0.013)	0.161 (0.012)	0.060 (0.35)

Table 8.5 Results of the multiple regression analysis

8.4.6 Sensitivity analyses

Overall, 196 patients were included in the sensitivity analysis of patients fulfilling the initial criteria for switching to the POC INR monitoring: 114 for the POC INR cohort and 82 for the laboratory INR cohort. Reasons for exclusion were: severe diseases (five patients in the POC INR cohort and eight patients in the laboratory INR cohort); higher target INR (one patient in each group); unstable INR (39 patients in the POC INR cohort and 63 patients in the laboratory INR cohort). Results of the sensitivity analysis confirmed the results of the main analysis, with the exception of the anticoagulant treatment satisfaction subscale of the PACT-Q2, which was not statistically significant (Table 8.6).

	POC INR cohort (n=114)	**Laboratory INR cohort (n=82)**	**P value**
DASS scores, median (IQR)			
• Sections 1 and 2: limitations	13 (11-19)	14 (11-21)	0.56
• Section 3: hassles/burdens	11 (9-16)	16 (12-21)	<0.001
• Section 4: psychological impact	20 (16-24)	23 (19-27)	0.001
• Overall DASS	46.5 (38-60)	55.5 (46-64)	<0.001
PACT-Q2 scores, median (IQR)			
• Sections B and C: convenience	88.5 (80.8-94.2)	82.7 (75.0-90.4)	0.005
• Section D: anticoagulant treatment satisfaction	64.3 (60.7-75.0)	64.3 (57.1-75.0)	0.43

Table 8.6 Results of the DASS and the PACT-Q2 questionnaires in the sensitivity analysis of patients fulfilling the initial criteria for POC switching

Another sensitivity analysis was performed considering separately the Maltese and the English versions of the PACT-Q2. For both languages, the POC INR cohort obtained better scores in the convenience subscale, while the anticoagulant treatment satisfaction subscale was not statistical significant when considering only the English

version (Table 8.7) Taken together, the results of the sensitivity analyses suggested that the POC monitoring has a greater impact in improving the convenience, while the overall anticoagulant treatment satisfaction might be influenced also by other variables.

	POC INR cohort	Laboratory INR cohort	P value
Maltese version of the PACT-Q2			
• Patients, n	83	80	
• Sections B and C: convenience score, median (IQR)	88.5 (78.8-94.2)	84.6 (73.1-90.4)	0.020
• Section D: anticoagulant treatment satisfaction score, median (IQR)	64.3 (60.7-71.4)	60.7 (57.1-71.4)	0.041
English version of the PACT-Q2			
• Patients, n	76	74	
• Sections B and C: convenience score, median (IQR)	88.5 (80.8-98.1)	82.7 (71.2-90.4)	<0.001
• Section D: anticoagulant treatment satisfaction score, median (IQR)	67.9 (60.7-75.0)	64.3 (57.1-78.6)	0.35

Table 8.7 Separate analysis of the Maltese and English versions of the PACT-Q2

8.5 Discussion

This study compared patients' satisfaction associated with two different ways of INR monitoring: the standard laboratory INR vs. the use of POC coagulometers by healthcare professionals in anticoagulation clinics. The POC INR group was found to be more satisfied, as represented by the scores obtained in the overall DASS, in the hassles/burdens and psychological impact subscales, and in the convenience and anticoagulant treatment satisfaction subscales of the PACT-Q2.

The POC coagulometers represent an accurate and effective alternative to the standard INR monitoring. Several studies reported an overall good accuracy of the POC devices

when compared to the standard laboratory INR or to global coagulation assays (Donaldson et al., 2010; Meneghelo et al., 2015; Plesch & van den Besselaar, 2009; Riva et al., 2017). Two recent meta-analyses reported that PST and PSM are associated with a reduction of thromboembolic complications (Bloomfield et al., 2011; Heneghan et al., 2012). Furthermore, the possibility of self-testing at home was greatly appreciated by the patients. The THINRS study randomized 2922 patients to clinic-testing or POC self-testing, and reported higher satisfaction in the latter, with a difference of -2.4 points in the overall DASS score (p=0.002) at 2-year follow-up (Matchar et al., 2010). Another randomized trial showed that patients in the self-management group, compared to patients in the standard INR management group, had an increase in the "general treatment satisfaction" and a decrease in the "daily hassles" and in the "psychological distress" (Verret et al., 2012). Furthermore, a recent study reported that among 92 patients switched to POC self-testing at home 85 (92%) were "much" or "completely" satisfied by the use of POC coagulometers, while only 36 (39%) were "much" or "completely" satisfied by INR monitoring at the thrombosis centre (Barcellona et al., 2018). While the use of POC devices for self-testing at home can reduce the hassles associated with long travelling and waiting time, there are contexts in which the POC coagulometers are used by healthcare professionals in anticoagulation clinics (Zammit et al., 2011). However, it is still unclear whether the same degree of satisfaction is associated with the use of POC devices in these contexts. A small study compared 30 VKA patients monitored with the standard laboratory INR (vein-testing group) with 46 patients assigned to the POC INR monitoring (POC-testing group), the latter consisting mainly of patients with physical disabilities, difficult venous access, tight working schedule or long distance travelling (Gafou et al., 2007). The DASS was translated into Greek and culturally adapted, therefore

resulting in 27 questions with six possible answers. No statistically significant difference was found in neither the DASS total score (vein-testing 71.05 vs. POC-testing 72.37, p=0.738), nor in the DASS subscales or the single DASS items (Gafou et al., 2007). However, the number of patients that could be evaluated for the DASS total score was influenced by the high number of questionnaires with at least one missing item (33%).

In the present study patients' satisfaction associated with different INR monitoring strategies was compared through the use of two psychometric questionnaires and an adequately powered sample size. It was found that the overall satisfaction was higher in the POC INR cohort, but also that the hassles/burdens were lower (on the specific DASS subscale) and that the convenience was higher (on the specific PACT-Q2 subscale). Although the population characteristics were not completely balanced between the two study cohorts, the sensitivity analyses confirmed the results of the primary analysis. Furthermore, the multiple regression models for the DASS total score and the PACT-Q2 convenience confirmed the important contribution of POC monitoring to patients' satisfaction and convenience associated with the anticoagulant treatment. Other factors positively associated with patients' satisfaction were male sex and increasing age, while previous bleeding and previous experience of unsuitable blood specimens was negatively associated. Conversely, the fact of having a stable anticoagulation control, expressed by the high TTR, did not appear to impact on patients' satisfaction. Negative perception of the QoL has already been reported in the literature in patients of young age (Almeida et al., 2011) and with previous bleeding episodes (Lancaster et al., 1991). A number of studies highlighted that women have a lower health-related QoL (Casais et al., 2005; Cherepanov et al., 2010; Corbi et al., 2011; Hajian-Tilaki et al., 2017) and several explanations were provided. Differences

in sociodemographic and socioeconomic status (such as education or income) might play a role (Cherepanov et al., 2010), as well as the higher prevalence in women of anxiety disorders (Pigott, 2003), which might have resulted in increased fear of possible anticoagulation-related complications. Furthermore, it has been reported that men give less importance to healthcare (Corbi et al., 2011) and have a lower health-care seeking behaviour (A. E. Thompson et al., 2016).

The main strengths of this study are the use of psychometric questionnaires that were rigorously translated and validated (Chapter 7) and the completeness of data, without any missing answers. Furthermore, the patients were enrolled from different locations in the Maltese island, therefore this sample is likely to be generalisable to the overall Maltese anticoagulated population.

However, this study has also some limitations that need to be acknowledged. First, there was a different response rate in the two cohorts (94.3% of questionnaires returned in the POC INR group vs. 67.1% in the laboratory INR group). The high response rate in this study could be partly due to the fact that it was calculated on the number of patients who accepted to participate and partly to the fact that face-to-face recruitment was used (Sitzia & Wood, 1998). However, for the laboratory INR group a larger number of patients was approached and recruited, in order to reach the planned sample size for the analysis of the questionnaires. This can be partly explained by the busy and crowded context of the Anticoagulation Clinic at Mater Dei Hospital. Whether the non-response rate could have influenced these results, it is a matter for debate, since it has been reported that non-respondents are less likely to be satisfied (Kelley et al., 2003) and the laboratory INR group was less satisfied than the POC INR group. Second, a selection bias cannot be completely excluded, since the initial local protocol for POC switching included only patients without severe comorbidities.

However, the sensitivity analysis of patients fulfilling the initial criteria for switching confirmed higher convenience and less hassles/burdens of the POC monitoring. Third, some variables which could have influenced patients' satisfaction were not available, such as journey time or waiting time. Furthermore, although differences in the baseline characteristics of the two study groups could have influenced the degree of satisfaction, the important role of the POC monitoring was confirmed in the regression models after adjusting for other variables. Finally, the cross-sectional design did not allow to evaluate changes in patients' satisfaction over time.

8.6 Conclusion

The results of this study suggested that the use of POC coagulometers by healthcare professionals in anticoagulation clinics, together with a dedicated time slot and immediate warfarin dose adjustment, is associated with a better QoL for anticoagulated patients. These findings are particularly relevant in the local context, as a feedback for the recently introduced POC system, but also in the international literature. In fact, the availability of instant INR results can allow the immediate management of patients with extremely out-of-range values or patients needing an interventional procedure.

Chapter 9 :

Anticoagulation Control with the Point-of-care INR

9.1 Introduction

VKA need to be monitored with the INR, which represents the intensity of the anticoagulation. Their dosage is periodically adjusted in order to maintain the INR within an established therapeutic range, derived from the balance between the prevention of thromboembolic events and the avoidance of haemorrhagic complications. The TTR, which is the proportion of time spent within the INR therapeutic range, is an indirect measure of the anticoagulation control. In fact, it is known to correlate with the incidence of thromboembolic and bleeding events (Björck et al., 2016; S. Haas et al., 2016; Wan et al., 2008). In order to maximise the benefit of the VKA, the TTR should be ≥ 70% (Lip et al., 2018). Furthermore, increasing TTR is associated with increasing benefit, which is particularly evident in patients with TTR ≥ 80% (Lehto et al., 2017). For these reasons, a TTR < 65% is considered "poor anticoagulation control" (National Institute for Health and Care Excellence, 2014b) and for these patients the 2018 ACCP guidelines recommend interventions to improve the TTR (such as more frequent testing, reviewing adherence or counselling) or switching to a DOAC (Lip et al., 2018).

The use of POC coagulometers for INR self-testing was associated with a 42% relative risk reduction of major thromboembolic events and a 26% relative risk reduction of mortality (Bloomfield et al., 2011). Better TTR was also reported in the self-testing group, compared to the clinic-testing group (Matchar et al., 2010). However, whether the same effect applies to the use of POC coagulometers by healthcare professionals is still a matter of debate. A recently published retrospective analysis of more than 1900 patients reported that the TTR was significantly lower during POC INR monitoring vs. laboratory INR monitoring (Biedermann et al., 2016). However, in this research two independent cohorts of patients constituted the study population and the

INR target ranges were wider than internationally recommended (Biedermann et al., 2016).

9.2 Aim

The aim of this study was to compare the anticoagulation control, expressed as TTR, associated with these two different ways of monitoring VKA treatment: the POC INR monitoring and the standard laboratory INR monitoring.

9.3 Methods

9.3.1 Study population

In Malta, the POC INR monitoring was started in May 2014 at the Anticoagulation Clinics of different Health Centres spread around the island. The first Health Centre to adopt this new system was Rabat Health Centre (RHC), followed by Qormi Health Centre (QHC), Birkirkara Health Centre (BKHC), Paola Health Centre (PHC), and Floriana Health Centre (FHC) in 2014; Cospicua Health Centre (CHC) and Gzira Health Centre (GHC) in 2015. The Health Centre in the sister island Gozo was also started in the second half of 2015, while more Health Centres have been activated in the following years. In these Anticoagulation Clinics, POC INR testing is usually performed by nurses, immediately followed by warfarin dose adjustment by the attending general practitioners. In the local context patients are usually started on standard laboratory INR monitoring at the Anticoagulation Clinic at Mater Dei Hospital, and subsequently switched to a POC INR Anticoagulation Clinic.

Inclusion criteria for this study were consecutive adult patients on VKA treatment attending the POC INR Anticoagulation Clinics at seven Health Centres (RHC, QHC,

BKHC, PHC, FHC, CHC, GHC) in the years 2014-2015. Patients with less than two POC INR measurements and with INR target ranges different from the most commonly used ranges 2.0-3.0 or 2.5-3.5 were excluded.

9.3.2 Study design

The study design was an observational retrospective cohort study. From a retrospective review of clinical notes and laboratory test results, the following information were retrieved: the number of INR tests, the INR values, the indication for anticoagulation, the target INR range, and the number of hospital admissions. Follow-up was started on the day of the first POC INR and concluded at the end of December 2017. Furthermore, three time frames were considered for each patient (where available):

1) The first 12 months with the POC INR monitoring ("initial-POC" period);
2) The last 12 months with the standard laboratory INR monitoring ("pre-POC" period);
3) All the results of the year 2017 ("stable-POC" period).

This study was approved by the University of Malta Research and Ethics Committee (protocol 07/2016, Appendix B). The need for informed consent was waived, due to the observational design of this study.

9.3.3 INR measurements

For the determination of the POC INR, the coagulometer CoaguChek XS Plus (Roche Diagnostics International Ltd, Germany) was used. For the determination of the laboratory INR until December 2015, the INR was performed using the automated coagulation analysers Sysmex CS-2100i or CA-1500 (Siemens Healthcare

Diagnostics Products GmbH, Germany) and the Dade® Innovin® reagent (Siemens Healthcare Diagnostics Products GmbH, Germany).

9.3.4 Statistical analysis

Continuous variables were expressed as mean (SD) or median (IQR), according to data distribution. Categorical variables were expressed as counts and percentages. The TTR was calculated according to the Rosendaal method (Rosendaal et al., 1993), considering only the INR performed as outpatients. The time during hospitalisations was removed from the TTR calculation (except for access to Accident and Emergency Department only) to avoid possible VKA interruptions or switching to parenteral anticoagulation, which could not be identified elsewhere. If more than one outpatient INR test was performed on the same day, only the first INR was included in the TTR calculation. The overall POC-TTR was considered from the first POC INR, until the end of December 2017, death or switching to laboratory INR, whichever came first.

Furthermore, a comparison among the three time frames was performed: the initial-POC period, the pre-POC period, and the stable-POC period. In this comparison only patients with all three time frames available were included (e.g. at least 12 months of laboratory INR monitoring before switching to the POC, still alive and on POC INR monitoring at the end of 2017). The non-parametric Friedman test for repeated-measures was used to evaluate the differences among the three time frames. Post-hoc analysis to examine the actual difference was performed using the non-parametric Wilcoxon signed-rank test for each pairwise combination, using a Bonferroni adjustment for the significant p value (<0.017).

Sensitivity analyses of the TTR and the number of days between two INR tests in the three time frames, were performed by age categories (< 60 years, 60 to ≤ 70 years, 70

to ≤ 80 years, ≥ 80 years), by sex (females, males), by clinical indication for warfarin (AF, VTE, heart valve replacement), by INR target range (2.0-3.0, 2.5-3.5), and by warfarin treatment duration (≤ 2 years, > 2 years). For the analysis reported in this chapter, the statistical programs STATA/SE v.12 (StataCorp LP, College Station, TX, USA) was used. The median difference (with 95% CI) was calculated using the somersd package and the cendif program, which allows to calculate robust confidence intervals for the median differences, considering unequal variances and paired measurements (Newson, 2000, 2002).

9.4 Results

9.4.1 Study population

Overall, 1555 patients started the POC INR monitoring at seven Health Centres (RHC, QHC, BKHC, PHC, FHC, CHC, GHC) in the years 2014-2015. The first 700 patients in alphabetical order were analysed. Median age at the time of starting the POC INR was 70 years (IQR 65-77), and there was a slight prevalence of male sex (56.6%). The most common indication for warfarin was AF (72.4%), followed by mechanical heart valve replacement (15.6%) and VTE (12.6%). At the time of starting the POC INR, 76.8% of patients have been already on warfarin for more than two years. Baseline characteristics of the patients included in the comparison of the three time frames were similar (Table 9.1).

	All patients (n=700)	Patients included in the comparison of the three time frames (n=471)
Age (years), median (IQR)	70 (65-77)	70 (65-75)
Sex, n (%)		
• Females	304 (43.4%)	197 (41.8%)
• Males	396 (56.6%)	274 (58.2%)
Clinical indication for warfarin*, n (%)		
• AF	507 (72.4%)	325 (69.0%)
• VTE	88 (12.6%)	57 (12.1%)
• Heart valve replacement	109 (15.6%)	91 (19.3%)
• Others	19 (2.7%)	13 (2.8%)
INR target range, n (%)		
• 2.0-3.0	656 (93.7%)	438 (93.0%)
• 2.5-3.5	44 (6.3%)	33 (7.0%)
Warfarin treatment duration**, n (%)		
• ≤ 3 months	22 (3.2%)	NA
• > 3 to ≤ 6 months	20 (2.9%)	NA
• > 6 to ≤ 9 months	25 (3.6%)	NA
• > 9 months to ≤ 1 year	16 (2.3%)	1 (0.2%)
• > 1 year to ≤ 2 years	79 (11.3%)	60 (12.7%)
• > 2 years	535 (76.8%)	410 (87.1%)

Table 9.1 Baseline characteristics of the population
*more than one option was possible ** data available only in 697 patients

9.4.2 Time within the therapeutic range

The median overall POC-TTR, obtained from the INR values of 700 patients, was 72.2% (IQR 62.1-80.8%). During a median follow-up of 1019.5 days (IQR 763.5-1148.0), the median number of INR tests was 40 (IQR 28-51). This data corresponds, on average, to one INR every 23.2 days (IQR 19.4-27.8).

The analysis of the three time frames included 471 patients (Table 9.2). There was a statistically significant increase of the TTR in the three time frames (Friedman test = 36.769, p <0.001). The post-hoc analysis with the Wilcoxon signed-rank tests and the Bonferroni correction (significance level set at p <0.017) revealed a statistically significant difference in all three comparisons (time 1 vs. time 2 p<0.001; time 2 vs.

time 3 p=0.014; time 1 vs. time 3 p<0.001). The median difference in the TTR between time 2 vs. time 1 was 5.1% (95% CI, 2.2-7.0%), between time 3 vs. time 1 was 6.6% (95% CI, 3.7-8.9%) and between time 3 vs. time 2 was 2% (95% CI, 0-3.4%).

There was also a significant increase in the number of days between two INR tests (Friedman test = 220.173, p<0.001). The post-hoc analysis revealed that the difference was only in time 1 vs. time 2 (p<0.001) and time 1 vs. time 3 (p<0.001), while there was no difference in time 2 vs. time 3 (p=0.12). The median difference in the number of days between two INR tests between time 2 vs. time 1 was 5.37 days (95% CI, 4.20 to 6.61), between time 3 vs. time 1 was 5.50 days (95% CI, 4.85 to 6.07) and between time 3 vs. time 2 was 0.44 days (95% CI, -0.74 to 1.47).

	Time 1: Pre-POC period	**Time 2: Initial-POC period**	**Time 3: Stable-POC period**
TTR (%)	70.0 (58.4-80.0)	74.2 (60.5-85.2)	77.2 (63.1-88.0)
Number of INR tests	20 (16-24)	15 (12-18)	14 (11-18)
Follow-up duration (days)	354 (346-358)	349 (335-358)	333 (315-345)
Number of days between two INR tests	17.4 (14.6-21.4)	23.6 (18.8-28.8)	23.6 (18.9-29.8)
Number of hospitalisations	0 (0-1)	0 (0-1)	0 (0-1)

Table 9.2 Results of the three time frames: the pre-POC period, the initial-POC period, and the stable-POC period
Results are reported as median (IQR).

9.4.3 Analysis of TTR categories

TTR values were also analysed in three categories, as performed in a previous study (Razouki et al., 2014): high TTR (> 70%), moderate TTR (50-70%), low TTR (< 50%). During the overall POC time, 396 (56.6%) of the 700 patients were in the high TTR category, 238 (34.0%) in the moderate TTR category and 66 (9.4%) in the low TTR category.

The analysis of the three time frames is reported in Figure 9.1. There was a progressive increase in the percentage of patients in the high TTR category (from 49.5% in the pre-POC period, to 59.2% in the initial-POC period, to 63.9% in the stable-POC period) and a progressive decrease in the percentage of patients in the other two TTR categories (p<0.001).

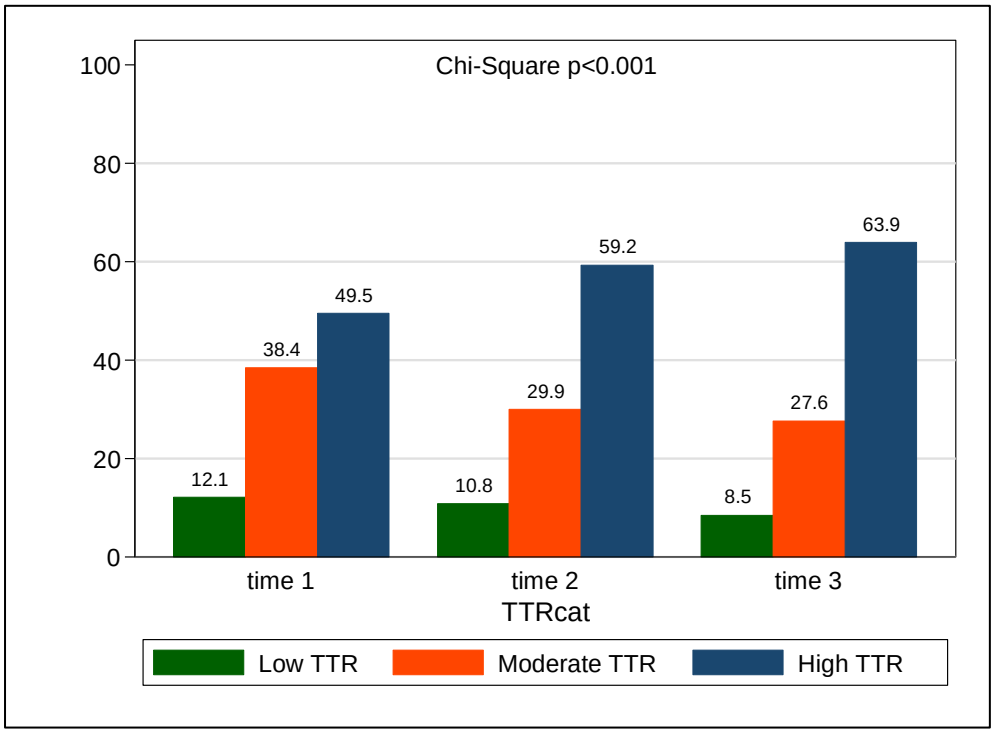


Figure 9.1 TTR categories in the three time frames: pre-POC (time 1), initial-POC (time 2), stable-POC (time 3)

9.4.4 Sensitivity analyses

The results of the sensitivity analysis for the TTR confirmed the trend shown in the main analysis, with the exception of the following categories: patients with VTE (p=0.20) and patients with INR target range 2.5-3.5 (p=0.27), as reported in Table 9.3. The results of the sensitivity analysis for the number of days between two INR tests confirmed the trend shown in the main analysis for all categories (Table 9.4).

	Time 1: Pre-POC period	Time 2: Initial-POC period	Time 3: Stable-POC period	P value (Friedman test)
By age categories				
• < 60 years (n=53)	70.0 (54.6-76.0)	74.2 (63.4-86.1)	75.3 (61.4-84.0)	0.016
• 60 to ≤ 70 years (n=176)	71.5 (59.7-80.9)	72.5 (61.4-83.0)	79.1 (65.7-88.3)	<0.001
• 70 to ≤ 80 years (n=189)	69.8 (59.5-79.2)	74.5 (60.5-86.5)	78.4 (65.5-89.1)	<0.001
• ≥ 80 years (n=53)	68.8 (56.5-79.4)	74.6 (58.5-82.6)	65.0 (52.7-81.6)	0.053
By sex				
• Females (n=197)	68.9 (58.4-80.2)	74.2 (60.9-85.6)	74.7 (62.2-85.7)	0.032
• Males (n=274)	71.9 (58.7-79.6)	74.0 (60.4-84.7)	79.0 (64.3-90.2)	<0.001
By clinical indication for warfarin				
• AF (n=325)	69.7 (59.2-78.3)	73.6 (60.5-84.6)	76.3 (62.7-87.1)	<0.001
• VTE (n=57)	75.0 (62.8-84.6)	80.1 (64.7-88.3)	78.7 (67.0-90.7)	0.20
• Heart valve replacement (n=91)	63.3 (54.2-79.2)	71.6 (56.1-83.0)	76.5 (62.8-90.2)	0.002
By INR target range				
• 2.0-3.0 (n=438)	71.2 (60.3-80.3)	74.3 (62.1-85.5)	77.6 (64.4-88.0)	<0.001
• 2.5-3.5 (n=33)	56.3 (41.8-67.0)	69.6 (42.7-81.0)	66.4 (51.1-82.4)	0.27
By warfarin treatment duration				
• ≤ 2 years (n=61)	65.1 (52.4-77.3)	74.1 (63.4-85.8)	76.4 (66.8-88.3)	<0.001
• > 2 years (n=410)	70.6 (59.9-80.3)	74.2 (60.0-85.2)	77.2 (62.5-88.0)	<0.001

Table 9.3 Sensitivity analysis of the TTR in the three time frames
Results are reported as median (IQR).

	Time 1: Pre-POC period	Time 2: Initial-POC period	Time 3: Stable-POC period	P value (Friedman test)
By age categories				
• < 60 years (n=53)	16.3 (13.4-21.1)	23.7 (19.4-29.0)	22.2 (18.4-28.0)	<0.001
• 60 to ≤ 70 years (n=176)	17.6 (14.4-22.1)	24.2 (18.6-29.3)	24.1 (19.5-30.6)	<0.001
• 70 to ≤ 80 years (n=189)	17.6 (15.3-21.4)	23.7 (19.2-28.5)	23.1 (18.6-31.6)	<0.001
• ≥ 80 years (n=53)	18.1 (15.1-21.1)	21.8 (17.3-25.8)	24.6 (17.3-28.4)	<0.001
By sex				
• Females (n=197)	17.9 (14.6-21.1)	22.8 (18.7-27.4)	23.6 (18.8-29.4)	<0.001
• Males (n=274)	17.3 (14.6-21.7)	23.9 (19.1-29.6)	23.8 (18.9-30.5)	<0.001
By clinical indication for warfarin				
• AF (n=325)	18.1 (15.1-21.9)	24.1 (19.3-28.8)	24.0 (19.0-30.4)	<0.001
• VTE (n=57)	17.4 (14.2-22.4)	24.1 (19.2-32.8)	23.7 (17.6-29.5)	<0.001
• Heart valve replacement (n=91)	15.2 (13.2-19.8)	20.8 (16.7-26.3)	21.5 (18.6-28.6)	<0.001
By INR target range				
• 2.0-3.0 (n=438)	17.5 (14.9-21.4)	23.7 (19.0-29.0)	24.0 (19.0-30.2)	<0.001
• 2.5-3.5 (n=33)	14.5 (13.0-19.8)	20.5 (16.7-25.8)	20.0 (17.9-27.3)	<0.001
By warfarin treatment duration				
• ≤ 2 years (n=61)	16.5 (13.7-19.5)	22.9 (18.1-25.7)	22.2 (18.5-30.0)	<0.001
• > 2 years (n=410)	17.6 (14.8-21.8)	23.7 (18.9-29.1)	23.9 (18.9-29.8)	<0.001

Table 9.4 Sensitivity analysis of the number of days between two INR tests in the three time frames
Results are reported as median (IQR).

9.5 Discussion

This study analysed a cohort of VKA patients switched, during the years 2014-2015, to POC INR monitoring performed by healthcare professionals in the context of the anticoagulation clinics. It was found that the introduction of the POC coagulometers was associated with both an increase in the TTR and in the number of days between the INR tests. The median TTR was 70% during the year before the switching to POC INR, 74.2% in the first year of POC INR monitoring and 77.2% when considering the INR results of the year 2017. This increase was statistically significant for all three pairwise comparisons. Furthermore, the proportion of patients in the high TTR category (defined as TTR > 70%) showed a statistically significant progressive increase in these three time frames (from 49.5% to 63.9%). There was also a progressive increase in the number of days between two INR tests (from 17.4 to 23.6 days), which was statistically significant only between the pre-POC period and the other two measurements, while there was no difference between the initial-POC and the stable-POC period.

The POC coagulometers are portable devices which can allow immediate INR results from capillary blood obtained with a finger-prick. When used by patients themselves for self-testing they were associated with better TTR (Matchar et al., 2010) and lower incidence of thromboembolic events and mortality (Bloomfield et al., 2011), compared to the standard clinic-testing. However, the POC coagulometers can also be used by healthcare professionals in the anticoagulation clinics, where they can allow immediate warfarin dose adjustment and immediate management of extremely out-of-range values or patients with anticoagulant-related complications. It was previously demonstrated that the POC coagulometers are accurate devices compared to other INR assays (Chapter 3), and that their use in anticoagulation clinics is associated with better

QoL for anticoagulated patients (Chapter 8). However, whether their use in anticoagulation clinics can also improve the TTR is still a matter of debate, since contrasting results have been reported recently. A Canadian study compared 74 VKA patients managed by pharmacists with routine laboratory INR in 2008 with 72 patients managed by a pharmacist-led POC-INR clinic in 2010, of whom 32 patients were present in both study cohorts (Rossiter et al., 2013). The authors found that the introduction of the POC system was associated with a 6.3% increase of the TTR in the overall population, and a 12.7% increase when considering only those patients with consistent warfarin use during the study period (Rossiter et al., 2013). Another group of researchers reviewed 150 VKA patients before and after the introduction of the POC coagulometers in a pharmacist-managed anticoagulation clinic in the USA (Challen et al., 2015). They reported an increase of the TTR of 7.8% (Challen et al., 2015), even though in this study the TTR was not calculated using the linear interpolation method recommended by Rosendaal et al. (1993). More recently, Biederman et al. (2016) compared two independent cohorts of VKA patients monitored in an anticoagulation clinic in the Netherlands: 1973 VKA patients managed with the standard laboratory-INR monitoring in the year 2012 and 1959 VKA patients managed with POC-INR monitoring in the year 2013. They found that the median TTR was significantly lower in the POC-cohort vs. the laboratory-cohort (77.9% vs. 81.0%, $p<0.001$) and that the proportion of patients with poor TTR (defined as TTR <60%) was significantly increased in the POC-cohort (14.5% vs. 10.7%, $p<0.001$). However, in this study wider therapeutic ranges were considered (2.0-3.5 for low-intensity and 2.5-4.0 for high-intensity anticoagulation), which can partly explain the very high median TTR values reported (Biedermann et al., 2016).

In the current analysis of 471 patients pre- and post-POC implementation, the median TTR increased from 70.0% to 74.2%. These TTR results are very good and in line with previously reported TTR values in other cohort studies. For instance, a group of researchers analysed the INR data of more than 28,000 AF patients from Swedish registries, and reported a mean individual TTR of 68.6% (± 22.6%) (Björck et al., 2016). Similarly, another study analysed the INR data of 5210 AF patients included in the US Outcomes Registry for Better Informed Treatment of Atrial Fibrillation (ORBIT-AF) and found a mean TTR of 65% (± 20%) and a median TTR of 68% (IQR 53-79%) (Pokorney et al., 2015).

With regards to the median number of INR tests during a 1-year period, in the present study there was a reduction from 20 tests in the pre-POC period to 15 tests in the initial-POC period (which corresponds to a decrease from one INR test every 17.4 days to one INR test every 23.6 days). Previously, some authors analysed more than 54,000 AF patients on warfarin from Finnish registries and reported a median of 16.6 INR tests in a year and a median TTR of 67% (Lehto et al., 2017). Another study, instead, analysed more than 22,000 AF patients on acenocoumarol in a Spanish region and reported a mean of 14 INR tests in a year and a mean TTR of 63% (García-Sempere et al., 2019). Results of the current study are more similar to Lehto et al. (2017), partly due to the fact that all patients were on warfarin, while acenocoumarol has a shorter half-life and is also more unstable, and partly due to the fact that a closer INR monitoring could have contributed to a better TTR.

The main strength of this study includes the fact that the same population has been included in the three time frames, by performing a pre-post analysis in which each patient acted as its own control. However, this study has also some limitations which need to be acknowledged. First, in order to be included in the analysis of the three time

frames, patients needed to have performed laboratory INR monitoring for at least 12 months before switching to the POC, and needed to be still alive and on POC monitoring at the end of 2017. These criteria could have created a survival bias, since patients with early VKA discontinuation or early mortality were excluded. Second, this calculation considered only the number of assays with a proper INR results, excluding unsuitable blood specimens (e.g. haemolysed, clotted or insufficient samples) or errors in the finger-prick. Third, it was not possible to identify all potential VKA discontinuations for interventional procedures, but it was decided to exclude the hospitalisation time in order to account for this limitation. Similar approaches have been adopted in previous studies to eliminate the potential interference by VKA discontinuation, such as removing also one week before and after the hospitalisation (McAlister et al., 2018). Fourth, differences between the pre-POC and the POC time frames could also be due to the different types of physicians operating in the two settings, being the Anticoagulation Clinics at the Health Centres led mainly by general practitioners and general practitioner trainees. Finally, these study results could be improved by correlating the TTR with the occurrence of anticoagulant-related clinical events during follow-up.

9.6 Conclusion

The results of this study suggested that the introduction of POC coagulometers used by healthcare professionals for VKA monitoring in anticoagulation clinics is associated with a 5.1% increase of the TTR in the short term (initial-POC period) and a 6.6% increased of the TTR in the long term (stable-POC period). There was also a reduction in the number of INR assays, corresponding to an increase of approximately five days in the time between two INR tests. These results should be confirmed by

evaluating a larger sample size and by correlating the TTR with the anticoagulant-related clinical events during follow-up.

Chapter 10 :

Conclusions

10.1 General overview of findings

This thesis provided more evidence in some of the grey areas in the management of anticoagulation and VTE, from both a laboratory and a clinical perspective. Since the POC coagulometers represent an attractive alternative to the standard laboratory INR, their accuracy for monitoring VKA patients was evaluated in Chapter 3. One of the most commonly used POC, the CoaguChek XS Plus (Roche Diagnostics), was found to have a very good correlation with the other INR assays (a photo-optical automated coagulometer, an electromechanical automated coagulometer, and the manual tilt-tube technique) and with the thrombin generation, as a global coagulation assay. Therefore, the POC INR should be considered as an accurate and valid alternative to the standard laboratory monitoring of the INR in VKA patients.

The accuracy and the relative importance of several laboratory tests as potential biomarkers of acute VTE (two different D-dimer assays, the thrombin generation assay performed with the CAT, the procoagulant phospholipid-dependent clotting time, and the human soluble P-selectin) was subsequently assessed (Chapter 4). The diagnosis of VTE currently requires a composite of clinical pre-test probability, D-dimer and specific imaging tests. D-dimer has very high sensitivity and negative predictive value, therefore it can rule out the suspicion of VTE; however, due to its low specificity, specific imaging techniques are required to confirm the diagnosis of VTE. It was confirmed that the D-dimers are the main biomarkers for VTE; the thrombin generation showed only a limited relative importance; while the PPL did not appear to have any role in VTE diagnosis. The sP-selectin showed a good predictive value and improved the diagnostic accuracy of the D-dimers when used in combination. Therefore, the latter might be considered as an important emergent biomarker, although it is still hampered by high costs and some technical difficulties.

The sensitivity of two global coagulation assays, the thrombin generation performed with the CAT and the thromboelastography, to several oral and parenteral anticoagulants (namely, three direct factor Xa inhibitors, three direct thrombin inhibitors, three indirect factor Xa inhibitors and warfarin) was evaluated in Chapter 5. Global coagulation assays have the peculiarity that they can allow the evaluation of the different phases of coagulation (initiation, propagation and termination) and, with some modifications, they can also evaluate fibrinolysis. Results showed that the CAT was more sensitive to the presence of the DOAC than the routine coagulation assays (APTT and PT/INR) and the specific chromogenic assays (anti-Xa or DTT). The TEG was insensitive to apixaban, while a prolongation of the R time appeared to be a good marker for the presence of edoxaban, rivaroxaban and dabigatran. Furthermore, there appeared to be some differences in the final clot strength among the different anticoagulants, suggesting that the warfarinised plasma with INR 3.24 and 4.11 has less stiff fibrin clots than apixaban, dabigatran and rivaroxaban at the tested concentrations (apixaban 89 ng/ml and 128 ng/ml, dabigatran 92 ng/ml and 148 ng/ml, rivaroxaban 118 ng/ml and 174 ng/ml).

Chapter 6 focused on the analysis of the reversal strategies for the oral anticoagulants (VKA or DOAC). Different generic reversal strategies are currently available (such as FFP, 3- or 4-factor PCC, activated PCC, rVIIa), however the availability of these products can vary in different countries. The use of FFP for VKA reversal *ex vivo* was found to be associated with a significant reduction of the INR and the CAT parameters; however, a complete normalisation of the haemostatic balance was not obtained in any patient. By analysing the effect of different reversal agents for DOAC reversal *in vitro*, it was found that different concentrations of the reversal agents PCC or rVIIa might be needed in order to normalise the coagulation profile, based on the DOAC plasma

concentrations. The reversal effect of FFP was limited by the high volume required in order to normalise the haemostatic balance. Finally, by investigating the neutralising effect of DOAC Stop® *in vitro*, it was confirmed that it can normalise the basic coagulation assays (APTT and PT/INR). However, a potential for false negative results in patients with lupus anticoagulant and a trend towards a reduction of the plasma levels of several coagulation factors also emerged, suggesting a certain level of DOAC Stop® binding.

From a clinical perspective, the psychometric properties (reliability and validity) of the Maltese translations of the DASS and the PACT-Q questionnaires were assessed in Chapter 7. The correlation between patients' satisfaction and adherence to chronic treatment is well recognised nowadays; however, there was no validated questionnaire available in the Maltese language specifically assessing the QoL of anticoagulated patients. Thus, these two psychometric questionnaires assessing patients' satisfaction with the anticoagulant treatment were translated in Maltese and validated. The Maltese translation of the DASS showed good reliability and an acceptable level of validity, while the Maltese translation of the PACT-Q2 showed good reliability and validity. Therefore, the Maltese DASS and the Maltese PACT-Q2 emerged as valid and reliable instruments to assess the level of satisfaction of Maltese-speaking anticoagulated patients.

Chapter 8 analysed whether the use of the POC coagulometers by healthcare professionals in the setting of the anticoagulation clinics can improve the satisfaction of VKA patients. INR monitoring can be performed through venepuncture and laboratory coagulometers (laboratory INR) or through finger-prick and POC coagulometers (POC INR). One of the main advantages of the POC INR is a dedicated time slot for each patient with the immediate availability of the INR result and warfarin

dose adjustment. The POC INR group obtained higher scores in the overall DASS, in the hassles/burdens and the psychological impact subscales of the DASS, and in the convenience and the anticoagulant treatment satisfaction subscales of the PACT-Q2, indicating that the POC INR group was more satisfied than the laboratory INR group. In Chapter 9 the use of the POC coagulometers by healthcare professionals for INR monitoring was correlated with the time spent within the therapeutic INR range, which is an indirect measurement of the anticoagulation control. The POC devices, when used for PST, were associated with better TTR and reduced clinical events; however, it remained controversial whether the same effect could be obtained with their use in the anticoagulation clinics. From the analysis of the INR results of a cohort of VKA patients in three different time frames (pre-POC, initial-POC and stable-POC period), it was found that the introduction of the POC coagulometers was associated not only with an increase in the individual TTR, but also with a reduction in the number of INR tests. Furthermore, the advantages of the POC devices were evident both in the short term (initial-POC period) and in the long term (stable-POC period), compared to the pre-POC time frame.

10.2 Practical implications

The findings of these studies present several implications for real life clinical practice. First, it was demonstrated that the use of the POC coagulometers for INR monitoring in the anticoagulation clinics is an accurate alternative to the standard laboratory INR, is associated with better patients' satisfaction and can also improve the anticoagulation control. In most anticoagulation clinics around the world, INR monitoring is still performed through blood sample collection early in the morning and subsequent analysis in coagulation laboratories, therefore the INR results are available only in the

afternoon. This standard way of INR monitoring (laboratory INR) is associated with a delay in patients management, long waiting time for the patients in the anticoagulation clinics and, sometimes, a deferred collection of the INR results. For instance, at the Anticoagulation Clinic at Mater Dei Hospital warfarin dose adjustment is communicated by phone for significantly out-of-range INR values, while INR booklets are usually sent by post, therefore reaching the patients days later. The use of POC coagulometers by healthcare professionals in the anticoagulation clinics can allow the availability of immediate INR results, which is particularly relevant in case of extremely out-of-range values or in patients undergoing interventional procedures. These results, demonstrating both the accuracy of the POC devices and the improvement in patients' satisfaction and anticoagulation control, need to be disseminated since they can support a wider use of the POC coagulometers, locally and internationally. These results are particularly relevant in the local context, since the POC have been introduced in Malta only a few years ago as part of a decentralisation program. Previously, warfarin monitoring was centralised at the anticoagulation clinic at Mater Dei Hospital. With the advent of the POC devices, several anticoagulation clinics spread around the Maltese island started offering the possibility of VKA monitoring with the use of the POC INR. Since VKA will still remain the anticoagulant of choice for patients with several conditions (such as valvular AF, mechanical heart valves, renal failure or other contraindications to the DOACs), these results can support the propagation of the use of the POC devices.

Second, for the first time validated Maltese translations of two psychometric questionnaires that specifically evaluate patients' satisfaction associated with the anticoagulant treatment were provided. The Maltese versions of the DASS and the PACT-Q can be used by healthcare professionals in clinical practice, in order to assess

the degree of satisfaction of Maltese speaking anticoagulated patients. Anticoagulation is a long-term treatment for most clinical indications and is subjected to a significant drop in adherence over time. The evaluation of the QoL might be particularly relevant for patients with low anticoagulation control (e.g. low TTR) or low adherence. Different healthcare professionals, not limited to doctors and nurses, should be educated on the importance of considering patients' satisfaction. Identifying which aspects can cause more hassles and burdens is the preliminary step in order to promote specific interventions, with the aim of increasing the adherence and the anticoagulation control.

Third, these preliminary results on the effects of different reversal strategies for patients on oral anticoagulant treatment can improve the management of anticoagulated patients. In this study, the use of FFP for VKA reversal, although resulting in a significant reduction of the INR, did not obtain a complete normalisation of the haemostatic balance, suggesting that the local reversal strategy with FFP for VKA-treated patients might need to be revised. For DOAC-treated patients, when specific antidotes are not available, activated PCC, PCC and rVIIa at appropriate dosages could be potentially reasonable approaches; however, future management studies are needed to strengthen these findings. These results are particularly relevant in the local context, where 3- and 4-factor PCCs are not available and VKA reversal is performed with vitamin K and FFP. For the DOACs, reversal of the factor Xa-inhibitors is performed with rVIIa, while the specific antidote idarucizumab is available for dabigatran.

10.3 Recommendations for future research

Based on the results of these studies, some areas of research are worth pursuing. First, the evaluation of the fibrin clot strength showed some differences among the different anticoagulants, with the clot formed under dabigatran, apixaban and rivaroxaban being apparently stiffer than the ones formed in the warfarinised plasma with INR greater than three. These findings suggested a different anticoagulant effect of the VKAs and the DOACs; however, they would need to be confirmed in future studies. The *in vitro* TEG-TPA model unfortunately had some limitations, resulting in a wide variability of the lysis parameters. Future research should aim at creating more stable TEG-TPA models, for instance by diluting TPA in serum albumin, or by using TEG activators (such as TF to elicit the extrinsic pathway or kaolin to elicit the intrinsic pathway). The fibrinolysis could also be evaluated through the use of other methodologies, such as direct analysis of fibrin clot structure, permeability analysis and confocal microscopy.

Second, the availability of validated Maltese translations of two psychometric questionnaires assessing the anticoagulant-related QoL means that they could be used in future national or international research projects assessing patients' satisfaction or barriers to the anticoagulant treatment. In particular, the PACT-Q has been already translated in more than 40 languages and used in several RCTs. The availability of the Maltese translations can translate in the possibility of the anticoagulated Maltese population to be part of large international studies.

Third, these results showed that several assays currently used only for research purposes could potentially improve the clinical management of anticoagulated patients. Confirmation by future research studies will strengthen these results. For instance, the sP-selectin could be used in combination with the D-dimer in the

diagnostic algorithm for VTE, with the aim to reduce the number of required imaging tests, but its role needs to be confirmed in large management studies. The thrombin generation, measured with the CAT, appeared to be more sensitive to the presence of low concentrations of the DOACs than the specific chromogenic assays. The CAT is currently used only as a research test, due to its high inter-laboratory variability, the manual preparation of the plates and the amount of time required for the results. However, automated thrombin generation analysers, which can solve part of these limitations, have been recently developed and the detection of the DOACs could represent a possible area of further research. Conversely, the CAT did not appear to be a suitable assay to evaluate the efficacy of different reversal agents for the oral anticoagulant treatments, since the concentrations used *in vivo* could not be exactly reproduced *in vitro* due to substrate consumption.

10.4 Recommendations for clinical practice and policy makers

Based on the results of this thesis and its practical implications, the following recommendations can be made for clinical practice and policy makers. First, since the POC coagulometers can improve patients' satisfaction and the anticoagulation control, a more widespread use of these devices in the anticoagulation clinics worldwide can be recommended. This approach would reduce the time wasted by the patients and the staff working in the anticoagulation clinics, while waiting for the INR results to be assayed in the coagulation laboratories. Locally, policy makers could consider introducing POC coagulometers also at Mater Dei Hospital, in order to reduce the delay between blood collection and INR results and to speed up the management of VKA patients.

Second, since the use of FFP for VKA reversal did not obtain a complete normalisation of the haemostatic balance, the local reversal strategy for VKA would need to be revised with the use of higher doses of FFP or other more efficient reversal products. For instance, 3- and 4-factor PCCs are currently used as first line reversal agents for warfarin-related major bleeding in several countries, including the UK and Italy, and increasing doses are administered based on the initial INR values. Policy makers should consider the introduction of 3-factor or 4-factor PCCs in Malta, since they appeared to be effective both for VKA and DOAC reversal.

Third, since the use of the binding agent DOAC Stop® was associated with the potential for artefactual results in some coagulation assays (such as lupus anticoagulant and factor assays), caution should be applied when interpreting the results obtained after sample processing with the DOAC Stop® and the use of this binding agent should be discouraged in routine clinical practice, until more evidence becomes available.

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Nicoletta Riva

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Appendices

Appendix A – Publications

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ORIGINAL ARTICLE

WILEY ISLH International Journal of Laboratory Hematology

A comparative study using thrombin generation and three different INR methods in patients on Vitamin K antagonist treatment

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Summary

Introduction: Vitamin K antagonist (VKA) treatment requires routine monitoring using the international normalized ratio (INR). However, different INR assays may vary in their results. The aim of this study was to assess the agreement of three different INR methods, compared with thrombin generation, in patients on VKA treatment.

Methods: Sixty patients attending the Anticoagulation Clinic at Mater Dei Hospital (Msida, Malta) for VKA monitoring between August and September 2015 were enrolled. The INR was tested using a point-of-care (POC) device (CoaguChek XS Plus, Roche Diagnostics) for both capillary and venous blood samples, a photo-optical (Sysmex CS-2100i/CA-1500, Siemens) and a mechanical clot detection system (Thrombolyzer XRC, Behnk Elektronik). All assays used human recombinant thromboplastin as reagent. Thrombin generation was performed using the calibrated automated thrombogram.

Results: There was a negative curvilinear correlation between the endogenous thrombin potential and different INR assays ($r \leq -.75$) and a strong positive linear correlation between the CoaguChek XS Plus on capillary samples and the other INR methodologies ($r \geq .96$).

Conclusion: All different INR assays showed good correlation with the thrombin generation potential. The POC INR showed one of the highest correlation coefficients with thrombin generation, confirming the POC devices as an accurate, valid alternative to laboratory INR in VKA patients.

KEYWORDS

accuracy, international normalized ratio, point-of-care systems, thrombin generation, warfarin

1 | INTRODUCTION

Vitamin K antagonists (VKAs) have a narrow therapeutic window, several food and drug interactions and a variable anticoagulant response, which explain the need for periodical anticoagulation monitoring and dose adjustment.[1] As VKAs inhibit the synthesis of vitamin K-dependent coagulation factors (factors II, VII, IX and X), they are monitored using laboratory tests that assess the extrinsic pathway of the coagulation cascade. The prothrombin time (PT) measures the time to clot formation of citrated plasma, after recalcification and addition of thromboplastin to trigger coagulation, and is usually expressed as international normalized ratio (INR). The WHO-recommended method for PT testing in relation to VKA therapy is the manual tilt-tube technique,[2] but currently most PT determinations are performed using automated coagulation analysers, such as photo-optical or electromechanical coagulometers. Furthermore, in the last two decades,

several portable coagulometers, also known as point-of-care (POC) devices, have been developed for the self-care of patients prescribed with VKAs.[3] More recently, we saw the advent of global coagulation assays, such as thrombin generation, which may have the potential to better evaluate all phases of coagulation.[4]

Several studies indicated an excellent correlation between photo-optical and electromechanical coagulation analysers,[5,6] while the comparison between POC and laboratory or manual INRs showed a certain variability in the results, with potential clinical disagreement and differences in VKA dosing.[7-10] However, it is not known which test actually correlates better with the overall blood coagulation potential, because these three INR methods have never been compared simultaneously with global coagulation assays.

The aim of this study was to assess the agreement of three different INR assays, compared with thrombin generation, in patients on VKA treatment.

2 | MATERIAL AND METHODS

2.1 | Study population

Consecutive adult patients attending the Anticoagulation Clinic at Mater Dei Hospital (in Msida, Malta) for warfarin monitoring were screened. We included 30 patients deemed eligible for POC monitoring according to the local protocol (target INR≤3.0 and at least three consecutive INRs within the therapeutic range, absence of antiphospholipid syndrome, liver disease, severe renal failure, active cancer or dual antiplatelet therapy) and 30 random patients, in order to cover a broader range of INR values. Patients were recruited during the months of August and September 2015.

The study was reviewed and approved by the University of Malta Research and Ethics Committee, and written informed consent was obtained from all patients before inclusion.

2.2 | Sample collection and tests performed

2.2.1 | Laboratory INR

From each patient, one venous blood sample was collected using a 10-mL syringe and a 21G needle, to fill in three coagulation tubes, each containing 2 mL of whole blood and sodium citrate 0.109M/3.2% (Vacuette, Greiner Bio-One). One tube was processed according to the standard system at Mater Dei Hospital at the time of this study. This tube was centrifuged for 10 minutes at 2500 g and plasma was analysed using a photo-optical clot detection system (Sysmex CS-2100i or CA-1500, Siemens Healthcare Diagnostics) and human recombinant thromboplastin (Dade Innovin; Siemens Healthcare Diagnostics Products GmbH). We had previously tested with both Sysmex analysers 33 samples with various INRs, ranging from 0.9 to 4.45, and found no statistical difference in the PT and the INR between the two analysers (data not shown).

The two remaining tubes underwent double centrifugation (2500 g for 10 min twice) with plasma separation, to obtain platelet poor plasma (PPP) within a 2-hour time frame from phlebotomy. They were stored in 300-μL aliquots at −80°C. It has previously been demonstrated that freezing plasma does not affect INR testing.[11]

Afterwards, one scientist tested the INR on thawed PPP using a mechanical clot detection system (Thrombolyzer XRC; Behnk Elektronik GmbH & Co. KG, Norderstedt, Germany) and the same human recombinant thromboplastin (Dade Innovin; Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany).

The INR is calculated dividing the patient's PT by the mean of the PTs of adult normal subjects, to the power of the thromboplastin's International Sensitivity Index (ISI),[12] according to the following formula:

$$\mathrm{INR} = \left(\frac{\text{patient PT}}{\text{mean normal PT}}\right)^{\mathrm{ISI}}$$

INR calibration was performed locally, on each analyser, using a calibrator kit (PT-Multi Calibrator; Siemens Healthcare Diagnostics Products GmbH) composed of five lyophilized calibration plasmas.

2.2.2 | Point-of-care testing

All 60 patients were tested using the CoaguChek XS Plus (Roche Diagnostics) coagulometer. Quality control (QC) analysis for POC was performed at the beginning of each testing day. One researcher performed all the tests. Both capillary and venous blood samples were tested with the CoaguChek XS Plus. Capillary blood samples were obtained by finger prick and applied on a test strip within 10 seconds. Noncitrated venous blood samples were obtained from the syringe used to draw the venous blood, after filling the coagulation tubes and after discharging few blood drops. The same CoaguChek XS Plus coagulometer was used throughout study. Two lots of test strips were used during the study (233 430-11 and 202 053-11). As per manufacturer's instructions, the CoaguChek XS Plus system utilizes human recombinant thromboplastin with ISI=1.0.[13]

2.2.3 | Thrombin generation

Frozen aliquots were shipped to the Coagulation Laboratory at the Royal Hallamshire Hospital (in Sheffield, United Kingdom) in dry ice. Thrombin generation was performed using the calibrated automated thrombogram (CAT), according to the method described by Hemker et al..[14]

Prior to this analysis, samples were thawed in a water bath at 37°C for 5 minutes. Afterwards, 80 μL of PPP was added to 20 μL of tissue factor trigger at a concentration of 5pM (PPP reagent, Thrombinoscope BV, Maastricht, the Netherlands) in a 96-well plate. All samples were tested in duplicate, and one calibrator (Thrombin Calibrator, activity 580 nM) well was run in parallel. Three 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 1 hour 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.

2.3 | Statistical analysis

We collected information regarding demographic characteristics of the population, past medical history, details of the warfarin treatment and concomitant medications.

Continuous variables were expressed as mean with standard deviation (SD) or median with interquartile range (IQR); categorical variables were expressed as counts and percentages. Continuous variables were compared using the Student's *t* test or the Mann-Whitney U test; categorical variables were compared using the Chi square or Fisher's exact tests, as appropriate. The correlation between different laboratory tests was evaluated using the non-parametric Spearman's rank correlation test, according to data distribution, and the correlation coefficients (*r*) were calculated. The mean INRs obtained with different methodologies were compared using one-way repeated measures ANOVA with Bonferroni's post hoc correction.

The statistical agreement between different INR methodologies was evaluated creating Bland-Altman plots (or difference plots) with the mean of the two measurements on the *x*-axis and the difference between the two values on the *y*-axis.[15] The estimated mean bias is the mean difference between the two values, and the 95% limits of agreement are computed as mean bias±1.96 SD.[15]

In order to evaluate the clinical agreement and to estimate the percentage of INR values which might have resulted in a different clinical management, the INR values were categorized as above, within or below the INR therapeutic range (2.0-3.0 for patients with atrial fibrillation, venous thromboembolism and aortic valve replacement; and 2.5-3.5 for patients with mitral valve replacement).

Data analysis was performed using the statistical software STATA SE 12 (StataCorp LP, College Station, TX, USA). Two-tailed *P* values<.05 were considered statistically significant.

3 | RESULTS

3.1 | Study population

Sixty patients were enrolled in this study. Mean (SD) age was 68.5 (11.5) years, and 26 (43.3%) were males. The most common indications for warfarin treatment were atrial fibrillation (63.3%) and venous thromboembolism (26.7%), followed by mechanical heart valve replacement (8.4%). The majority of patients (73.3%) were on oral anticoagulant treatment for more than a year. The current median (IQR) dose of warfarin was 4 (3-5) mg. Comorbidities and concomitant medications in our population are summarized in Table 1. None of these patients had known antiphospholipid syndrome.

TABLE 1 Baseline characteristics of the population

	N. of patients=60
Age (y), mean (SD)	68.5 (11.5)
Male sex, n (%)	26 (43.3%)
Indication for anticoagulant treatment:	
Atrial fibrillation, n (%)	38 (63.3%)
Venous thromboembolism, n (%)	16 (26.7%)
Aortic valve replacement, n (%)	4 (6.7%)
Mitral valve replacement, n (%)	1 (1.7%)
Cerebrovascular accident, n (%)	1 (1.7%)
Duration of the anticoagulant treatment:	
≤3 months, n (%)	6 (10.0%)
3-6 months, n (%)	6 (10.0%)
6-12 months, n (%)	4 (6.7%)
>1 y, n (%)	44 (73.3%)
Current warfarin dose (mg), median (IQR)	4 (3-5)
Comorbidities:	
Hypertension, n (%)	49 (81.7%)
Diabetes mellitus, n (%)	22 (36.7%)
Dyslipidaemia, n (%)	32 (53.3%)
Coronary artery disease, n (%)	18 (30.0%)
Hypothyroidism, n (%)	8 (13.3%)
Previous stroke, n (%)	3 (5.0%)
Chronic obstructive pulmonary disease, n (%)	5 (8.3%)
Malignancy, n (%)	8 (13.3%)
Smokers: current, n (%)/previous, n (%)	5 (8.3%)/13 (21.7%)
Obesity, n (%)	29 (48.3%)
Concomitant medications:	
Antiplatelet*, n (%)	5 (8.3%)
Steroids, n (%)	1 (1.7%)
Statins, n (%)	35 (58.3%)
ACE inhibitors or ARBs, n (%)	42 (70.0%)
Diuretics, n (%)	34 (56.7%)
Beta blockers, n (%)	22 (36.7%)
Calcium channel blockers, n (%)	11 (18.3%)
Digoxin, n (%)	14 (23.3%)
Levothyroxine, n (%)	8 (13.3%)
Proton pump inhibitors, n (%)	10 (16.7%)
Metformin, n (%)	17 (28.3%)

ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blockers; IQR, interquartile range; SD, standard deviation.
*Antiplatelet therapy refers to aspirin or clopidogrel; none of the patients was receiving dual antiplatelet therapy.

3.2 | Different INR methodologies

Using the standard laboratory instrumentation in our Coagulation Laboratory (the Sysmex CS-2100i/CA-1500), mean (±SD) INR was 2.46 (±0.75), with a range from 1.37 to 4.92. Mean and median INR values measured with the other methodologies were slightly higher and are summarized in Table 2. Mean INR obtained using the Sysmex CS-2100i/CA-1500 analysers was significantly different from the CoaguChek XS Plus on capillary and venous samples and from the Thrombolyzer XRC results (*P* values<.001).

TABLE 2 Summary of INR measurements using different methodologies

Instrument (n of tests)	Mean INR (SD)	Median INR (IQR)	INR range
Sysmex CS-2100i/CA-1500 (60)	2.46 (0.75)	2.31 (1.95-2.74)	1.37-4.92
CoaguChek XS Plus (capillary blood) (60)	2.74 (0.86)	2.6 (2.2-3.1)	1.4-5.8
CoaguChek XS Plus (venous blood) (60)	2.74 (0.82)	2.6 (2.2-3.0)	1.4-5.7
Thrombolyzer XRC (59*)	2.71 (0.85)	2.52 (2.14-2.97)	1.34-5.33

INR, international normalized ratio; IQR, interquartile range; SD, standard deviation.
*Thrombolyzer results were available for 59 patients, as one patient had a difficult blood sampling and only a limited amount of plasma was available.

TABLE 3 Results of thrombin generation test in the overall population and in the comparison between patients with venous thromboembolism and atrial fibrillation

Overall population[a]			
Parameter	**Mean (SD)**	**Median (IQR)**	**Range**
Lag time (min)	6.35 (1.99)	6 (5.17-7.17)	3.47-13
Peak thrombin concentration (nM)	101.66 (44.51)	91.49 (72.51-121.2)	29.99-269.37
Time to peak (min)	9.23 (2.15)	8.83 (7.67-10.17)	5.97-16
Endogenous thrombin potential (nM/min)	596.75 (265.26)	547.5 (419-722.5)	186.5-1835
Velocity index (nM/min)	36.53 (17.76)	31.34 (25.22-47.91)	8.59-85.19
Comparison between patients with atrial fibrillation and venous thromboembolism[b]			
Parameter	**AF patients (n=38)**	**VTE patients (n=15)**	***P* value**
Lag time (min)	6.33 (5.33-7.67)	5.17 (4.8-6.33)	.08
Peak thrombin concentration (nM)	83.82 (69.05-121.2)	97.57 (77.37-135.39)	.43
Time to peak (min)	9.33 (8-10.65)	7.83 (7.67-9)	.06
Endogenous thrombin potential (nM/min)	508.5 (398-722.5)	547.5 (465-803)	.40
Velocity index (nM/min)	30.6 (24.02-42.72)	38.45 (25.45-47.98)	.44

AF, atrial fibrillation; IQR, interquartile range; SD, standard deviation; VTE, venous thromboembolism.
[a]Thrombin generation results were available for 59 patients, as the thrombin generation curve was not computable in one patient with VTE.
[b]All parameters are reported as median (IQR).

3.3 | Thrombin generation

The intra-assay coefficient of variation (CV) of thrombin generation was 4.3%. The interassay CV was 13.7% for the normal QC and 6.8% for the warfarin QC.

Thrombin generation results are summarized in Table 3. Patients with VTE had a slightly lower lag time and time to peak compared to patients with atrial fibrillation (AF), although this was not statistically significant (P=.08 and P=.06, respectively). This result was not explained by other variables that were comparable in the two groups (eg median INR 2.35 in AF patients *vs.* 2.3 in VTE patients, P=.99; median age 69.5 *vs.* 67 years, P=.54; warfarin treatment duration more than 1 year 71.7% *vs.* 73.3%, P=1.00; median TTR in the previous 3 months 67.8% *vs.* 67.0%, P=.77, respectively). There was no difference in the other parameters of the thrombin generation curve, as reported in Table 3.

3.4 | Correlation between thrombin generation and different INR methodologies

There was a negative curvilinear correlation between the ETP and the INR measured with the Sysmex CS-2100i/CA-1500 (r=−.75, P<.001), the CoaguChek XS Plus on capillary (r=−.80, P<.001) and venous blood (r=−.78, P<.001) and the Thrombolyzer XRC (r=−.78, P<.001), as shown in Figure 1.

3.5 | Comparison between INRs

A strong positive linear correlation was found between the CoaguChek XS Plus, tested on capillary samples, and the other INR methodologies, showing Spearman's r coefficients above .95 (Table 4 and Figure 2). The CoaguChek XS Plus tended to overestimate the INR by a mean of approximately 0.3 INR units, compared to the Sysmex CS-2100i/CA-1500. The agreement, represented by the Bland-Altman or difference plots, is reported in Figure 3.

From a clinical perspective, the INR values within the same clinical category, compared to the CoaguChek XS Plus on capillary samples, were 93.3% for the CoaguChek XS Plus on venous samples; 78.3% for the Sysmex CS-2100i/CA-1500; and 93.2% for the Thrombolyzer XRC. However, the disagreement between the two methods would never lead to antagonistic behaviour (such as dose increase *vs.* dose reduction or vice versa).

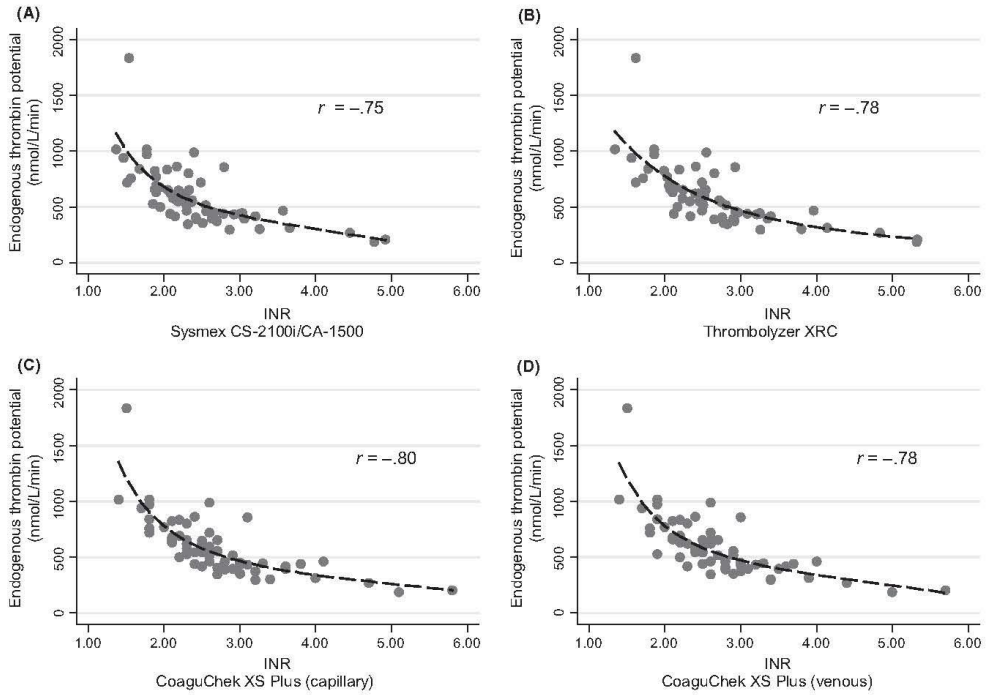

FIGURE 1 Correlation between the endogenous thrombin potential (ETP) and the INR, measured with the Sysmex CS-2100i/CA-1500 (A), the Thrombolyzer XRC (B), the CoaguChek XS Plus on capillary samples (C) and on venous samples (D)

TABLE 4 Agreement of the CoaguChek XS Plus on capillary blood samples, with the other INR methodologies

Comparison	Spearman's correlation coefficient *r* (*P* value)	INR difference, mean (±SD)	Magnitude of absolute difference, n (%)		
			<0.5	0.5-1.0	>1.0
CoaguChek XS Plus (capillary blood) vs.CoaguChek XS Plus (venous blood)	.9856 (<.001)	0.002 (0.11)	60 (100%)	0	0
CoaguChek XS Plus (capillary blood) vs. Sysmex CS-2100i/CA-1500	.9699 (<.001)	0.28 (0.18)	53 (88.3%)	7 (11.7%)	0
CoaguChek XS Plus (capillary blood) vs. Thrombolyzer XRC	.9646 (<.001)	0.04 (0.18)	58 (98.3%)	1 (1.7%)	0

INR, international normalized ratio; SD, standard deviation.

4 | DISCUSSION

To the best of our knowledge, this is the first time that three different INR assays (namely the CoaguChek XS Plus, the Sysmex CS-2100i/CA-1500 and the Thrombolyzer XRC) have been simultaneously compared with the thrombin generation assay. All the INR assays used human recombinant thromboplastin as reagent; therefore, the difference in results was mainly due to the different analysers.

We found a negative curvilinear relationship between the ETP measured by the CAT and the INR values, with Spearman's coefficients ranging between -0.80 and -0.75. A similar negative correlation was already reported by Gatt et al. in comparison with the Sysmex CA-1500.[16] In our study, thrombin generation showed a better correlation with the CoaguChek XS Plus and the Thrombolyzer XRC than with the Sysmex CS-2100i/CA-1500.

We also found a strong positive linear correlation between the CoaguChek XS Plus, tested on capillary samples, and the other INR methodologies, with all Spearman's coefficients above 0.95. The correlation was almost perfect for the CoaguChek XS Plus tested on capillary samples *vs.* venous samples with a mean (±SD) bias of 0.002 (±0.11) INR units, suggesting that this pre-analytical variable does not interfere with the INR values, if the test is correctly performed. Similar

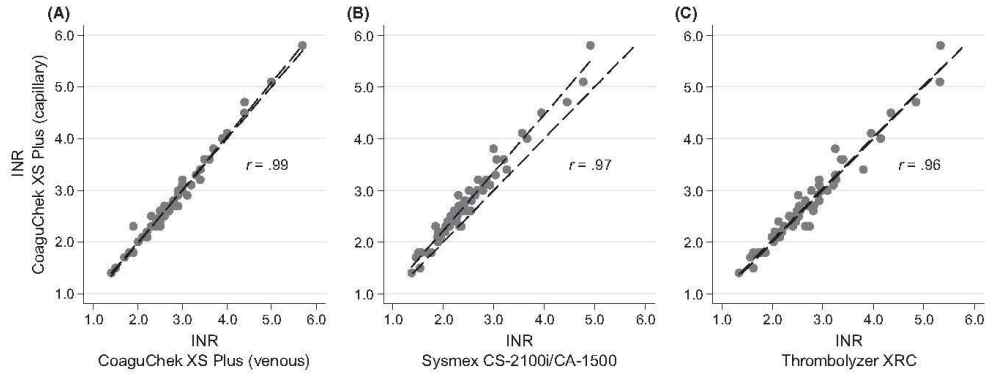

FIGURE 2 Correlation between the INR measured with the CoaguChek XS Plus on capillary samples and the CoaguChek XS Plus on venous samples (A), the Sysmex CS-2100i/CA-1500 (B) and the Thrombolyzer XRC (C). In each graph, the dashed line represents the perfect correlation, while the continuous line is the actual correlation between the two different INR methodologies

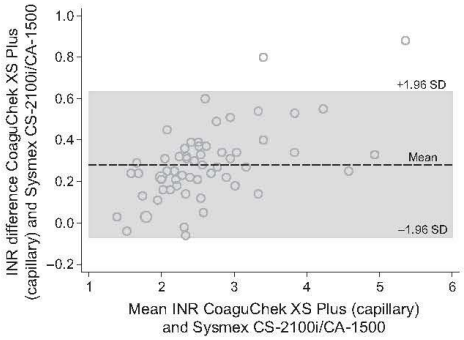

FIGURE 3 Bland-Altman plots representing the difference between the CoaguChek XS Plus on capillary samples and the Sysmex CS-2100i/CA-1500. The dashed line represents the mean difference, while the grey area defines the 95% limits of agreement

results were obtained by Plesch et al. who found a mean bias of less than±0.02 INR units between the capillary and venous sample, albeit using a different device, the CoaguChek XS.[17] In our study, the correlation was also very strong when the CoaguChek XS Plus was compared with the photo-optical (Sysmex CS-2100i/CA-1500) and the mechanical clot detection methods (Thrombolyzer XRC). Previous studies, that compared the CoaguChek XS Plus with photo-optical (Sysmex analysers) or mechanical clot detection methods (STAGO analysers), found correlation coefficients approximately 0.95-0.96[8,18,19]; however, the CoaguChek XS Plus had never been compared before with different laboratory techniques simultaneously.

Although the statistical agreement was very good, clinical disagreement between the CoaguChek XS Plus on capillary samples and the other INR assays ranged from 6.7% to 21.7% of patients, resulting in possibly different, but never antagonistic, warfarin management. A previously published study reported clinical disagreement in 26-29% of cases, but the management differed only by minor interventions.[9] Furthermore, considering that VKA patients managed with a POC device should be monitored in this way for a certain period of time, without continuously switching between POC and laboratory INR, this small difference is unlikely to negatively interfere with the clinical management of VKA patients.

Our findings have important implications in the international literature. Despite the recent discovery of the novel direct oral anticoagulants, VKAs will remain the treatment of choice for several categories of patients, such as those with valvular AF, mechanical heart valves or with severe renal insufficiency. Portable coagulometers, compared to traditional laboratory INR, are less invasive and can provide immediate results. Furthermore, POC can allow a more practical INR monitoring, because they can be used in different settings outside the hospital and they can also allow patient self-testing and self-management. Portable coagulometers therefore represent an alternative to standard laboratory INR, and our results can provide reassurance on the accuracy of the CoaguChek XS Plus device.

The strengths of our study include the simultaneous comparison of thrombin generation measured by the CAT with three different INR assays, all using the same thromboplastin in order to reduce possible variability due to this analytical variable. Furthermore, we decided to reduce variability by asking a single investigator to perform all the POC tests. However, there are also some limitations that need to be acknowledged. First, the small number of patients, although similar to previous studies,[17,18] resulted in a small number of INRs above 4.0, thus precluding the possibility of a sensitivity analysis in this patient subgroup. Second, despite the potential to better assess all phases of coagulation, thrombin generation is not yet considered a validated test for monitoring anticoagulation. However, we chose to compare different INR methodologies with thrombin generation because the latter is known to show more variation in VKA patients and has the potential to better identify small differences in test accuracy, than simply comparing different INR methodologies among each other. Third, all POC

measurements were performed by a trained scientist, and our results might not be generalizable, for example, to all patients performing INR self-testing.

In conclusion, our study showed that the relationship between INR results and thrombin generation does not differ depending on the assay used for INR measurement. Despite not being generally considered as the "gold standard", the POC INR showed one of the highest correlation coefficients with thrombin generation, therefore confirming the POC devices as an accurate and valid alternative to laboratory INR in VKA patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

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, S.Meli, K.Hickey, D.Zammit and C.Calamatta contributed to acquisition, analysis and interpretation of data. M.Makris, S.Kitchen and W.Ageno contributed to interpretation of data and critical revision of the manuscript. All authors provided final approval of the manuscript.

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Biomarkers for the diagnosis of venous thromboembolism: D-dimer, thrombin generation, procoagulant phospholipid and soluble P-selectin

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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 patients with VTE.

Aim To assess the accuracy of different coagulation tests (D-dimer, thrombin generation, phospholipid-dependent (PPL) clotting time, soluble P-selectin (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 calibrated automated thrombogram), STA Procoag PPL (Diagnostica Stago) and sP-selectin (Affymetrix; eBioscience). VTE was objectively confirmed by compression ultrasonography, CT 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). Patients with VTE showed significantly higher sP-selectin concentrations compared with 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.

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 CT 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 thrombin generation, procoagulant phospholipid-dependent clotting time and soluble P-selectin (sP-selectin)) that are currently used only for research purposes but might contribute to identify a prothrombotic predisposition. So far, some alterations of the thrombin generation parameters have been reported in patients with VTE, although at a small extent.[6–8] Studies evaluating patients with acute DVT showed increased values of sP-selectin compared with 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 sP-selectin), and their relative importance, as biomarkers of acute VTE.

MATERIALS AND METHODS

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) and samples from patients with VTE confirmed by compression ultrasonography, CT 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 anonymised 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 (CAT)), 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 online supplementary material. 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.

Statistical analysis

Continuous variables were expressed as mean±SD or as median with 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% 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.

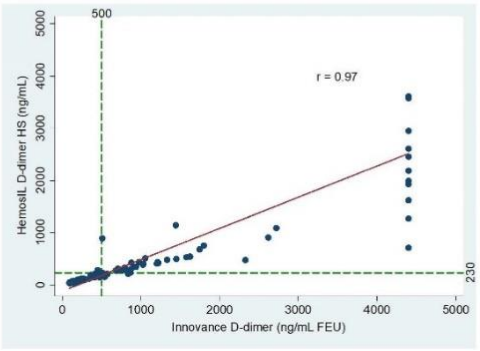

Figure 1 Correlation between the Innovance D-dimer and the HemosIL D-dimer HS. The vertical dashed line represents the cut-off for the Innovance D-dimer (500 ng/mL fibrinogen equivalent units (FEU)), while the horizontal dashed line represents the cut-off for the HemosIL D-dimer HS (230 ng/mL). D-dimer 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.

The correlation between different laboratory tests was evaluated using non-parametric 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 that might have resulted in a different clinical management, the D-dimer values were categorised as positive or negative, according to the manufacturers' cut-offs (500 ng/mL fibrinogen equivalent units (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

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 26.9)
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 (−176.7 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 (s)	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.
PPL, procoagulant phospholipid; VTE, venous thromboembolism.

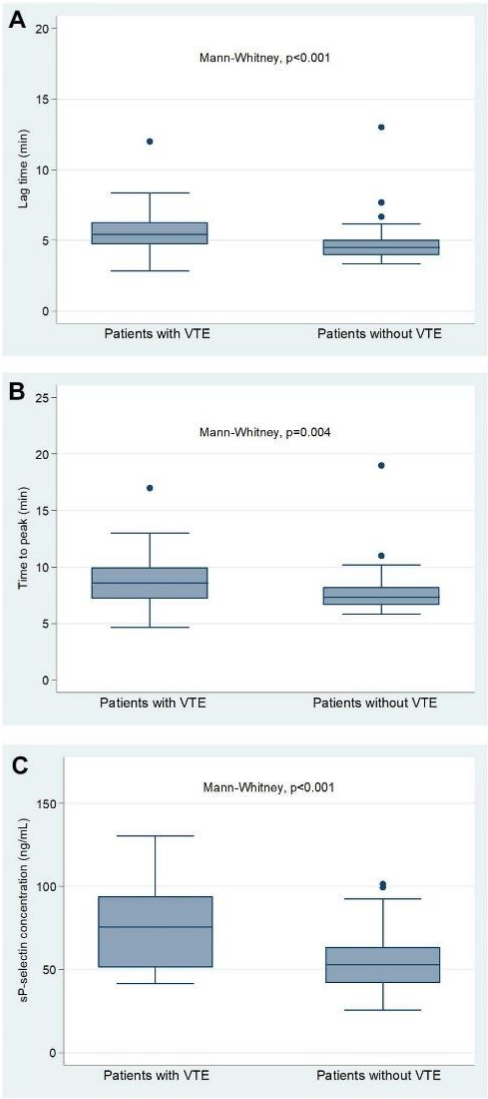

Figure 2 Biomarkers significantly different in patients with and without venous thromboembolism (VTE): lag time (A) and time to peak (B) on the thrombin generation curve, soluble P-selectin concentration (C).

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, phospholipid-dependent 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 three out of nine 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 V.12 (StataCorp LP, College Station, Texas, USA), SAS V.9.4 (SAS Institute, Cary, North Carolina, USA) and R software packages (Party and randomForest).[13–15] Two-tailed p values less than 0.05 were considered statistically significant.

RESULTS

Study population

Overall, 100 samples were collected. Median (IQR) age was 59.0 (41.3–70.2) years, and 47% were men. 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 (men 45.5% vs 47.8%, respectively, p=0.83).

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 categorisation 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 the patients with VTE had a negative D-dimer.

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 with 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 with those with negative D-dimer.

Table 2 Results of thrombin generation, procoagulant phospholipid-dependent clotting time and soluble P-selectin in the three 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 (s)	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 Dunn's test and reported as follows.
*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 1 vs group 2.
§P<0.05 for the comparison group 1 vs group 3.
¶P<0.01 for the comparison group 2 vs group 3.
DD, D-dimer; FEU, fibrinogen equivalent units; PPL, procoagulant phospholipid; VTE, venous thromboembolism.

Procoagulant phospholipid-dependent clotting time

There was no difference in the phospholipid-dependent clotting time between patients with and without VTE (table 1), or between patients with positive versus negative D-dimer (table 2). These results did not change when the phospholipid-dependent clotting time was expressed as ratio (tables 1–2).

Soluble P-selectin

The median concentration of sP-selectin was significantly higher in patients with VTE compared with 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 versus negative D-dimer (table 2).

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); phospholipid-dependent 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).

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 to 0.87); lag time (AUC 0.73; 95% CI 0.61 to 0.85) and time to peak (AUC 0.68; 95% CI 0.56 to 0.81).

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

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 4A,B). In the model without D-dimers, sP-selectin concentration was the most relevant biomarker (figure 4C,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' cut-off, 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).

DISCUSSION

In this study, we evaluated several tests, namely two D-dimers, the thrombin generation, the phospholipid-dependent clotting time and the sP-selectin, as potential biomarkers of VTE. Our results suggest that the sP-selectin concentration has a good

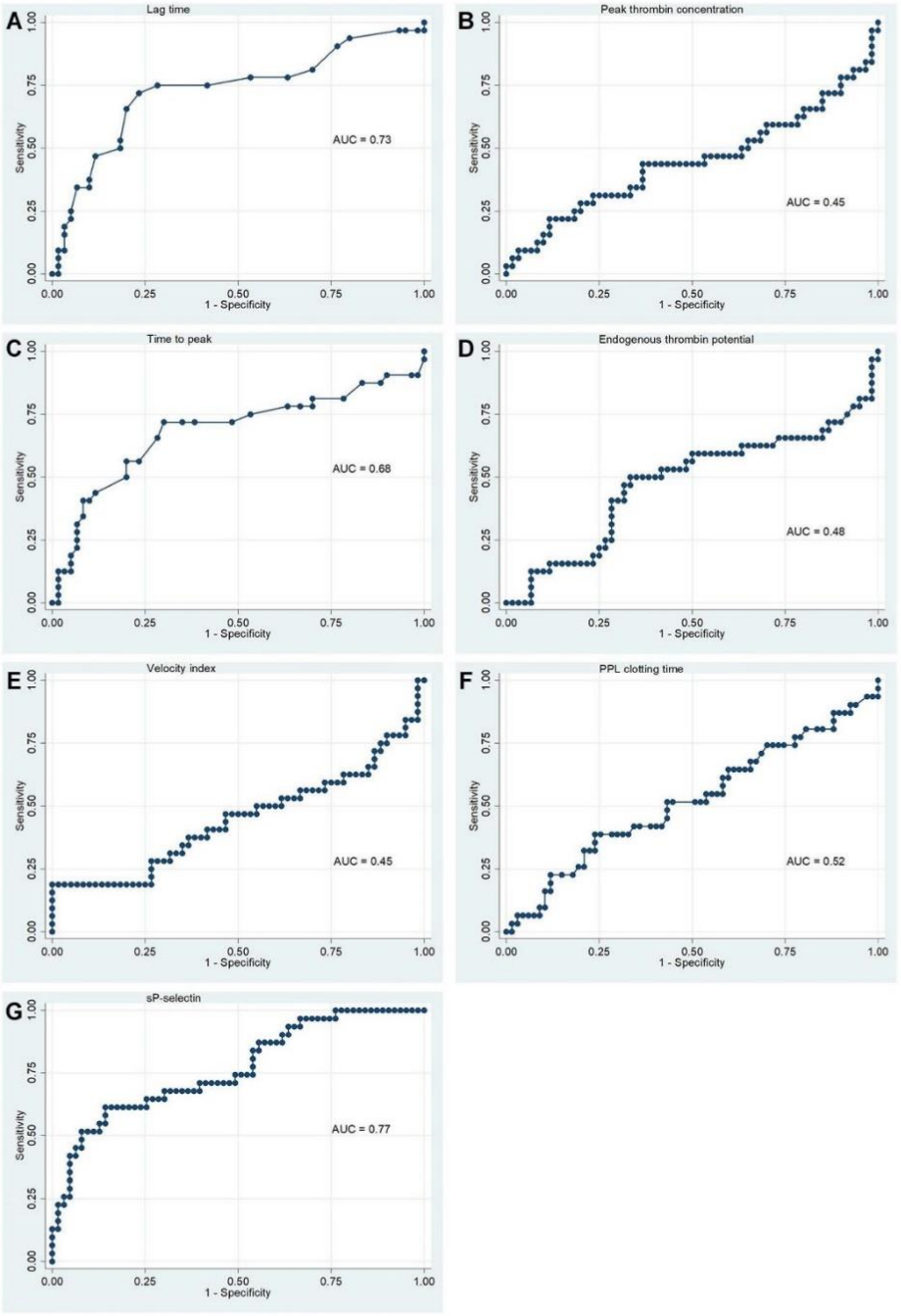

Figure 3 Receiver operating characteristic curve for different biomarkers of venous thromboembolism: lag time (A), peak thrombin concentration (B), time to peak (C), endogenous thrombin potential (D), velocity index (E), PPL clotting time (F), sP-selectin (G). AUC, area under the receiver operating characteristic curve; PPL, procoagulant phospholipid.

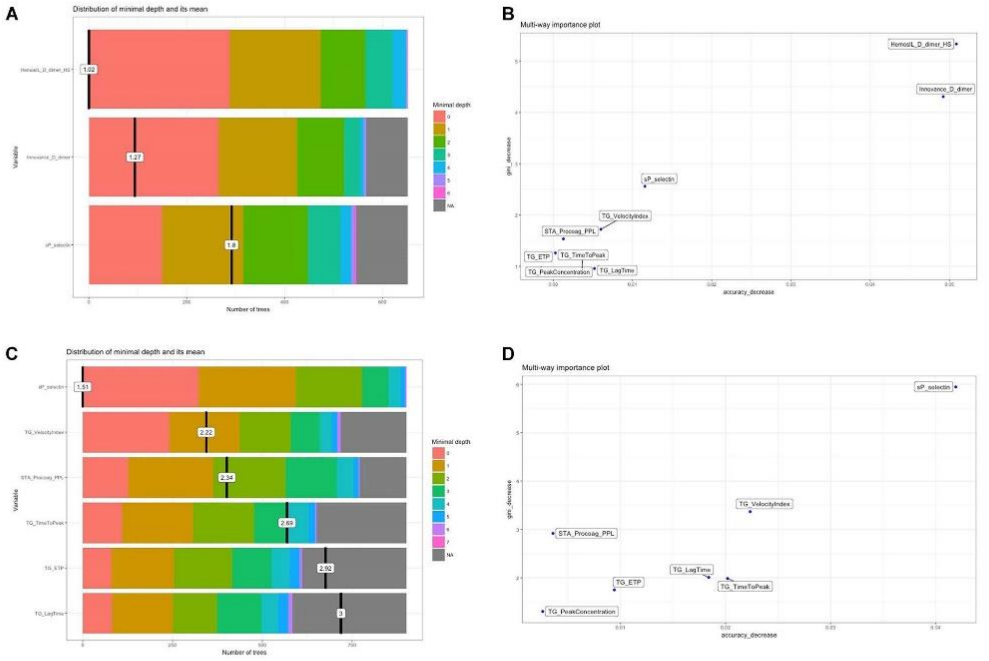

Figure 4 Random forest and multiway importance plot for the model including all potential biomarkers of venous thromboembolism (A, B) and for the model excluding the D-dimers (C, D). In the random forest method,1000 decision trees were grown to form the random forest. A random subset of variable was used at each split point (three random variables out of nine 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 (eg, the variance in a node). The higher decrease of accuracy and Gini index means higher variable importance. ETP, endogenous thrombin potential; TG, thrombin generation.

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 hypothesised 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 with other biomarkers, suggesting a limited application of this test to VTE diagnosis.

Our study, for the first time, evaluated the phospholipid-dependent clotting time in the setting of VTE diagnosis. The phospholipid-dependent 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 cannot

detect their functional activity.[19] Considering the great correlation between the phospholipid-dependent clotting time and flow cytometry,[11 19] we hypothesised that the phospholipid-dependent 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, which 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 with 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 versus 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 versus 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 a 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 demographic 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 patient 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 patients with VTE.[7 8] On the other hand, it is unknown whether the delayed freezing time could have influenced the phospholipid-dependent 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 phospholipid-dependent clotting time. However, these data should be confirmed in large management studies.

Take home messages

- 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 soluble P-selectin (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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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.

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ORIGINAL RESEARCH

Validation and psychometric properties of the Maltese version of the Duke Anticoagulation Satisfaction Scale (DASS)

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Purpose: Assessing treatment satisfaction can guide specific interventions to improve anticoagulation adherence and reduce adverse outcomes. We aimed to assess the psychometric properties (reliability and validity) of the Maltese translation of the Duke Anticoagulation Satisfaction Scale (DASS).

Patients and methods: The DASS explores three dimensions (limitations, hassles/burdens, psychological impact). The translation process included forward and backward translations. Reliability was evaluated through internal consistency and reproducibility. Validity was evaluated through floor/ceiling effect, convergent/discriminant validity, construct validity, and known-group validity.

Results: The Maltese version of the DASS, administered to 174 patients on warfarin for different clinical indications, showed good reliability (Cronbach's alpha 0.87; intraclass correlation coefficient for test retest 0.73). Floor effect was identified mainly in the limitations and hassles/burdens subscales. Significant positive correlations were found between the DASS total score and its subscales (limitations 0.80, hassles/burdens 0.85, psychological impact 0.68). Female sex, shorter warfarin treatment duration (≤5 years), previous hospitalization and history of bleeding were associated with lower satisfaction.

Conclusion: Psychometric properties of the Maltese DASS were comparable to the original English version. The Maltese version of the DASS is a valid and reliable instrument that can be used by health care professionals to assess the level of satisfaction of Maltese-speaking anticoagulated patients.

Keywords: atrial fibrillation, psychometrics, quality of life, surveys and questionnaires, venous thromboembolism, warfarin

Introduction

As anticoagulant therapy is a chronic treatment for most clinical indications, it can affect patients' quality of life and satisfaction.[1–3] Vitamin K antagonists (VKA) are one of the oral anticoagulant medications currently available for the treatment and secondary prevention of venous thromboembolism (VTE) and for stroke prevention in patients with atrial fibrillation (AF).[4] VKAs require routine laboratory monitoring in order to maintain the international normalized ratio (INR) within the therapeutic target range and have several food/drug restrictions, therefore contributing to the burden of these medications.[2,4]

Health-related quality of life (QoL) is an important aspect in the decision-making process and can be measured through the use of generic scales and condition-specific

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scales. Generic scales, e.g., the 36-item Short Form[5] or the 12-item Short Form,[6] can be applied to patients with different conditions, such as rheumatological, orthopedic and hematological diseases.[7–9] Specific scales measure aspects of QoL that are related to a particular condition, for instance, the Duke Anticoagulation Satisfaction Scale (DASS) and the Perception of Anticoagulant Treatment Questionnaire (PACT-Q) measure the QoL of anticoagulated patients.[10,11] Understanding the degree of satisfaction associated with the anticoagulant treatment allows for specific interventions to focus on increasing the anticoagulation adherence and reduce adverse clinical outcomes. However, there was no such scales assessing the satisfaction of anticoagulated patients that had been validated in the Maltese language.

We chose to translate the DASS and the PACT-Q because they have been used in several studies enrolling patients with various clinical indications to the anticoagulant treatment.[10,12–19] The aim of this study was to assess the psychometric properties (reliability and validity) of the Maltese version of the DASS. The psychometric properties of the Maltese version of the PACT-Q have been reported in a separate paper.[20]

Materials and methods

The DASS

The DASS explores three dimensions: limitations (9 items), hassles and burdens (8 items) and psychological impact (5 positive items and 3 negative items).[10] Therefore, the DASS has 25 items, each with 7 possible responses (not at all, a little, somewhat, moderately, quite a bit, a lot, and very much). Six items require reverse-coding prior to analysis. The final score can range from 25 to 175, with lower scores representing greater satisfaction and less hassles and burdens.[10]

Permission to translate and use the DASS was obtained from the corresponding author of the original DASS paper.[10] According to published guidelines,[21,22] different people, all bilingual in English and Maltese (a professional translator, a health psychologist, and a speech and language pathologist), were involved in the translations of the DASS, with two forward translations from English to Maltese and a backward translation from Maltese to English. For the pilot testing of the Maltese version of the DASS, the questionnaire was completed and discussed with 5 patients on long-term oral anticoagulant treatment (not included in this analysis).

Study population

The Maltese version of the DASS was completed by 174 patients on warfarin treatment for different clinical indications, enrolled from the Anticoagulation Clinics at 5 Health Centres (Cospicua, Floriana, Mosta, Qormi, Rabat) and at Mater Dei Hospital (Msida) in Malta. INR testing at the Health Centres is performed using point-of-care devices, while at Mater Dei Hospital is performed using traditional venepuncture. In both settings, warfarin dose adjustment is decided by the attending physicians. Patients with cognitive impairment, dementia or major psychiatric disorders (such as schizophrenia) were excluded.

The questionnaires were distributed by two authors (NR, CBX) between July 2017 and February 2018 and patients were also asked to fill in a form on sociodemographic data (age, sex, living situation, level of education, working status, and self-reported history of any bleeding). Questionnaires were identified using a code, to ensure anonymity. The researchers had the list with the correspondence between the code and the demographic details, which was used to contact the patients only in case of missing values. Therefore, there were no missing answers in our cohort. From a review of clinical notes, we also collected information on clinical indication and duration of the anticoagulant treatment, INR on the day of enrollment, INR results and any hospitalization in the previous year.

For the purpose of this validation study, we also considered 157 patients who completed the English version of the DASS, enrolled from the same Anticoagulation Clinics during the same time frame. Malta is a bilingual country, where Maltese is the national language and English is considered a co-official language.[23] The choice of whether to complete the Maltese or the English version of the DASS was left at the discretion of each patient.

A randomly chosen sample of 40 patients was retested after 7–14 days: 20 patients who initially completed the Maltese version and 20 patients who initially completed the English version. Half of each group was retested in the same language (to estimate the intra-language correlation), while the other half was retested in the other language (to estimate the cross-language correlation).

This study was approved by the University of Malta Research and Ethics Committee (Ref No 07/2016) and all patients signed an informed consent form before inclusion.

Statistical analysis

Continuous variables were expressed as mean ± SD and compared using the Student's independent samples *t*-test;

categorical variables were expressed as counts and percentages and compared using the Chi-square or the Fisher's exact tests, as appropriate.

For the calculation of the DASS score, six items (3h, 4a, 4b, 4f, 4h and 4j) were reverse-coded, as reported in the original publication.[10]

Reliability of the Maltese version of the DASS was evaluated through internal consistency and reproducibility.[24] The internal consistency (correlation between different items on the same scale or subscale) was assessed using the Cronbach's alpha coefficient, with a value ≥0.70 considered acceptable.[25]

Reproducibility was assessed in the subgroup of patients who participated in the test–retest in the same language and the intraclass correlation coefficients (ICC) were calculated (intra-language correlation for the Maltese–Maltese test–retest and the English–English test–retest). Commonly cited cut-off for ICC considers acceptable values between 0.60 and 0.74.[26] We evaluated the cross-language test–retest correlation in those patients who participated in the test–retest in the other language. We calculated the raw cross-language correlation and the adjusted cross-language correlation (adjusted for score unreliability by dividing the raw cross-language test–retest correlation by the square-root of the product of the Maltese–Maltese and English–English test–retest intra-language correlation).[27,28] For the cross-language correlation, the English–Maltese and Maltese–English test–retest groups were pooled together, as previously done.[28]

Validity of the Maltese version of the DASS was evaluated through floor and ceiling effect, convergent and discriminant validity, construct validity, and known-group validity. Floor effect occurs when a significant proportion of respondents (>15%) achieves the lowest score; vice versa, ceiling effect occurs when a significant proportion of respondents (>15%) achieves the highest score, meaning that there is more variance that the questionnaire is able to capture (limited content validity).[29]

Convergent and discriminant validity was assessed through factor analysis. An exploratory factor analysis with varimax rotation was performed to examine the structure of the DASS. In the confirmatory factor analysis the following parameters were calculated: root mean square error of approximation (RMSEA), where values ≤0.05 correspond to "good fit," and values ≤0.08 correspond to "acceptable fit"; standardized root mean squared residual (SRMR), where values ≤0.05 correspond to "good fit," and values ≤0.10 correspond to "acceptable fit"; goodness-of-fit index (GFI), adjusted goodness-of-fit index (AGFI) and comparative fit index (CFI), where values ≥0.90 are considered "acceptable fit."[30]

Construct validity was evaluated by assessing the Pearson's correlation between different subscales and between each subscale and the overall DASS (scale-subscale validity).[31]

Known-group validity was evaluated by assessing the correlation (Pearson's or point-biserial correlation as appropriate) between the overall DASS score and the following covariates, some of which have been previously shown to correlate with patients' satisfaction[10,12,13,32,33]: increasing age; male sex; living alone; level of education primary school only; full-time or part-time paid employment; AF as clinical indication to anticoagulant treatment; warfarin treatment duration >5 years; INR in range at enrollment; high time within therapeutic range (TTR ≥70%, calculated according to the Rosendaal method[34]) in the previous 12 months; any hospitalization in the previous 12 months; self-reported history of any bleeding on warfarin.

We planned a sample size of at least 150 patients, given that published recommendations considered adequate a sample size of at least 50 patients[29] and that previous validation studies enrolled approximately 100 patients.[35,36]

Statistical analysis was performed using the statistical software STATA SE v.12 (StataCorp LP, College Station, TX, USA) and SAS v. 9.4 (SAS Institute Inc, Cary, NC, USA), with two-tailed $P<0.05$ considered statistically significant.

Results

Study population

Baseline characteristics of the enrolled patients, including sociodemographic details, are reported in Table 1. The comparison between patients who completed the Maltese and the English version of the DASS showed some differences: there was a predominance of female ($P=0.01$) and primary school level of education ($P<0.001$) among those subjects who completed the Maltese version of the DASS; while full- or part-time employment ($P<0.001$) and heart valve replacement as indication to warfarin ($P=0.02$) were more common among those subjects who completed the English version of the DASS. While the overall DASS score was similar in the two cohorts, patients who completed the Maltese version obtained higher scores in the psychological impact subscale (mean ± SD 23.4±6.2 for the Maltese version vs 21.3±7.4 for the English version, $P=0.006$), corresponding to lower anticoagulation satisfaction.

Table 1 Baseline characteristics of the study population

	Patients who completed the Maltese questionnaire (n=174)	Patients who completed the English questionnaire (n=157)	*P*-value
Age (years), mean (SD)	70 (10.1)	69.8 (10.2)	0.87
Females, n (%)	92 (52.9)	61 (38.9)	0.01
Living situation, n (%)			0.82
Living with family members	138 (79.3)	124 (79.0)	
Living alone	31 (17.8)	30 (19.1)	
Other	5 (2.9)	3 (1.9)	
Level of education, n (%)			<0.001
Primary school	108 (62.1)	36 (22.9)	
Secondary school	48 (27.6)	67 (42.7)	
College or above	18 (10.3)	54 (34.4)	
Employment status, n (%)			<0.001
Full-time or part-time paid employment	18 (10.3)	25 (15.9)	
Retired/pension	103 (59.2)	114 (72.6)	
Other (homemaker/housewife, unemployed)	53 (30.5)	18 (11.5)	
Clinical indications to warfarin, n (%)[a]			
Atrial fibrillation	122 (70.1)	97 (61.8)	0.11
Venous thromboembolism	30 (17.2)	23 (14.7)	0.52
Heart valve replacement	25 (14.4)	38 (24.2)	0.02
Other	25 (14.4)	9 (5.7)	0.32
Warfarin treatment duration, n (%)			0.15
≤5 years	98 (56.3)	76 (48.4)	
>5 years	76 (43.7)	81 (51.6)	
INR at enrollment, n (%)			0.31
In range	99 (56.9)	98 (62.4)	
Other (above or below range)	75 (43.1)	59 (37.6)	
High TTR (≥70%) in the previous year, n (%)[b]	96 (56.5)	89 (59.7)	0.56
Any hospitalisation in the previous year, n (%)[b]	86 (50.6)	77 (51.7)	0.85
Self-reported history of any bleeding, n (%)	63 (36.2)	50 (31.9)	0.40
Site of enrollment, n (%)			0.96
Health centers	86 (49.4)	78 (49.7)	
Mater Dei Hospital	88 (50.6)	79 (50.3)	
DASS results, mean (SD)			
Overall score	56.7 (18.5)	53.6 (16.9)	0.18
Limitations subscale	17.1 (9.4)	17.0 (8.0)	0.92
Hassles/burdens subscale	16.2 (8.0)	15.3 (6.7)	0.27
Psychological impact subscale	23.4 (6.2)	21.3 (7.4)	0.006

Notes: [a]More than one option is possible. [b]Data available only in 170 patients who completed the Maltese version and 149 patients who completed the English version.
Abbreviations: INR, international normalized ratio; TTR, time within therapeutic range; DASS, Duke Anticoagulation Satisfaction Scale.

Internal consistency

The internal consistency of the Maltese translation of the DASS was good with the following Cronbach's alpha coefficients: 0.87 for the overall DASS total score; 0.86 for the limitations subscale (9 items); 0.84 for the hassles and burdens subscale (8 items); 0.65 for the positive (5 items) and 0.64 for the negative (3 items) psychological impact subscales. When the 8 items of the psychological impact subscale were considered together, Cronbach's alpha was 0.57.

The English version of the DASS also shows good internal consistency in our cohort: overall DASS Cronbach's alpha 0.85; limitations subscale 0.82; hassles and burdens subscale 0.79; positive psychological impact 0.79; negative psychological impact 0.56; overall psychological impact 0.71.

Details of the internal consistency analysis are reported in Table 2. No significant increase or decrease of the Cronbach's alpha coefficients was observed when each question was deleted.

Reproducibility

ICC for the intra-language correlation was very good, being 0.73 for the DASS total score in the Maltese–Maltese test–retest and 0.85 in the English–English test–retest. Further details of the intra-language correlation, including DASS subscales, are reported in Table S1.

ICC for the cross-language correlation for the DASS total score was 0.31 and the adjusted cross-language correlation was 0.39. When analyzed separately, ICC for the English–Maltese test–retest was 0.59, while ICC for the Maltese–English test–retest was 0.

Floor and ceiling effect

When we analyzed the response distribution for each item of the Maltese translation of the DASS, a significant floor effect was identified, mainly in questions pertaining to the limitations and hassles/burdens subscales (Table 3). Except for one question, no significant ceiling effect was detected. However, a significant floor effect was observed also in the English version of the DASS in our study (Table S2) and in the original publication of the DASS.[10]

We subsequently analyzed the results of the overall DASS score and each subscale. In the Maltese version floor effect was 0% for the overall DASS, 15.5% for limitations, 3.5% for hassles/burdens, and 0% for psychological impact (1.2% for positive and 12.1% for negative psychological impact). In the English version floor effect was 0% for the overall DASS, 12.1% for limitations, 14.7% for hassles/burdens, and 1.9% for psychological impact (6.4% for positive and 14.0% for negative psychological impact). Ceiling effect was 0% for all subscales in both languages.

Factor analysis

Results of the confirmatory factor analysis were unsatisfactory. For the Maltese version of the DASS, RMSEA and SRMR (both 0.13) were slightly above the acceptable value (≤0.08 and ≤0.10, respectively); whereas GFI (0.64), AGFI (0.57) and CFI (0.67) were all below the adequate fit level (≥0.90). However, we obtained similar unsatisfactory results for the English version of the DASS in our study cohort: RMSEA 0.10, SRMR 0.14, GFI 0.66, AGFI 0.65, CFI 0.63. Detailed results of the factor analysis and the rotated factor pattern, in comparison to previously published studies,[10,13] is reported in Table 4.

Correlation scale-subscales

We found a statistically significant positive correlation between the DASS total score and its main subscales. For the Maltese version correlation coefficients were 0.80 for limitations, 0.85 for hassles and burdens, and 0.68 for psychological impact. For the English version correlation coefficients were 0.74, 0.82, and 0.74, respectively. Details of the correlation between each subscale are reported in Table S3.

Known-group validity

The Maltese version of the DASS showed a significant positive correlation with previous hospitalization and previous bleeding events, a significant negative correlation with longer anticoagulant treatment duration, and a borderline negative correlation with male sex. These findings suggest that female sex, shorter treatment duration (≤5 years), previous hospitalization and history of bleeding are associated with lower satisfaction.

The English version of the DASS showed a significant negative correlation with increasing age and male sex, and a significant positive correlation with paid-employment status and previous bleeding. These findings suggest that young age, female sex, full- or part-time paid employment and history of bleeding are associated with lower satisfaction.

However, for all the significant sociodemographic and clinical characteristics the correlation with the DASS total score was in the same direction for both languages, although sometimes lacking of statistical significance (Table 5).

Table 2 Internal consistency of the Maltese and English versions of the DASS in our study cohort

DASS	Item	Maltese version			English version		
		Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted	Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted
DASS score (overall)		0.87			0.85		
Limitations subscale		0.86			0.82		
	1a		0.60	0.85		0.54	0.79
	1b		0.63	0.85		0.59	0.79
	1c		0.72	0.84		0.48	0.80
	1d		0.70	0.84		0.63	0.79
	1e		0.72	0.84		0.66	0.78
	2a		0.46	0.86		0.46	0.80
	2b		0.31	0.88		0.43	0.81
	2c		0.60	0.85		0.47	0.81
	2d		0.70	0.84		0.56	0.79
Hassles/burdens subscale		0.84			0.79		
	3a		0.59	0.82		0.51	0.76
	3b		0.73	0.80		0.61	0.74
	3c		0.70	0.81		0.57	0.75
	3d		0.48	0.83		0.66	0.74
	3e		0.80	0.79		0.70	0.73
	3f		0.56	0.82		0.38	0.78
	3g		0.78	0.79		0.74	0.73
	3h		0.004	0.88		0.12	0.86
Psychological impact (positive) subscale		0.65			0.79		
	4a		0.34	0.63		0.60	0.74
	4b		0.54	0.55		0.69	0.72
	4f		0.38	0.65		0.49	0.79
	4h		0.48	0.59		0.60	0.75
	4j		0.42	0.59		0.55	0.76
Psychological impact (negative) subscale		0.64			0.56		
	4d		0.41	0.62		0.39	0.45
	4g		0.55	0.40		0.45	0.33
	4i		0.41	0.59		0.30	0.56

Abbreviation: DASS, Duke Anticoagulation Satisfaction Scale.

Discussion

In our study, for the first time, the DASS was translated into the Maltese language and we administered this questionnaire to a group of patients on warfarin treatment for different clinical indications. We evaluated the reliability and the validity of the Maltese translation in comparison to the psychometric properties of the DASS previously reported in the literature and to the original English version completed by a different group of patients in our study cohort. We found that the Maltese DASS has good reliability and an acceptable level of validity.

Table 3 Response distribution for each DASS item of the Maltese translation and summary statistics

DASS item	Response category (%)							Mean (SD) in our study (Maltese version)	Mean (SD) in our study (English version)	Mean (SD) in the study by Samsa et al[10]
	1	2	3	4	5	6	7			
1a	**74.1**	13.2	1.7	3.5	4.0	2.9	0.6	1.61 (1.31)	1.59 (1.28)	1.84 (1.37)
1b	**75.9**	12.1	3.5	2.9	1.7	3.5	0.6	1.55 (1.25)	1.50 (1.12)	1.36 (0.99)
1c	**60.3**	16.1	6.3	2.9	4.6	6.3	3.5	2.08 (1.76)	2.04 (1.51)	1.69 (1.36)
1d	**76.4**	12.6	2.3	2.3	1.7	3.5	1.2	1.55 (1.30)	1.46 (1.14)	1.84 (1.78)
1e	**72.4**	12.6	3.5	5.8	1.2	3.5	1.2	1.66 (1.35)	1.62 (1.19)	1.88 (1.31)
2a	**39.1**	24.7	13.2	9.8	8.1	4.6	0.6	2.39 (1.54)	2.10 (1.35)	2.60 (1.66)
2b	**70.1**	13.2	2.3	4.6	2.3	4.6	2.9	1.81 (1.60)	2.14 (1.59)	1.97 (1.89)
2c	**46.6**	19.5	10.9	4.0	9.2	5.8	4.0	2.43 (1.82)	2.71 (1.96)	3.02 (2.12)
2d	**55.2**	20.1	8.1	8.1	2.3	4.0	2.3	2.03 (1.55)	1.86 (1.21)	2.20 (1.43)
3a	**63.2**	17.8	5.8	5.2	2.3	4.0	1.7	1.84 (1.47)	1.80 (1.19)	1.78 (1.22)
3b	**46.6**	19.0	9.8	11.5	4.6	5.8	2.9	2.37 (1.72)	2.15 (1.49)	2.09 (1.25)
3c	**62.1**	20.1	5.2	6.3	3.5	2.9	0.0	1.78 (1.29)	1.66 (1.16)	1.65 (1.09)
3d	**74.1**	10.3	5.2	2.9	4.0	3.5	0.0	1.63 (1.30)	1.78 (1.08)	1.76 (0.97)
3e	**57.5**	16.1	7.5	6.3	5.2	6.3	1.2	2.09 (1.64)	1.85 (1.35)	1.76 (1.24)
3f	**69.0**	14.4	7.5	3.5	4.6	0.6	0.6	1.64 (1.20)	1.33 (0.90)	1.37 (0.90)
3g	**52.3**	23.0	5.8	7.5	5.2	5.2	1.2	2.10 (1.57)	1.94 (1.19)	1.81 (1.17)
3h	7.5	52.3	23.0	5.2	4.0	4.6	3.5	2.74 (1.39)	2.77 (1.98)	2.90 (2.19)
4a	4.0	31.6	30.5	16.7	4.6	10.3	2.3	3.26 (1.44)	2.54 (1.50)	2.32 (1.67)
4b	4.0	40.2	28.2	17.2	4.0	4.6	1.7	2.98 (1.26)	2.64 (1.46)	2.78 (1.66)
4d	**20.7**	15.5	10.9	18.4	14.9	14.9	4.6	3.55 (1.89)	2.87 (1.77)	2.55 (1.64)
4f	7.5	19.0	12.1	10.3	8.6	14.9	**27.6**	4.49 (2.12)	4.01 (2.01)	4.15 (2.08)
4g	**58.7**	14.9	6.9	9.2	4.6	5.2	0.6	2.04 (1.56)	2.24 (1.48)	2.00 (1.34)
4h	11.5	59.2	18.4	6.9	1.2	1.2	1.7	2.37 (1.08)	2.41 (1.35)	2.55 (1.60)
4i	**55.8**	20.1	6.3	10.9	3.5	2.9	0.6	1.97 (1.41)	1.68 (1.22)	1.75 (1.23)
4j	10.9	45.4	24.1	11.5	2.3	2.9	2.9	2.69 (1.32)	2.91 (1.93)	2.42 (1.73)

Note: Numbers in bold in the response category section indicate significant floor or ceiling effect.
Abbreviation: DASS, Duke Anticoagulation Satisfaction Scale.

The internal consistency was very good, with Cronbach's alpha coefficients for the overall DASS score and the subscales limitations and hassles/burdens above 0.80. The Cronbach's alpha coefficients were slightly below the acceptable cut-off for the positive and negative psychological impact subscales (0.65 and 0.64, respectively). However, lower coefficients were reported in another study evaluating the Brazilian-Portuguese version of the DASS (0.67 and 0.38, respectively)[13] and in the English version in our study cohort (0.79 and 0.56, respectively). The low number of items included in these two subscales (5 questions in the positive impact and 3 questions in the negative impact) might be contributing to this finding.

Test–retest reliability (intra-language correlation) of the DASS in our cohort was very good with an ICC of 0.73 for the Maltese version and 0.85 for the English version, which are above the desirable cut-off and similar to values reported in the literature.[10,32] Given the peculiarity of the Maltese population with high percentage of bilingual subjects,[37] we were also able to assess the cross-language correlation. Despite the rigorous process of translation, the ICC for the cross-language correlation was lower than the ICC for the intra-language correlation. However, this finding has been previously reported by several authors[27,38] and different solutions have been proposed. Wood et al hypothesized that it could be due to score unreliability and suggested to adjust the cross-language correlation by the intra-language correlation,[27] while Chung et al hypothesized that it could be due to poor bilingual proficiency and performed a sub-analysis of subjects with higher level of education.[38] In agreement with the study by Wood et al,[27] we obtained better cross-language correlation when applying the suggested adjustment. In our study, the number of retested patients with higher level of education was too low to perform a specific sub-analysis. However, when we analyzed separately the English–Maltese and Maltese–English test–retest, we

Table 4 Results of the 3-factor analysis in our study cohort, in comparison to previously published studies

	Our study (Maltese version)			Our study (English version)			Pelegrino et al[13] (Brazilian-Portugues version)			Samsa et al[10] (English version)		
Item	Limitations	Hassles/ burdens	Psychological impact	Limitations	Hassles/ burdens	Psychological impact	Limitations	Hassles/ burdens	Psychological impact	Limitations	Hassles/ burdens	Psychological impact
1a	**0.81**	0.23	0.11	**0.70**	0.14	0.05	**0.76**	0.12	−0.09	**0.68**	0.34	−0.07
1b	**0.75**	0.14	−0.05	**0.77**	0.23	0.07	**0.45**	0.18	0.16	**0.67**	0.30	−0.04
1c	**0.59**	0.36	−0.29	**0.43**	0.21	−0.05	**0.41**	0.17	−0.38	**0.50**	0.09	−0.04
1d	**0.86**	0.13	−0.06	**0.83**	0.10	−0.07	**0.56**	0.13	−0.19	**0.77**	0.13	−0.12
1e	**0.84**	0.31	−0.04	**0.84**	0.24	−0.03	**0.67**	0.11	−0.04	**0.81**	0.31	−0.06
2a	0.19	0.35	**−0.44**	0.19	**0.42**	−0.05	0.24	0.13	**−0.31**	**0.56**	0.32	−0.16
2b	0.16	0.07	**−0.43**	0.18	**0.38**	−0.004	0.07	**0.37**	−0.19	**0.43**	−0.06	0.06
2c	0.28	0.41	**−0.51**	0.26	**0.35**	−0.14	0.18	**0.38**	−0.27	**0.48**	0.22	−0.04
2d	0.45	**0.68**	−0.19	0.36	**0.59**	0.02	**0.47**	0.39	0.03	**0.75**	0.41	−0.02
3a	0.33	**0.61**	−0.14	0.34	**0.52**	0.09	0.30	**0.46**	0.23	0.51	**0.60**	−0.01
3b	0.09	**0.77**	0.10	0.08	**0.70**	0.08	0.15	**0.55**	0.05	0.34	**0.65**	0.06
3c	0.32	**0.73**	−0.10	0.24	**0.61**	0.17	0.21	**0.59**	0.22	0.19	**0.64**	0.08
3d	−0.22	**0.58**	0.34	−0.03	**0.72**	0.11	0.05	**0.60**	−0.06	0.26	**0.71**	−0.02
3e	0.14	**0.85**	0.16	0.04	**0.83**	0.17	0.05	**0.71**	0.15	0.12	**0.81**	0.00
3f	0.25	**0.55**	0.18	0.11	**0.41**	−0.03	0.10	**0.63**	0.07	0.27	**0.44**	−0.05
3g	0.10	**0.84**	0.09	0.04	**0.86**	0.06	0.04	**0.74**	0.08	0.20	**0.77**	0.06
3h	0.05	−0.04	**0.39**	−0.08	0.04	**0.52**	0.03	−0.07	**0.43**	0.04	−0.17	**0.51**
4a	0.10	0.15	**0.40**	0.02	0.05	**0.72**	−0.03	0.27	**0.55**	0.06	−0.06	**0.76**
4b	0.14	0.06	**0.54**	0.08	0.09	**0.82**	−0.11	0.16	**0.62**	0.01	0.11	**0.83**
4d	**0.39**	0.36	−0.005	0.31	**0.35**	0.06	0.41	0.17	**−0.53**	**0.58**	0.38	0.07
4f	−0.14	−0.05	**0.43**	−0.05	−0.02	**0.58**	0.01	0.20	**0.42**	−0.25	−0.07	**0.57**
4g	0.26	**0.50**	−0.11	0.26	**0.45**	0.07	**0.67**	0.02	0.14	0.29	**0.64**	0.03
4h	−0.05	0.14	**0.34**	0.06	0.30	**0.63**	0.09	0.20	**0.72**	−0.02	0.38	**0.74**
4i	0.23	**0.57**	−0.06	0.14	**0.32**	0.16	0.19	**0.48**	−0.07	0.07	**0.68**	0.06
4j	−0.004	0.06	**0.49**	0.01	−0.005	**0.61**	0.22	−0.24	**0.70**	−0.15	0.20	**0.70**

Note: Numbers in bold indicate the highest loading of each factor, which is therefore likely to explore that dimension.

Table 5 Correlation between satisfaction and sociodemographic or clinical characteristics

Variable	Correlation coefficient for DASS total score for the Maltese version (*P*-value)	Correlation coefficient for DASS total score for the English version (*P*-value)
Increasing age	−0.12 (0.12)	−0.25 (0.002)
Sex: male	−0.15 (0.05)	−0.22 (0.004)
Living situation: living alone	−0.02 (0.72)	0.06 (0.45)
Level of education: primary school only	−0.05 (0.54)	0.01 (0.86)
Employment status: full- or part-time paid employment	0.09 (0.37)	0.17 (0.04)
Clinical indication to anticoagulation: atrial fibrillation	−0.09 (0.23)	−0.08 (0.30)
Anticoagulant treatment duration: >5 years	−0.18 (0.02)	−0.05 (0.55)
INR in range at enrollment	−0.06 (0.41)	−0.01 (0.94)
High TTR (≥70%) in the previous year	−0.01 (0.86)	−0.05 (0.53)
Any hospitalization in the previous year	0.17 (0.03)	0.14 (0.09)
Self-reported history of any bleeding on warfarin	0.20 (0.01)	0.20 (0.01)

Note: Since lower DASS total scores represent greater satisfaction, a negative correlation means greater satisfaction.
Abbreviations: INR, international normalized ratio; TTR, time within therapeutic range; DASS, Duke Anticoagulation Satisfaction Scale.

observed poor results only in the latter. This finding suggests that those patients who initially chose to complete the Maltese version of the questionnaire were probably less confident in English, in line with the hypothesis by Chung et al.[38]

While ceiling effect was found only in one question, a significant floor effect was observed in both the Maltese and the English versions in our study, as well as in the original publication of the DASS,[10] suggesting that it is more likely to be an intrinsic characteristic of the questionnaire itself rather than a weakness of the Maltese translation.

Results of the confirmatory factor analysis were not satisfactory: RMSEA and SRMR were slightly above the acceptable limit; while GFI, AGFI and CFI were significantly below the acceptable cut-off. However, the fit parameters were not reported in previous studies, making it impossible to compare these results. Therefore, we performed an additional exploratory factor analysis which showed that the factors load on the three subscales was similar to previous publications.[10,13]

Regarding construct validity, a significant positive correlation was found between the overall DASS score and its subscales, in line with the fact that the DASS total score is the sum of its three subscales (limitations, hassles and burdens, and psychological impact).

For the known-group validity, both versions of the DASS in our study correlated with sex and history of bleeding. The Maltese version correlated also with anticoagulant treatment duration and hospitalization in the previous year, while the English version correlated also with age and employment status. Differences in the study population between patients who completed the Maltese and the English version of the DASS might have contributed to these slightly different results. Similarly, previous studies reported that younger age[10,13] and working status[12,32] were associated with lower satisfaction, suggesting that the burden of anticoagulant therapy and INR monitoring is greater for young working people, compared to older retirees. In addition, hospitalization for bleeding correlated with lower satisfaction in the studies by Samsa et al[10] and Radaideh et al.[32] Although in our study we used two different definitions (hospitalization in the previous year and self-reported history of any bleeding), they showed the same correlation with the DASS total score. Finally, higher treatment satisfaction was reported by those patients on VKA treatment for more than 5 years.

Anticoagulant therapy is the mainstay for the treatment of VTE and for stroke prevention in patients with AF or mechanical heart valves. In Malta around 1% of the population is anticoagulated,[39] corresponding to approximately 4000 people. However, to the best of our knowledge, there was no specific quality of life instrument for anticoagulated patients that had been validated in the Maltese language. Assessing the QoL is important in order to improve the quality of care for anticoagulated patients. It has been shown that satisfaction correlates with adherence and INR control[40,41] and that, in turn, the TTR correlates with thromboembolic and bleeding complications.[42] By identifying limitations and hassles associated with VKA treatment, health care professionals can provide specific educational interventions to improve the burden associated with this treatment. Furthermore, they can also

reinforce the positive aspects which are salient for each patient. In our research project, we decided to translate the DASS and the PACT-Q, because they are both psychometric questionnaires that can be specifically used to assess the quality of life of anticoagulated patients. Therefore, they were administered at the same time to the same patients' population (results of the validation of the PACT-Q2 are reported elsewhere).[20] The PACT-Q2 is shorter (20 questions to be answered on a 5-point Likert scale), however the items of the two dimensions "convenience" and "treatment satisfaction" have to be scored separately. Conversely, the DASS is slightly longer (25 questions to be answered on a 7-point scale) but has the advantage that can provide one overall final score. The DASS was originally developed in the English[10] and, so far, it has been translated and validated in Greek,[12] Brazilian-Portuguese,[13] Malay[32] and Turkish.[33] In our study, it was translated in Maltese and tested in 157 patients who completed the Maltese version and in 174 patients who completed the English version. The availability of a large number of patients enrolled from the same setting in a short time frame, who completed the questionnaire in two different languages is a peculiarity of our study. In fact, both English and Maltese are official languages in Malta and it has been estimated that more than two thirds of the population are bilingual,[37] although there is the prevalence of one language over the other in certain areas (e.g., Maltese predominates as spoken language, while English is mainly used in written communications).[23] In our study patients were enrolled from different locations around the Maltese island, therefore our sample is likely to be generalizable to the overall anticoagulated Maltese population. Furthermore, although we included only patients on VKA, at the time of enrollment the overwhelming majority of anticoagulated patients in Malta were on warfarin, since the novel direct oral anticoagulants were not centrally funded.

The main strengths of our study are the completeness of data (no missing answers) and the rigorous analysis to validate the Maltese version of the DASS. Furthermore, a group of patients in our study cohort completed the original English version of the DASS, therefore allowing a comparison of the psychometric properties of the two versions in the same population.

However, our study has also some limitations which need to be acknowledged. First, only patients on VKA treatment were enrolled; therefore, our results might not be generalizable to patients treated with other oral anticoagulants. Second, the number of patients included in the test–retest was smaller than the generally recommended size by Terwee et al[29] for the calculation of the ICC.

Conclusion

The Maltese version of the DASS, administered to patients receiving oral anticoagulation with VKA, showed good reliability and an acceptable level of validity. These findings were comparable to the original English version. The results of our study suggest that the Maltese DASS is a valid and reliable instrument to assess the level of satisfaction of Maltese-speaking anticoagulated patients. It can be therefore used by health care professionals working in the setting of anticoagulation clinics or in future research studies assessing patients' satisfaction and barriers to anticoagulant treatment.

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Disclosure

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Table S1. Score differences on re-administration of the DASS (time 2 minus time 1) and intraclass correlation coefficients for the intra-language correlation

	Mean score difference (SD)	Min score difference	Max score difference	ICC
Maltese-Maltese				
DASS total score	3.5 (8.9)	-6	24	0.73
DASS limitations subscale	1.5 (3.5)	-3	9	0.69
DASS hassles/burdens subscale	1 (3.6)	-4	8	0.87
DASS psychological impact subscale	1 (3.8)	-3	10	0.77
English-English				
DASS total score	4.7 (9.9)	-10	19	0.85
DASS limitations subscale	2.8 (6.1)	-3	15	0.80
DASS hassles/burdens subscale	2.3 (2.5)	0	6	0.80
DASS psychological impact subscale	-0.4 (4.7)	-10	6	0.71

Abbreviations: ICC, intraclass correlation coefficient; SD, standard deviation.

Table S2. Response distribution for each DASS item of the English version in our study

DASS item	Response category (%)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1a	**76.4**	8.3	3.8	5.7	3.2	1.9	0.6
1b	**74.5**	15.3	3.2	3.2	1.9	0.6	1.3
1c	**56.7**	15.9	9.6	7.0	7.6	1.9	1.3
1d	**78.3**	12.7	1.3	3.8	0.6	2.6	0.6
1e	**67.5**	19.8	4.5	3.2	2.6	1.9	0.6
2a	**42.7**	33.1	7.0	10.2	3.8	2.6	0.6
2b	**49.0**	26.1	7.6	5.1	5.7	3.8	2.6
2c	**38.2**	24.2	8.9	4.5	12.7	3.8	7.6
2d	**53.5**	26.1	7.6	7.6	3.8	1.3	0.0
3a	**54.1**	29.3	7.0	4.5	3.2	1.3	0.6
3b	**46.5**	25.5	9.6	8.9	5.7	1.9	1.9
3c	**65.0**	20.4	5.7	3.2	5.1	0.0	0.6
3d	**52.2**	31.2	6.4	7.0	2.6	0.6	0.0
3e	**58.0**	23.6	5.1	6.4	2.6	4.5	0.0
3f	**80.3**	15.3	0.0	1.9	1.3	0.6	0.6
3g	**46.5**	31.2	10.8	6.4	3.2	1.9	0.0
3h	**33.1**	28.7	10.2	10.2	1.9	5.7	10.2
4a	**29.3**	28.7	19.1	12.7	4.5	3.2	2.6
4b	**24.2**	31.9	18.5	13.4	6.4	5.1	0.6
4d	**27.4**	29.3	6.4	15.9	12.1	4.5	4.5
4f	11.5	17.8	16.6	13.4	10.2	14.7	**15.9**
4g	**41.4**	28.0	9.6	13.4	3.8	1.3	2.6
4h	**28.0**	33.8	20.4	10.8	3.2	1.9	1.9
4i	**65.0**	21.0	2.6	6.4	3.8	0.0	1.3
4j	**28.7**	26.8	13.4	10.8	5.7	5.7	8.9

Note: Numbers in bold in the response category section indicate significant floor or ceiling effect.

Table S3. Correlation between the DASS total score and its subscales

	Limitations (9 items)	Hassles / burdens (8 items)	Psychological impact (8 items)	Positive psychological impact (5 items)	Negative psychological impact (3 items)
Maltese version					
Limitations	1.00				
Hassles/burdens	0.48*	1.00			
Psychological impact	0.24*	0.51*	1.00		
Positive psychological impact	-0.13	0.20#	0.80*	1.00	
Negative psychological impact	0.57*	0.59*	0.63*	0.03	1.00
DASS total score	0.80*	0.85*	0.68*	0.29*	0.75*
English version					
Limitations	1.00				
Hassles/burdens	0.43*	1.00			
Psychological impact	0.22#	0.50*	1.00		
Positive psychological impact	0.03	0.31*	0.90*	1.00	
Negative psychological impact	0.43*	0.53*	0.57*	0.14	1.00
DASS total score	0.74*	0.82*	0.74*	0.53*	0.66*

Note: *$p \leq 0.001$; #$p < 0.05$.

Patient Preference and Adherence

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ORIGINAL RESEARCH

Reliability and validity of the Maltese version of the Perception of Anticoagulant Treatment Questionnaire (PACT-Q)

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Patient Preference and Adherence

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Purpose: Anticoagulant therapy has an impact on the health-related quality of life, as it is a chronic treatment for most clinical indications and also requires some lifestyle changes. Since there was no validated questionnaire available in the Maltese language, the aim of our study was to translate and validate the Perception of Anticoagulant Treatment Questionnaire (PACT-Q2).

Patients and methods: The PACT-Q2 explores two dimensions (convenience and anticoagulant treatment satisfaction). Forward and backward translations were performed. The Maltese version of the PACT-Q2 was administered to 174 patients on warfarin treatment enrolled from different anticoagulation clinics in Malta. Reliability was assessed through internal consistency (Cronbach's alpha) and test-retest (intraclass correlation coefficient [ICC]). Validity was assessed through floor/ceiling effect, factor analysis (root mean square error of approximation [RMSEA], standardized root mean squared residual [SRMR], goodness-of-fit index [GFI], adjusted goodness-of-fit index [AGFI], comparative fit index [CFI]), subscales correlation and known-group validity.

Results: Reliability was very good for the convenience subscale (Cronbach's alpha 0.86, ICC 0.87), but less good for the satisfaction subscale (Cronbach's alpha 0.62, ICC 0.40). Floor effect was 0%; ceiling effect was low (6.3% convenience, 1.2% satisfaction). Fit parameters were close to acceptable cut-offs (RMSEA =0.09, SRMR =0.10, GFI =0.82, AGFI =0.78, CFI =0.79). There was no correlation between the two subscales (r=0.01, p=0.83). Patients with history of bleeding showed lower convenience (r= 0.16, p=0.08) and lower satisfaction (r=−0.21, p=0.01).

Conclusions: Our results support the finding that the Maltese translation of the PACT-Q2 is a valid and reliable instrument.

Keywords: atrial fibrillation, psychometrics, quality of life, surveys and questionnaires, venous thromboembolism, warfarin

Introduction

Anticoagulant therapy is the mainstay treatment for the primary and secondary prevention of thromboembolic complications in patients with atrial fibrillation (AF), venous thromboembolism (VTE) and mechanical heart valve replacement.[1] However, since it is a chronic treatment for most clinical indications, it can affect the health-related quality of life.[2,3] For instance, vitamin K antagonists (VKA), such as warfarin, have several food and drug interactions and require dose adjustment, therefore mandating periodic blood testing of the international normalized ratio (INR).[1]

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Since patients' negative beliefs related to medications can result in non-adherence to chronic treatment and therefore reduced effectiveness,[4,5] specific questionnaires have been developed to assess the satisfaction associated with the anticoagulant treatment. These include the Perception of Anticoagulant Treatment Questionnaire (PACT-Q),[6] the Duke Anticoagulation Satisfaction Scale (DASS),[7] the Anti-Clot Treatment Scale (ACTS),[8] the Deep Venous Thrombosis Quality of Life questionnaire (DVTQOL)[9] and the Pulmonary Embolism Quality of Life Questionnaire (PEmb-QoL).[10] However, there was no validated questionnaire available in the Maltese language.

We chose to translate the PACT-Q and the DASS because they have already been translated into several languages and applied to patients with a broad range of clinical indications to the anticoagulant treatment.[7,11–15] The aim of this study was to assess the psychometric properties (reliability and validity) of the Maltese version of the PACT-Q. The psychometric properties of the Maltese version of the DASS have been reported in a separate paper.

Materials and methods

The perception of anticoagulant treatment questionnaire (PACT-Q)

The PACT-Q is divided into two parts: the PACT-Q1 measures the expectations associated with the anticoagulant treatment and is administered prior to treatment initiation, while the PACT-Q2 measures the convenience and the satisfaction and is administered during anticoagulant treatment.[6,11] In the PACT-Q2, the "Convenience" dimension comprises 13 items (from the combination of the original sections B "Convenience" and C "Burden of Disease and Treatment"), while the "Anticoagulant Treatment Satisfaction" dimension comprises 7 items (section D).[11] All items can be answered according to a 5-point Likert scale (not at all, a little, moderate, a lot, extremely). During the analysis, the items of "Convenience" are reversed, summed and rescaled on a 0–100 scale; the items of "Anticoagulant Treatment Satisfaction" are summed and rescaled on a 0–100 scale. Therefore, higher total scores correspond to higher convenience/satisfaction.[11]

Permission to translate and use the PACT-Q was obtained from Sanofi Aventis/Mapi Research Trust. The linguistic validation process followed published guidelines,[16,17] with two forward translations from English to Maltese and a backward translation from Maltese to English, performed by different people (a professional translator, a health psychologist, and a speech and language pathologist), all bilingual in English and Maltese. A pilot testing was initially performed by completing and discussing the questionnaire with 5 patients on long-term oral anticoagulant treatment (not included in the analysis).

Study population

We administered the Maltese version of the PACT-Q2 to 174 patients receiving warfarin treatment. They were enrolled from the Anticoagulation Clinics at Mater Dei Hospital (Msida) and at 5 Health Centers (Cospicua, Floriana, Mosta, Qormi, Rabat) in Malta. Blood samples for INR testing at Mater Dei Hospital are collected using traditional venepuncture and INR is performed using laboratory coagulometers, whilst at the Health Centres INR is tested using point-of-care devices. Patients with cognitive impairment, dementia or major psychiatric disorders (such as schizophrenia) were excluded.

Two authors (NR, CBX) distributed the questionnaires between July 2017 and February 2018. Since we considered patients already receiving the anticoagulant treatment, only the PACT-Q2 was administered. Patients were also asked to complete a form on sociodemographic data. To ensure anonymity, questionnaires were identified using a code. In case of missing answers, the researchers associated the code with the provided demographic details and patients were contacted by phone.

During the same period, 157 patients on warfarin enrolled from the same Anticoagulation Clinics completed the original English version of the PACT-Q2.

A random sample of 40 patients underwent the following test-retest after 1–2 weeks (10 patients for each type): Maltese–Maltese; English–English; Maltese–English; English–Maltese.

This study was approved by the University of Malta Research and Ethics Committee (Ref No 07/2016) and all patients signed a written informed consent form before inclusion.

Statistical analysis

Continuous variables were reported as mean±standard deviation (SD), while categorical variables were reported as counts and percentages. Continuous variables were compared using the Student's independent samples *t*-test, while categorical variables were compared using the Chi-square or the Fisher's exact tests, as appropriate.

We evaluated the reliability of the Maltese version of the PACT-Q2 through internal consistency and test–retest.[18] The Cronbach's alpha coefficient was used to assess the internal consistency, with a value ≥0.70 indicating high internal consistency.[19]

A test–retest was performed to assess reproducibility, and we calculated the intraclass correlation coefficients (ICC) for the intra-language correlation (Maltese-Maltese and English–English test–retest). Values between 0.60 and 0.74 are considered acceptable.[20] For the cross-language correlation (Maltese–English and English–Maltese test-retest pooled together), we calculated the raw and the adjusted cross-language correlation (dividing the raw cross-language correlation by the square-root of the product of the intra-language correlations, to adjust for score unreliability).[21,22]

We evaluated the validity of the Maltese translation of the PACT-Q2 through floor and ceiling effect, factor analysis, construct validity and known-group validity. Floor and ceiling effect occur when more than 15% of the respondents achieve the lowest or the highest possible score, respectively.[23]

In the factor analysis, convergent and discriminant validity were evaluated. The convergent validity criterion was considered met when the correlation between each item and its dimension was ≥0.40, while the discriminant validity criterion was considered met when each item showed higher correlation with its dimension than the other.[24] We conducted an exploratory factor analysis with varimax rotation to examine the structure of the PACT-Q2. A subsequent confirmatory factor analysis provided the following fit parameters: root mean square error of approximation (RMSEA ≤0.05 good fit, ≤0.08 acceptable fit); standardized root mean squared residual (SRMR ≤0.05 good fit, ≤0.10 acceptable fit); goodness-of-fit index (GFI), adjusted goodness-of-fit index (AGFI) and comparative fit index (CFI), with values ≥0.90 considered acceptable.[25]

To examine construct validity, the Pearson's correlation between different subscales was assessed.[26] Known-group validity was assessed through Pearson's correlation between the score of each PACT-Q2 subscale and the following variables: increasing age; male sex; living alone; primary school education only; paid employment; atrial fibrillation; anticoagulant treatment duration (>5 years); INR in the therapeutic range at enrolment; time-within-therapeutic-range (TTR, calculated according to the Rosendaal method)[27] ≥70% in the previous year; hospitalization in the previous year; history of any bleeding during anticoagulant treatment (self-reported).

A sample size of at least 150 patients was planned, since recommendations suggest at least 50 patients[23] and previous validation studies enrolled around 100 patients.[28,29]

The statistical software STATA SE v.12 (StataCorp LP, College Station, TX, USA) and SAS v. 9.4 (SAS Institute Inc, Cary, NC, USA) were used for statistical analysis, with two-tailed p-values<0.05 considered statistically significant.

Results

Study population

Baseline characteristics of the study population are summarized in Table 1. The comparison between patients who completed the Maltese and the English version of the questionnaires has been already reported.

There was no difference in the mean convenience score between the two cohorts (mean±SD 82.2±16.1 for the Maltese version vs 84.0±13.7 for the English version, p=0.28), while patients who completed the Maltese version showed a trend toward lower satisfaction score (65.2±11.5 vs 67.6±14.6,

Table 1 Characteristics of patients who completed the Maltese version of the PACT-Q2

N of patients	174
Age (years), mean (SD)	70 (10.1)
Males, n/N (%)	82/174 (47.1%)
Living alone, n/N (%)	31/174 (17.8%)
Primary school education only, n/N (%)	108/174 (62.1%)
Paid employment (full- or part-time), n/N (%)	18/174 (10.3%)
Anticoagulant indications: atrial fibrillation, n/N (%)	122/174 (70.1%)
Anticoagulant treatment duration: >5 years, n/N (%)	76/174 (43.7%)
INR in range at enrolment, n/N (%)	99/174 (56.9%)
Good anticoagulation control (TTR ≥70%) in the previous 12 months, n/N (%)	96/170 (56.5%)
Hospitalisation in the previous 12 months, n/N (%)	86/170 (50.6%)
History of bleeding (self-reported), n/N (%)	63/174 (36.2%)

Abbreviations: INR, international normalized ratio; SD, standard deviation; TTR, time within therapeutic range.

respectively, p=0.09), corresponding to lower anticoagulant treatment satisfaction.

Internal consistency

The internal consistency of the Maltese translation of the PACT-Q2 was good for the convenience subscale with Cronbach's alpha coefficient of 0.86. Cronbach's alpha was 0.62 for the satisfaction subscale, which is slightly below the standard acceptable cut-off of 0.70. However, the satisfaction subscale has only 7 items and one item (D2) showed poor correlation with the overall satisfaction subscale, showing both a low item-total correlation of ~0.3 and an increase of Cronbach's alpha when deleted. However, D2 corresponds to the question "Do you feel that your anticoagulant treatment has decreased your symptoms?", which might have a negative answer also in satisfied patients, if anticoagulation is used for stroke prevention in atrial fibrillation or mechanical heart valves, and therefore does not have any impact on patients' symptoms.

The English version of the PACT-Q2 also showed good internal consistency in our cohort with the following Cronbach's alpha coefficients: 0.86 for the convenience subscale and 0.75 for the satisfaction subscale (Table 2).

Reproducibility

In the Maltese–Maltese test–retest, the ICC for the intra-language correlation was very good for the convenience subscale (0.87), but low (0.40) for the satisfaction subscale. In the English–English test–retest, ICC was 0.87 for the convenience subscale and 0.60 for the satisfaction subscale (Table S1).

For the cross-language correlation, the corresponding ICC was 0.51 and 0.52 and the adjusted ICC was 0.59 and 1.06 for the convenience and satisfaction subscales, respectively. When analyzed separately, ICC for the English–Maltese test–retest was 0.76 and 0.68 for the convenience and satisfaction subscales, while ICC for the Maltese–English test–retest was 0.43 and 0.41, respectively.

Floor and ceiling effect

When we analyzed the response distribution for each item of the PACT-Q2, reversing the items of the convenience subscale, a significant ceiling effect was observed for most of the questions. A significant floor effect was found only for question D2. The Maltese and the English version of the PACT-Q2 showed similar results in our study (Table S2).

We subsequently analyzed the results of each PACT-Q2 subscale: for the Maltese version, ceiling effect was 6.3% for convenience and 1.2% for satisfaction; for the English version, ceiling effect was 9.6% for convenience and 1.3% for satisfaction. Floor effect was 0% for all subscales in both languages.

Factor analysis

Results of the confirmatory factor analysis were acceptable. For the Maltese version of the PACT-Q2, SRMR=0.10 was within the acceptable limits; RMSEA=0.09 was slightly above the reference, while GFI=0.82, AGFI=0.78 and CFI=0.79 were slightly below the reference values (Table 3).

The rotated factor pattern is reported in Table S3. The convergent validity criterion was met by all items of the Maltese PACT-Q2, except B10, B11, C2 (for the convenience subscale) and D2, D3 (for the satisfaction subscale). All items met the discriminant validity criterion.

Correlation scale-subscales

There was no correlation between the convenience and the satisfaction subscales in the Maltese version of the PACT-Q2 (r=0.01, p=0.83), while a weak positive correlation was found in the English version (r=0.33, p<0.001).

Known-group validity

The Maltese version of the PACT-Q2 showed a negative correlation with previous bleeding. The correlation was statistically significant for the satisfaction subscale and borderline for the convenience subscale (Table 4).

In the English version of the PACT-Q2, the convenience subscale showed a significant positive correlation with increasing age and male sex, and a significant negative correlation with full- or part-time paid employment, hospitalization and history of bleeding. The subscale satisfaction gave similar results, which were statistically significant only for male sex.

These findings suggest that advanced age and male sex are associated with greater satisfaction/convenience, while paid employment, hospitalization and previous bleeding are associated with lower satisfaction/convenience.

Discussion

To the best of our knowledge, this is the first time that the PACT-Q2 has been translated and validated in the Maltese language. The results of our study suggest that the Maltese version of the PACT-Q2 is a valid and reliable instrument.

Table 2 Internal consistency of the Maltese and English versions of the PACT-Q2

PACT-Q2		Maltese version			English version		
	Item	Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted	Cronbach's alpha coefficient	Item-total correlation	Cronbach's alpha if item deleted
Convenience		0.86			0.86		
	B1		0.63	0.84		0.56	0.84
	B2		0.72	0.83		0.68	0.84
	B3		0.60	0.84		0.65	0.84
	B4		0.65	0.84		0.53	0.85
	B5		0.49	0.85		0.51	0.85
	B6		0.57	0.84		0.54	0.84
	B7		0.58	0.84		0.53	0.84
	B8		0.54	0.84		0.61	0.84
	B9		0.65	0.84		0.62	0.84
	B10		0.32	0.86		0.23	0.86
	B11		0.34	0.86		0.39	0.86
	C1		0.42	0.85		0.52	0.84
	C2		0.36	0.85		0.55	0.84
Satisfaction		0.62			0.75		
	D1		0.40	0.56		0.41	0.73
	D2		0.31	0.66		0.28	0.79
	D3		0.31	0.59		0.36	0.74
	D4		0.33	0.59		0.53	0.71
	D5		0.35	0.58		0.61	0.69
	D6		0.48	0.57		0.72	0.68
	D7		0.56	0.56		0.69	0.68

Table 3 Results of the confirmatory factor analysis

Fit parameters	Reference values[25]	Maltese version of the PACT-Q2	English version of the PACT-Q2
RMSEA	≤0.08	0.09	0.07
SRMR	≤0.10	0.10	0.08
GFI	≥0.90	0.82	0.80
AGFI	≥0.90	0.78	0.84
CFI	≥0.90	0.79	0.88

Abbreviations: Legend, AGFI, adjusted goodness-of-fit index; CFI, comparative fit index; GFI, goodness-of-fit index; RMSEA, root mean square error of approximation; SRMR, standardized root mean squared residual.

The psychometric properties were very good for the convenience subscale, whereas they were slightly lower for the satisfaction subscale.

The PACT-Q is a specific questionnaire that evaluates the quality of life of anticoagulated patients through simple questions. It was rigorously developed, translated in several languages and used in a number of studies enrolling patients with different clinical indications.[14,30–32] While the PACT-Q1 assesses the expectations of the anticoagulant treatment, the PACT-Q2 evaluates the satisfaction and is used for patients already receiving the anticoagulant treatment. Patient-reported outcomes should always be considered, because of the relationship between low satisfaction, poor adherence and treatment failure.[33–35] In our study, we translated the PACT-Q2 in Maltese and administered it to 174 patients on warfarin for different clinical indications, including atrial fibrillation, heart valve replacement and venous thromboembolism. A peculiarity of our study is the fact that during the same time-frame, the original English version of the PACT-Q2 was administered to 157 patients at the same centers, therefore allowing a comparison of the psychometric properties. This study design was possible because Malta is a bilingual country where both Maltese and English are official languages.[36]

We found that the reliability of the Maltese translation of the PACT-Q2 was very good for the convenience subscale (Cronbach's alpha 0.86, ICC 0.87), while it was less so for the satisfaction subscale (Cronbach's alpha 0.62, ICC 0.40). However, the satisfaction subscale showed lower reliability also in the English version of the PACT-Q2 (Cronbach's alpha 0.75, ICC 0.60 in our study; Cronbach alpha 0.76 in the study by Prins et al).[11] This finding can be partly explained by the lower number of items (13 questions in the convenience subscale vs 7 questions in the satisfaction subscale) and partly by a response bias. Response bias is common in patient-reported outcomes and occurs when participants' responses are influenced by their belief of which answers are socially acceptable or which answers are expected by the researchers.[37] The satisfaction subscale might have been particularly susceptible to response bias, due to the fact that several questions (D4-D7) ask directly the level of satisfaction with different aspects of the anticoagulant treatment (the level of independence, the appointments, the anticoagulant drug and the overall satisfaction). Participants might have felt more obliged to show that they were satisfied with the service, appointments,

Table 4 Correlation between the PACT-Q2 and sociodemographic or clinical characteristics

Variable	Correlation coefficient for the Maltese version (*p*-value)		Correlation coefficient for the English version (*p*-value)	
	Convenience	Satisfaction	Convenience	Satisfaction
Increasing age	0.05 (0.53)	0.02 (0.81)	0.34 (<0.0001)	0.14 (0.08)
Male sex	0.09 (0.26)	0.03 (0.68)	0.27 (0.001)	0.19 (0.02)
Living alone	0.05 (0.54)	−0.11 (0.14)	−0.07 (0.37)	0.05 (0.55)
Primary school education only	−0.01 (0.95)	−0.07 (0.36)	−0.02 (0.84)	−0.11 (0.16)
Paid (full- or part-time) employment	−0.09 (0.24)	0.04 (0.56)	−0.22 (0.006)	−0.15 (0.07)
Atrial fibrillation	−0.01 (0.94)	0.04 (0.58)	0.09 (0.27)	0.01 (0.91)
Anticoagulant treatment duration >5 years	0.03 (0.73)	0.07 (0.36)	0.05 (0.57)	0.01 (0.92)
INR in range at enrolment	0.05 (0.49)	0.05 (0.43)	0.07 (0.36)	−0.15 (0.07)
Good anticoagulation control (TTR ≥70%) in the previous 12 months	0.06 (0.43)	0.12 (0.12)	0.04 (0.64)	−0.03 (0.71)
Hospitalisation in the previous 12 months	−0.10 (0.21)	−0.03 (0.70)	−0.18 (0.03)	0.05 (0.58)
History of bleeding (self-reported)	−0.16 (0.08)	−0.21 (0.01)	−0.17 (0.03)	−0.12 (0.11)

Note: A negative correlation means lower satisfaction.
Abbreviations: INR, international normalized ratio; TTR, time within therapeutic range.

anticoagulant drug rather than risk reprisal on their treatment, even though the informed consent specified anonymity of data. Furthermore, although the retest was performed within two weeks, changes in the level of satisfaction might have occurred due to intercurrent clinical complications or differences of experience of service provision during following appointments for INR testing.

Validity of the Maltese translation of the PACT-Q2 was good. We observed a significant ceiling effect for most of the PACT-Q2 items when analyzed individually. However, when we considered the two subscales, floor effect was 0% and ceiling effect did not exceed 10% (being 6.3% for convenience and 1.2% for satisfaction). These results were even better than the original study of the PACT-Q2 which reported a ceiling effect of 22.1% for convenience and 3.3% for satisfaction.[11] Results of the factor analysis were good, with fit parameters close to the acceptable cut-offs. Furthermore, all items met the discriminant validity criterion, while the convergent validity was met by all items except B10, B11, C2 (convenience subscale) and D2, D3 (anticoagulant treatment satisfaction subscale). Although previous studies did not report the fit parameters (RMSEA, SRMR, GFI, AGFI and CFI), items B10-B11 and D2-D3 did not meet the convergent validity criterion also in the original study by Prins et al.[11] Correlation between the two PACT-Q2 subscales was weak, as previously reported,[11] confirming that they cover different dimensions. The results of the known-group validity analysis showed that patients with history of bleeding had lower satisfaction. Although previous validation studies of the PACT-Q did not evaluate this group,[11,12] lower scores on the convenience dimension of the PACT-Q2 were reported after bleeding events in a prospective study enrolling 807 atrial fibrillation patients on warfarin.[31] Furthermore, lower satisfaction in anticoagulated patients with history of bleeding was already reported in validation studies of other specific questionnaires.[7,38]

Our study population shows some differences when compared to previous PACT-Q validation studies. Mean age was older (70 years), compared to 65 years in the study by Prins et al[11] and 58 years in the study by Mohamed et al.[12] We enrolled patients on oral anticoagulant treatment with VKA, while Prins et al[11] considered also patients on treatment with idraparinux, which is a parenteral drug injected subcutaneously once weekly. Finally, we had a higher proportion of patients with primary school level of education (62%), compared to the study by Mohamed et al[12] where only 28% had only primary school education or no education at all.

Our study has also some limitations which need to be acknowledged. First, we enrolled patients who were already on anticoagulant treatment; therefore, we could validate only the PACT-Q2. Second, although the PACT-Q has been developed for patients receiving different types of anticoagulants (oral or parenteral),[6] we enrolled only patients on warfarin which was the main oral anticoagulant treatment in Malta at the time of patients enrolment. Nonetheless, the strengths of our study include the completeness of data, without any missing answers, and the rigorous process of translation and analysis.

Conclusion

Our results support the finding that the Maltese translation of the PACT-Q2 is a valid and reliable instrument, which can be used by health-care professionals when assessing Maltese-speaking anticoagulated patients.

Copyright

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Disclosure

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Supplementary material

Table S1 Details of the intra-language correlation: score differences on re-administration of the PACT-Q2 (time 2 minus time 1) and intraclass correlation coefficients

	Mean score difference (SD)	Min score difference	Max score difference	ICC
Maltese–Maltese				
PACT-Q2 convenience	−0.1 (3.2)	−6	6	0.87
PACT-Q2 satisfaction	0.1 (4.1)	−7	8	0.40
English–English				
PACT-Q2 convenience	0.9 (3.0)	−3	7	0.87
PACT-Q2 satisfaction	2.3 (3.6)	−1	+10	0.60

Note: For both subscales, the original scores were considered (not rescaled). Items in the convenience subscale were reversed.
Abbreviations: ICC, intraclass correlation coefficient; SD, standard deviation.

Table S2 Response distribution for each PACT-Q2 item and summary statistics

PACT-Q2 item	Maltese version						English version					
	Response category (%)					Mean (SD)	Response category (%)					Mean (SD)
	1	2	3	4	5		1	2	3	4	5	
B1*	1.2	5.2	4.0	12.1	**77.6**	4.6 (0.9)	0.6	1.3	5.7	14.0	**78.3**	4.7 (0.7)
B2*	2.9	6.3	8.6	20.1	**62.1**	4.3 (1.1)	0.0	5.1	6.4	16.6	**72.0**	4.6 (0.8)
B3*	4.0	5.8	6.3	24.1	**59.8**	4.3 (1.1)	0.0	2.6	9.6	26.1	**61.8**	4.5 (0.8)
B4*	5.2	12.6	8.6	24.1	**49.4**	4.0 (1.2)	2.6	11.5	19.1	26.8	**40.1**	3.9 (1.1)
B5*	2.9	10.3	15.5	21.8	**49.4**	4.0 (1.2)	1.3	8.3	19.8	29.3	**41.4**	4.0 (1.0)
B6*	2.9	8.1	4.0	10.3	**74.7**	4.5 (1.1)	0.6	2.6	7.6	21.0	**68.2**	4.5 (0.8)
B7*	1.7	4.6	9.8	18.4	**65.5**	4.4 (1.0)	1.9	3.8	8.3	26.1	**59.9**	4.4 (0.9)
B8*	1.2	4.0	7.5	14.4	**73.0**	4.5 (0.9)	1.3	6.4	8.9	27.4	**56.1**	4.3 (1.0)
B9*	0.6	4.0	4.6	6.9	**83.9**	4.7 (0.8)	0.6	1.9	2.6	17.2	**77.7**	4.7 (0.7)
B10*	5.2	8.1	5.2	17.8	**63.8**	4.3 (1.2)	1.3	3.2	3.8	13.4	**78.3**	4.6 (0.8)
B11*	11.5	17.8	29.5	24.1	**27.0**	3.4 (1.4)	8.3	17.8	19.1	26.1	**28.7**	3.5 (1.3)
C1*	2.3	4.0	6.9	10.9	**75.9**	4.5 (1.0)	0.0	4.5	10.8	14.0	**70.7**	4.5 (0.9)
C2*	2.3	8.6	10.9	25.3	**52.9**	4.2 (1.1)	0.6	2.6	7.0	28.0	**61.8**	4.5 (0.8)
D1	2.3	2.9	27.0	57.5	10.3	3.7 (0.8)	6.4	8.3	14.6	39.5	**31.2**	3.8 (1.2)
D2	**51.2**	14.9	6.9	16.7	10.3	2.2 (1.5)	**42.7**	16.6	19.1	14.0	7.6	2.3 (1.3)
D3	4.0	5.8	50.6	28.7	10.9	3.4 (0.9)	0.6	3.2	54.1	21.7	**20.4**	3.6 (0.9)
D4	0.6	2.9	12.6	72.4	11.5	3.9 (0.6)	0.6	1.9	11.5	64.3	**21.7**	4.0 (0.7)
D5	0.0	5.8	9.8	71.3	13.2	3.9 (0.7)	1.3	5.1	12.7	53.5	**27.4**	4.0 (0.9)
D6	0.0	0.6	6.3	81.6	11.5	4.0 (0.4)	1.3	0.6	7.0	68.8	**22.3**	4.1 (0.7)
D7	0.0	0.0	5.8	78.7	**15.5**	4.1 (0.5)	1.3	1.3	7.6	64.3	**25.5**	4.1 (0.7)

Notes: Numbers in bold in the response category section indicate significant floor or ceiling effect. * Items of the convenience subscale (B1 to C2) are reversed.

Table S3 Results of the 2-factor analysis

	Maltese version		English version	
Item	**Convenience**	**Satisfaction**	**Convenience**	**Satisfaction**
B1*	**0.69**	0.09	**0.63**	0.06
B2*	**0.78**	0.26	**0.69**	0.34
B3*	**0.64**	0.07	**0.69**	0.06
B4*	**0.70**	−0.03	**0.57**	−0.03
B5*	**0.53**	0.04	**0.53**	0.10
B6*	**0.60**	−0.08	**0.60**	0.16
B7*	**0.62**	0.32	**0.56**	0.18
B8*	**0.63**	0.13	**0.63**	0.25
B9*	**0.70**	0.07	**0.72**	0.05
B10*	**0.34**	0.09	**0.22**	0.15
B11*	**0.36**	0.07	**0.38**	0.11
C1*	**0.46**	−0.08	**0.55**	0.08
C2*	**0.38**	0.03	**0.58**	0.11
D1	−0.06	**0.42**	0.08	**0.37**
D2	−0.36	**0.29**	−0.07	**0.25**
D3	0.10	**0.27**	0.15	**0.39**
D4	0.20	**0.49**	0.38	**0.55**
D5	0.16	**0.58**	0.14	**0.75**
D6	0.03	**0.75**	0.17	**0.89**
D7	−0.10	**0.83**	0.18	**0.91**

Notes: Numbers in bold indicate the highest loading of each factor, which is therefore likely to explore that dimension. * Items of the convenience subscale (B1 to C2) are reversed.

Appendix B – Ethical approval

L-UNIVERSITÀ TA' MALTA
Msida - Malta
SKOLA MEDIKA
Sptar Mater Dei

UNIVERSITY OF MALTA
Msida - Malta
MEDICAL SCHOOL
Mater Dei Hospital

Ref No: **21/2015**

Friday, 26th June 2015

Dr Nicoletta Riva
90, Cardinal Xiberras Street,
Qormi QRM1021
Malta

Dear Dr Nicoletta Riva

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Bleeding Complications during Oral Anticoagulant Treatment and their Management in the Maltese Health Care System

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

E-MAIL: umms@um.edu.mt WEB: www.um.edu.mt/ms

L-UNIVERSITÀ TA' MALTA
Msida - Malta
SKOLA MEDIKA
Sptar Mater Dei

UNIVERSITY OF MALTA
Msida - Malta
MEDICAL SCHOOL
Mater Dei Hospital

Ref No: **07/2016**

Friday 13th May 2016

Dr Nicoletta Riva
90,
Cardinal Xiberras Street
Qormi (QRM1021)

Dear Dr Nicoletta Riva,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Main Project: Bleeding Complications during Oral Anticoagulant Treatment and their Management in the Maltese Health Care System

Clinical Study 3: Patients satisfaction associated with warfarin treatment in different setttings.

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

E-MAIL: umms@um.edu.mt WEB: www.um.edu.mt/ms

L-Università ta' Malta

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University of Malta
Msida MSD 2080, Malta

Tel: +356 2340 1879/1891/1167
umms@um.edu.mt

www.um.edu.mt/ms

Ref No: **07/2016**

Thursday 23rd November 2017

Dr Nicoletta Riva
90,
Cardinal Xiberras Street
Qormi QRM1021

Dear Dr Nicoletta Riva,

Please refer to your application to gather an extension of the ethics approval submitted to the Research Ethics Committee in connection with your research entitled:

Bleeding complications during Oral Anticoagulant Treatment and their Management in the Maltese Health Care System

Clinical Study 3: Patients satisfaction associated with warfarin treatment in different settings

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

Appendix C1 – English and Maltese versions of patient information sheets and consent forms for the laboratory study

PATIENT INFORMATION SHEET

Title of Main Project: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"
Laboratory Study 2: "Comparison between laboratory INR, POC-INR and thrombin generation in patients with stable anticoagulation control"

Dear Patient
We would like to invite you to take part in a study. Before you decide it is important for you to understand why this study is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part.

What is the purpose of the study?
The purpose of this study is to compare different blood tests in patients taking blood-thinning medication (the oral anticoagulant warfarin). Warfarin is usually monitored through the INR (international normalized ratio), which can be performed in the laboratory or with a point-of-care machine. We would like to analyse the results of INR tested in the laboratory, INR tested with a point-of-care machine and thrombin generation to see which test performs best. Thrombin generation is a newer test to evaluate the level of thinness of the blood.

Why have I been invited?
You have been asked to take part in this research study because you have been diagnosed with venous thromboembolism, a clot either in your legs or in the lungs, and you have been prescribed a blood-thinning medication (warfarin) to decrease your risk of getting another clot.

Do I have to take part?
It is up to you to decide whether or not to take part in the study. This information sheet will outline the study and will be given to you. We will then ask you to sign a consent form to show that you have understood the information and agreed to take part. If you decide to take part, you are still free to change your mind at any time until completion of the study, and you do not have to give a reason. Your decision will have no bearing on your medical treatment.

What will happen to me if I take part?
If you agree to take part in this study, the researcher will ask you to sign a consent form to state that you agreed to take part. After you have signed the consent form, the researcher will ask you some information

about your medical history and your tablets. Finally, you will have a blood sample taken (10 ml, which is approximately one full spoon), in order to compare the different blood test results.

What are the possible disadvantages and risks of taking part?

There are no disadvantages to you in taking part in this study. A small bruise can appear on your skin where the needle went in, as it might happen with every blood test.

What are the possible benefits of taking part?

We cannot promise that the study will have any benefits. However, our results might contribute to the discovery of the best way to monitor warfarin, thus helping patients who are on blood-thinning medications in the future.

What happens when the research study stops?

At the end of the study, the information you gave to the researcher and the results of your blood tests will be stored in an electronic database and analysed. However, your name will not appear anywhere in the research.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any point during the visit and up to two weeks following it.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to one of the researchers who will do their best to answer your questions (Dr. Nicoletta Riva or Dr. Alexander Gatt, see contact details below).

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. Your data will be stored in an electronic database; however, your name will not appear anywhere in the research and any material that can lead to your identity will not appear anywhere in the results or any publishable material. The data may also be looked at by representatives of regulatory authorities and by people authorised to check that the study is being carried out correctly. However, we all have a duty of confidentiality to you as a research participant and no identifiable material will be disclosed.

What will happen to the information I give?

As mentioned above, all information that is collected about you during the course of the research will be kept strictly confidential. The data will be kept in a secure location within the hospital and data stored on computers will be anonymised (using a numeric code) and the computers will be password protected. Only the researchers will have access to this data.

What will happen to the results of the research study?
At the end of the study, we hope to publish the results. You will not be identified in any report or publication. If you wish, we will send you a summary of our findings.

Who is organising and funding the research?
This research is being organised by the Department of Pathology, Faculty of Medicine and Surgery, University of Malta. No payments will be made to members of staff involved in this study.

Who has reviewed the study?
This study was reviewed by the University of Malta Research Ethics Committee. This is done to protect your safety, rights, well-being, and dignity.

Further information and contact details
If you have any questions about the study, please contact any of the person listed below. They will answer your questions or give you advice.

Chief Investigators:
Dr. Nicoletta Riva
Email: nicoletta.riva.14@um.edu.mt
Address: Medical School, Department of Pathology, Mater Dei Hospital, Msida MSD 2090

Dr. Alexander Gatt
Email: alexander.gatt@um.edu.mt
Tel: 25456318
Address: Hematology Laboratory, Mater Dei Hospital, Msida MSD 2090

Thank you for taking the time to read this Patient Information Sheet and considering whether to take part in the study.

Patient number _____

CONSENT FORM

Title of Main Project: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"

Laboratory Study 2: "Comparison between laboratory INR, POC-INR and thrombin generation in patients with stable anticoagulation control"

Name of Researchers: Dr. Nicoletta Riva, Dr. Alexander Gatt

1. I confirm that I have read and understood the information sheet (version 1; dated 09/01/2015) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, until the study is completed, without giving any reason and without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals working within the research team, Mater Dei Hospital or from regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature

1 copy for patient; 1 copy for researcher; 1 copy to be kept in hospital notes

English version 1.0 – date 09/01/2015

INFORMAZZJONI GHAL-PAZJENT

Titlu tal-Progett: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"

Laboratory Study 2: "Comparison between laboratory INR, POC-INR and thrombin generation in patients with stable anticoagulation control"

Għażiż Pazjent

Nixtiequ nistednuk biex tieħu parti fi studju. Qabel ma tiddeċiedi, huwa importanti li tifhem għala dan l-istudju qed isir u x'ser jinvolvi. Jekk jogħġbok ħu l'ħin neċessarju biex tifhem din l informazzjoni, u jekk tixtieq, tista' tiddiskuti ma ħaddieħor. Jekk jogħġbok tiddejjaqx issaqsina jekk hemm xi ħaġa li għalik mhix ċara jew jekk tixtieq iżjed informazzjoni. Hu l-ħin li għandek bżonn sabiex tiddeċiedi jekk trid tipparteċipa jew le.

X'inhu l-iskop tal-istudju?

L'iskop ta dan l istudju huwa li jqabbel testijiet differenti tad-demm f'pazzjenti li qed jieħdu mediċina li traqqaq id-demm (il-mediċina anti-koagulanti, il-warfarina). Il-warfarina, is-soltu, tiġi immoniterjata bit-test tal-INR li jista jiġi affetwat jew fil-laboratorju jew bil-magna tal-"point-of-care". Għaldaqstant aħna nixtiequ inqabblu ir-riżultati tal-INR meta jiġi eżaminat fil-laboratorju, bil-magna tal-"point-of-care" u it-"thrombin generation" biex naraw liema minn dawn testijiet tad-demm huwa l iktar effiċjenti. "Thrombin generation" huwa test iktar ġdid li qed jintuża biex jevalwaw kemm hu rqieq id-demm.

.

Għala ġejt mistieden/mistiedna?

Int ġejt mistoqsi tipparteċipa f din ir-riċerka għax int ġejt djanjostikat b'tromboemboliżmu venuż, jew f'saqajk jew fil-pulmun u ħabba f ħekk ġejt preskritt mediċina li traqqaq id-demm (bħal warfarina) biex tnaqqas ir-riskju ta embolu ieħor.

Għandi bżonn nipparteċipa?

Id-deċiżjoni hija f'idejk jekk tipparteċipax f'dan l'istudju jew le. Din l-formula ħa tiġi mogħtija lilek u ħa jkollha informazzjoni fuq l-istudju. Wara li taqra din l'informazzjoni, aħna ser insaqsuk tiffirma formula ta kunsens oħra, fejn int tkun qed turi li fhimt l'informazzjoni, u aċċettajt li tipparteċipa. Jekk int taċċetta li tipparteċipa, int xorta tista' tbiddel fhemtek meta trid, sa qabel ma jiġi konkluż l istudju, mingħajr ma għandek għalfejn tagħti raġun għala waqqaft. Id-deċiżjoni li tieħu mhux ħa jkollha effett fuq it-trattament mediku tiegħek.

X'ser jiġri minni jekk nipparteċipa?

Jekk int taċċetta li tipparteċipa f'dan l-istudju, ir-riċerkatriċi ser ssaqsik tiffirma formula ta kunsens fejn int turi li aċċettajt. Wara li tiffirma, ir-riċerkatriċi ser issaqsik ftit mistoqsijiet fuq l'istorja medika tiegħek u fuq l mediċini li qed tieħu. Wara, ser jittihidlek ftit demm (10ml, li huwa kwazi daqs imgħarfa waħda) biex jikkumparaw it-testijiet tad-demm differenti.

X'inhuma il-possibili żvantaġġi u riskji li tipparteċipa?

M'hemmx żvantaġġi meta tipparteċipa f'dan l'istudju. Tista tifformalek tbenġila żgħira meta jittieħed id-demm, iżda dan huwa komuni f'kull test tad-demm.

X'inhuma il-possibili vantaġġi li tipparteċipa?

Ma nistgħux inweduk illi hemm xi vantaġġi billi tipparteċipa. Iżda ir-riżultati ta dan l'istudju jistaw iwasslu biex insiru nafu l aħjar mod kif nistaw nsegwu il-warfarina. B'hekk nistaw intejbu is-servizz lil pazjenti t'ghada.

X'ser jiġri meta lis-studju jispiċċa?

Fl-aħħar tal-istudju, l'informazzjoni li tkun tajt lir-riċerkatriċi u r-riżultati tad-demm tiegħek ser ikunu miktubin elettronikament u analizzati. Iżda ismek mhux ser jidher imkien.

X'jigri jekk ma nkunx irrid inkompli bl-istudju?

Int liberu li tirtira mil-istudju meta trid waqt il-vista u sa ġimgħatejn wara.

X'jiġri f'kas ta problema?

Jekk int mħasseb b'xi aspett f'dan l-istudju, tiddejjaqx isaqsi biex titkellem ma wieħed mir-riċerkaturi, li ħa jgħamlu mil-aħjar li jistaw biex iwieġbu il-mistoqsijiet tiegħek (Dr. Nicoletta Riva jew Dr. Alexander Gatt, ara id-dettalji tagħhom isfel).

Il-parteċipazjoni tiegħhi f'dan l-istudju ħa tinżamm kunfidenzjali?

Iva. Ser nimxu ma prattika etika kif ukoll legali u kull informazzjoni fuqek, ser tinżamm kunfidenzjali. L-informazjoni fuqek ser tinżamm elettronikament; iżda ismek mhux ser jidher imkien fir-riċerka u kull materjal li jista' iwassal għal identità tiegħek mhux ser jidher fir-riżultati jew materjal li jiġi publikat. Din l-informazjoni tista tiġi reveduta minn nies awtorizzati biex jiċċekkjaw li l-istudju qed jimxi sew. Iżda, kollha kemm aħna marbutin b'konfidenzjalita lejk bħala parteċipant u l-identità tiegħek ser tiġi protetta.

X'ser jiġri bl-informazzjoni li ser nagħti?

Kif semmejna qabel, kull informazzjoni li ser tingabar, ser tinżamm kunfidenzjali. L informazzjoni ser tinżamm f'post protett ġewwa l'isptar u l informazzjoni maħżuna fuq kompjuters ser tkun kodifikata b-numri. Il-komputers ser ikunu wkoll protetti b'kodiċi. Ir-riċerkaturi biss ser ikollok aċċess għal dan l informazzjoni.

X'ser jiġri mir-riżultati ta dan l-istudju?
Fl-aħħar ta dan l-istudju, nixtiequ nippubblika ir-riżultati tagħna. Iżda ismek ser jinżamm kunfidenzjali. Jekk trid, nistgħu nibatulek sommarju tar-riżultati.

Min qed jorganizza u jħallas għal din ir-riċerka?
Din ir-riċerka qed tiġi organizzata mid-Dipartiment tal-Patoloġija, fil-Fakultà tal-Mediċina u Kirurġija, tal-Università ta Malta. L-ebda pagament mhu ser jingħata lil ħaddiema li qed jieħdu s'hemm f'dan l-istudju.

Minn min ġiet irriveduta din ir-riċerka?
Dan l-istudju ġie rivedut mil Kumitat tal-Etika fir-Riċerka, tal-Università ta Malta. Dan sar sabiex jipproteġi id drittijiet, saħħa u dinjita tiegħek.

Iżjed informazzjoni u kuntatti
Jekk għandek xi mistoqsijiet oħra fuq l-istudju, jekk joghġbok ikkuntattja xi persuna minn hawn taħt. Dawn ser iwieġbu jew jagħtu parir.

Investigatriċi kap:
Dr. Nicoletta Riva
Email: nicoletta.riva.14@um.edu.mt
Address: Medical School, Department of Pathology, Mater Dei Hospital, Msida MSD 2090

Dr. Alexander Gatt
Email: alexander.gatt@um.edu.mt
Tel: 25456318
Address: Hematology Laboratory, Mater Dei Hospital, Msida MSD 2090

Grazzi talli ħadt il-ħin tiegħek sabiex taqra dan id-dokument t'informazzjoni u tikkonsidra tipparteċipa fl-istudju.

Patient number _____

FORMULA TAL-KUNSENS

Titlu tal-proġett: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"

Laboratory Study 2: "Comparison between laboratory INR, POC-INR and thrombin generation in patients with stable anticoagulation control"

Isem tar-riċerkaturi: Dr. Nicoletta Riva, Dr. Alexander Gatt

1. Jien nikkonferma li qrajt u fhimt l informazzjoni (verżjoni 1; datata 09/01/2015) li ġejt mgħoti fuq dan l-istudju. Jien kelli l-opportunita li naħseb fuq din l-informazzjoni, nistaqsi mistoqsijiet, u dawn il-mistoqsijiet imwieġba. ☐

2. Jien nifhem li il-parteċipazzjoni tiegħi f'dan l-istudju hija volontarja u li jien liberu li nwaqqaf il-parteċipazzjoni tiegħi meta rrid, sa qabel ma jieqaf l-istudju, mingħajr ma nagħti spjegazzjoni u mingħajr ma jiġu affettwati id drittijiet mediċi u legali tiegħi. ☐

3. Jien nifhem li partijiet min-noti mediċi u informazzjoni miġbura, tista tiġi revista minn individwi li jagħmlu parti mit-tim tar-riċerkaturi ta Mater Dei jew awtoritajiet oħra. Jien nagħti permess għal dawn l-individwi biex ikollhom aċċess għan-noti mediċi tiegħi. ☐

4. Jien naċċetta li nipparteċipa f'dan l-istudju ☐

_____	_____	_____
Isem tal-Pazjent	Data	Firma
_____	_____	_____
Isem tal-persuna li qed jieħu il-kunsens	Data	Firma
_____	_____	_____
Isem tal-persuna li qed jieħu il-kunsens (jekk differenti mir-riċerkaturi)	Data	Firma

1 kopja għal-pazjent; 1 kopja għar-riċerkatur; 1 kopja għan-noti mediċi

Maltese version 1.0 – date 09/01/2015

Appendix C2 – English and Maltese versions of patient information sheets and consent forms for the clinical study

Patient number _____

PATIENT INFORMATION SHEET

Title of Main Project: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"

Clinical Study 3: "Patients satisfaction associated with warfarin treatment in different settings"

Dear Patient,

We would like to invite you to take part in a study. Before you decide it is important for you to understand why this study is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Please ask us if there is anything that is not clear or if you would like more information. Take your time to decide whether or not you wish to take part.

What is the purpose of the study?

This study will focus on patients taking warfarin, which is a blood-thinning medication. This medication is used to reduce the chances of getting certain diseases, such as thrombosis or stroke. The purpose of this study is to assess patients satisfaction associated with the use of warfarin.

Why have I been invited?

You have been asked to take part in this research study because you have been prescribed a blood-thinning medication (warfarin) and you attend either the Anticoagulation Clinic at Mater Dei Hospital or the Polyclinics spread around the Maltese island for blood monitoring.

Do I have to take part?

It is up to you to decide whether or not to take part in the study. This information sheet will outline the study and will be given to you. We will then ask you to sign a consent form to show that you have understood the information and agreed to take part. If you decide to take part, you are still free to change your mind at any time until completion of the study, and you do not have to give a reason. Your decision will have no bearing on your medical treatment.

What will happen to me if I take part?

- If you agree to take part in this study, the researcher will ask you to sign a **consent form** to state that you agreed to take part voluntarily. You will need to sign three copies of the consent form: one is for you to keep, one is for your hospital medical records and one is for the researchers.
- After you have signed the consent form, the researchers will ask you to fill in **two questionnaires**, which explore your satisfaction with the anticoagulant treatment (PACT-Q and DASS). There will be English or Maltese versions of the questionnaires available for you. It will take you approximately 20 minutes to

fill in the questionnaires. We will also ask you to provide some demographical information (such as age, sex, level of education). We hope that you will be willing to answer all questions.

- Finally, the researchers will collect some information about the **results of your blood tests** in the previous twelve months.

What are the possible disadvantages and risks of taking part?

There are no disadvantages for you in taking part in this study.

What are the possible benefits of taking part?

We cannot promise that the study will have any benefit. However, our results might contribute to improve the management of warfarin treatment in the future.

What happens when the research study stops?

At the end of the study, the information you gave to the researcher and the results of your blood test will be stored in an electronic database and analysed. However, your name will not appear anywhere in the research.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any point during the visit and up to two weeks following it.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to one of the researchers who will do their best to answer your questions (Dr. Nicoletta Riva or Dr. Alexander Gatt, see contact details below).

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. Your data will be stored in an electronic database; however, your name will not appear anywhere in the research and any material that can lead to your identity will not appear anywhere in the results or any publishable material. The data may also be looked at by representatives of regulatory authorities and by people authorised to check that the study is being carried out correctly. However, we all have a duty of confidentiality to you as a research participant and no identifiable material will be disclosed.

What will happen to the information I give?

As mentioned above, all information that is collected about you during the course of the research will be kept strictly confidential. The data will be kept in a secure location within the hospital and data stored on computers will be anonymised (using a numeric code) and the computers will be password protected. Only the researchers will have access to this data.

What will happen to the results of the research study?
At the end of the study, we hope to publish the results. You will not be identified in any report or publication. If you wish, we will send you a summary of our findings.

Who is organising and funding the research?
This research is being organised by the Department of Pathology, Faculty of Medicine and Surgery, University of Malta. No payments will be made to members of staff involved in this study.

Who has reviewed the study?
This study was reviewed by the University of Malta Research Ethics Committee. This is done to protect your safety, rights, well-being, and dignity.

Further information and contact details
If you have any questions about the study, please contact any of the person listed below. They will answer your questions or give you advice.

Chief Investigators:

Dr. Nicoletta Riva, PhD candidate
Email: nicoletta.riva.14@um.edu.mt
Tel: 2545 2518
Address: Medical School, Department of Pathology, Mater Dei Hospital, Msida MSD 2090

Dr. Alexander Gatt, Consultant Haematologist
Email: alexander.gatt@um.edu.mt
Tel: 2545 6318
Address: Haematology Laboratory, Department of Pathology, Mater Dei Hospital, Msida MSD 2090

Thank you for taking the time to read this Patient Information Sheet and considering whether to take part in the study.

Patient number _____

CONSENT FORM

Title of Main Project: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"

Clinical Study 3: "Patients satisfaction associated with warfarin treatment in different settings"

Name of Researchers: Dr. Nicoletta Riva, Dr. Alexander Gatt

1. I confirm that I have read and understood the information sheet (version 1.0; dated 26/10/2015) for the above study. I have had the opportunity to consider the information, ask questions, and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, until the study is completed, without giving any reason and without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals working within the research team, Mater Dei Hospital or from regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records. ☐
4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature
Name of Person taking consent (if different from researchers)	Date	Signature

1 copy for patient; 1 copy for researcher; 1 copy to be kept in hospital notes

English version 1.0 – date 26/10/2015

Patient number _____

INFORMAZZJONI GHAL-PAZJENT

Titlu tal-proġett: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"

Clinical Study 3: "Patients satisfaction associated with warfarin treatment in different settings"

Għażiż Pazjent

Nixtiequ nistednuk biex tieħu parti fi studju. Qabel ma tiddeċiedi, huwa importanti li tifhem għala dan l-istudju qed isir u x'ser jinvolvi. Jekk jogħġbok ħu l'ħin neċessarju biex tifhem din l informazzjoni, u jekk tixtieq, tista' tiddiskuti ma ħaddieħor. Jekk jogħġbok tiddejjaqx issaqsina jekk hemm xi ħaġa li għalik mhix ċara jew jekk tixtieq iżjed informazzjoni. Hu l-ħin li għandek bżonn sabiex tiddeċiedi jekk trid tipparteċipa jew le.

X'inhu l-iskop tal-istudju?

Dan l-istudju ser jiffoka fuq pazjenti li qed jieħdu l-warfarina, li hija mediċina li traqqaq id-demm. Din il-mediċina hija użata biex tnaqqas iċ-ċans li jkun hemm ċertu tip ta mard, bħat-trombosi (meta jaqad id-demm) u l- stroke. L-għan ta dan l-istudju huwa li naraw kemm il-pazzjent hu sodisfatt bl-użu tal-warfarina.

Għala ġejt mistieden/mistiedna?

Int ġejt mistieden/mistiedna tipparteċipa f din ir-riċerka għax int ġejt preskritt mediċina li traqqaq id-demm (warfarina) u tmur jew l-"Anticoagulation Clinic" tal-isptar Mater Dei jew xi polyclinic madwar Malta sabiex tieħu l-eżami tad-demm.

Għandi bżonn nipparteċipa?

Id-deċiżjoni hija f'idejk jekk tipparteċipax f'dan l'istudju jew le. Din l-formula ħa tiġi mogħtija lilek u ħa jkollha informazzjoni fuq l-istudju. Wara li taqra din l'informazzjoni, aħna ser insaqsuk tiffirma formula ta kunsens oħra, fejn int tkun qed turi li fhimt l'informazzjoni, u aċċettajt li tipparteċipa. Jekk int taċċetta li tipparteċipa, int xorta tista' tbiddel fhemtek meta trid, sa qabel ma jiġi konkluż l istudju, mingħajr ma għandek għalfejn tagħti raġun għala waqqaft. Id-deċiżjoni li tieħu mhux ħa jkollha effett fuq it-trattament mediku tiegħek.

X'ser jiġri minni jekk nipparteċipa?

Jekk int taċċetta li tipparteċipa f'dan l-istudju, ir-riċerkatriċi ser ssaqsik tiffirma formula ta kunsens fejn int turi li aċċettajt volontarjament. Int ikollok tiffirma tlett kopji tal-kunsens: waħda hija għalik, waħda hija għall-isptar, u waħda hija għar-riċerkaturi.

Wara li tiffirma, ir-riċerkaturi ser issaqsuk ukoll biex timla zewġ kwestjonarji, li jaraw is-sodisfazjon tiegħek bil-mediċina li traqqaq id-demm (PACT-Q u DASS). Ser ikun hemm verżjonijiet bl-Ingliż kif ukoll bil-Malti. Dawn il-kwestjonarji għandhom jehdulek 20 minuta biex timlihom. Ser nitolbuk ukoll timlilna naqra informazjoni fuqhek (bhall-eta, sess u livell ta edukazjoni). Ninkoraġġuk biex tirrispondi l-mistoqsijiet kollha.
Fl-aħħar, ir-riċerkaturi ser jiġbru x'informazjoni fuq ir-riżultati tad-demm tiegħek ta dawn l-aħħar tnax-il xhar.

X'inhuma il-possibili żvantaġġi u riskji li tipparteċipa?
M'hemmx żvantaġġi meta tipparteċipa f'dan l'istudju.

X'inhuma il-possibili vantaġġi li tipparteċipa?
Ma nistgħux inweduk illi hemm xi vantaġġi billi tipparteċipa. Iżda ir-riżultati ta dan l'istudju jistaw iwasslu biex insiru nafu l aħjar mod kif nistaw nsegwu il-mediċina li traqqaq id-demm.

X'ser jiġri meta lis-studju jispiċċa?
Fl-ahhar tal-istudju, l'informazzjoni li tkun tajt lir-riċerkatriċi u r-riżultati tad-demm tiegħek ser ikunu miktubin elettronikament u analizzati. Iżda ismek mhux ser jidher imkien.

X'jigri jekk ma nkunx irrid inkompli bl-istudju?
Int liberu li tirtira mil-istudju meta trid waqt il-vista u sa ġimgħatejn wara.

X'jiġri f'kas ta problema?
Jekk int mħasseb b'xi aspett f'dan l-istudju, tiddejjaqx isaqsi biex titkellem ma wieħed mir-riċerkaturi, li ħa jgħamlu mil-aħjar li jistaw biex iwieġbu il-mistoqsijiet tiegħek (Dr. Nicoletta Riva jew Dr. Alexander Gatt, ara id-dettalji tagħhom isfel).

Il-parteċipazjoni tiegħhi f'dan l-istudju ħa tinżamm kunfidenzjali?
Iva. Ser nimxu ma prattika etika kif ukoll legali u kull informazzjoni fuqek, ser tinżamm kunfidenzjali. L-informazjoni fuqek ser tinżamm elettronikament; iżda ismek mhux ser jidher imkien fir-riċerka u kull materjal li jista' iwassal għal identità tiegħek mhux ser jidher fir-riżultati jew materjal li jiġi publikat. Din l-informazjoni tista tiġi reveduta minn nies awtorizzati biex jiċċekkjaw li l-istudju qed jimxi sew. Iżda, kollha kemm aħna marbutin b'konfidenzjalita lejk bħala parteċipant u l-identità tiegħek ser tiġi protetta.

X'ser jiġri bl-informazzjoni li ser nagħti?
Kif semmejna qabel, kull informazzjoni li ser tingabar, ser tinżamm kunfidenzjali. L informazzjoni ser tinżamm f'post protett ġewwa l'isptar u l informazzjoni maħżuna fuq kompjuters ser tkun kodifikata b-

numri. Il-komputers ser ikunu wkoll protetti b'kodiċi. Ir-riċerkaturi biss ser ikollok aċċess għal dan l informazzjoni.

X'ser jiġri mir-riżultati ta dan l-istudju?
Fl-aħħar ta dan l-istudju, nixtiequ nippubblika ir-riżultati tagħna. Iżda ismek ser jinżamm kunfidenzjali. Jekk trid, nistgħu nibatulek sommarju tar-riżultati.

Min qed jorganizza u jħallas għal din ir-riċerka?
Din ir-riċerka qed tiġi organizzata mid-Dipartiment tal-Patoloġija, fil-Fakultà tal-Mediċina u Kirurġija, tal-Università ta Malta. L-ebda pagament mhu ser jingħata lil ħaddiema li qed jieħdu s'hemm f'dan l-istudju.

Minn min ġiet irriveduta din ir-riċerka?
Dan l-istudju ġie rivedut mil Kumitat tal-Etika fir-Riċerka, tal-Università ta Malta. Dan sar sabiex jipproteġi id drittijiet, saħħa u dinjita tiegħek.

Iżjed informazzjoni u kuntatti
Jekk għandek xi mistoqsijiet oħra fuq l-istudju, jekk jogħġbok ikkuntattja xi persuna minn hawn taħt. Dawn ser iwieġbu jew jagħtu parir.

Investigatriċi kap:
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Dr. Alexander Gatt, Consultant Haematologist
Email: alexander.gatt@um.edu.mt
Tel: 2545 6318
Address: Haematology Laboratory, Department of Pathology, Mater Dei Hospital, Msida MSD 2090

Grazzi talli ħadt il-ħin tiegħek sabiex taqra dan id-dokument t'informazzjoni u tikkonsidra tipparteċipa fl-istudju.

Patient number _____

FORMULA TAL-KUNSENS

Titlu tal-proġett: "Bleeding complications during oral anticoagulant treatment and their management in the Maltese Health Care System"

Clinical Study 3: "Patients satisfaction associated with warfarin treatment in different settings"

Isem tar-riċerkaturi: Dr. Nicoletta Riva, Dr. Alexander Gatt

1. Jien nikkonferma li qrajt u fhimt l informazzjoni (verżjoni 1.0; datata 26/10/2015) li ġejt mgħoti fuq dan l-istudju. Jien kelli l-opportunita li naħseb fuq din l-informazzjoni, nistaqsi mistoqsijiet, u dawn il-mistoqsijiet imwieġba. ☐

2. Jien nifhem li il-parteċipazzjoni tiegħi f'dan l-istudju hija volontarja u li jien liberu li nwaqqaf il-parteċipazzjoni tiegħi meta rrid, sa qabel ma jieqaf l-istudju, mingħajr ma nagħti spjegazzjoni u mingħajr ma jiġu affettwati id drittijiet mediċi u legali tiegħi. ☐

3. Jien nifhem li partijiet min-noti mediċi u informazzjoni miġbura, tista tiġi revista minn individwi li jagħmlu parti mit-tim tar-riċerkaturi ta Mater Dei jew awtoritajiet oħra. Jien nagħti permess għal dawn l-individwi biex ikollhom aċċess għan-noti mediċi tiegħi. ☐

4. Jien naċċetta li nipparteċipa f'dan l-istudju ☐

_____	_____	_____
Isem tal-Pazjent	Data	Firma
_____	_____	_____
Isem tal-persuna li qed jieħu il-kunsens	Data	Firma
_____	_____	_____
Isem tal-persuna li qed jieħu il-kunsens (jekk differenti mir-riċerkaturi)	Data	Firma

1 kopja għal-pazjent; 1 kopja għar-riċerkatur; 1 kopja għan-noti mediċi

Maltese version 1.0 – date 26/10/2015

Appendix D – Anticoagulant Pattern on Global Coagulation Assays: Additional Results

		Lag time	*ETP (nM*min)*	*Peak (nM)*	*Time to peak*	*Velocity index*
	Normal ranges	*5.0-10.3*	*1159-2317*	*71-401*	*7.4-16.2*	*NA*
Warfarinised plasma						
INR 2.22		10.06 (0.17)	659.5 (24.5)	127.30 (2.62)	12.56 (0)	51.13 (4.46)
INR 3.24		16.74 (0.83)	320.5 (16.5)	50.83 (1.09)	20.24 (0.67)	14.51 (0.38)
INR 4.11		18.41 (0.83)	257.0 (10.0)	37.86 (0.51)	22.08 (0.83)	10.31 (0.14)
Direct factor Xa inhibitors						
Apixaban	4 ng/ml	9.00 (0)	1465.5 (35.5)	135.49 (1.56)	14.17 (0.17)	26.26 (1.15)
	42 ng/ml	11.00 (0.33)	1154.5 (18.5)	80.32 (3.40)	15.00 (0.33)	20.08 (0.85)
	89 ng/ml	12.50 (0.17)	941.0 (22.0)	54.23 (0.65)	16.33 (0)	14.18 (0.79)
	128 ng/ml	11.67 (1.67)	1019.5 (208.5)	65.46 (22.5)	15.33 (2.0)	18.57 (7.82)
	179 ng/ml	14.33 (0.33)	723.5 (19.5)	35.38 (0)	18.33 (0.33)	8.85 (0)
	266 ng/ml	16.00 (0.33)	610.5 (7.5)	26.25 (0.07)	20.33 (0.33)	6.06 (0.02)
Edoxaban	0 ng/ml	9.00 (0.67)	1602.5 (16.5)	149.42	14.83 (1.17)	26.04 (4.92)
	15 ng/ml	12.00 (0)	1306.5 (15.5)	80.91 (0.10)	21.50 (0.17)	8.52 (0.14)
	51 ng/ml	13.33 (1.0)	1027.5 (72.5)	56.24 (6.55)	24.17 (1.50)	5.23 (0.85)
	85 ng/ml	14.00 (1.67)	1062.0 (232.0)	63.51 (22.21)	23.83 (3.83)	7.31 (3.87)
	113 ng/ml	16.83 (0.17)	646.5 (17.5)	30.84 (0.71)	28.83 (0.17)	2.57 (0.06)
	188 ng/ml	17.00 (1.33)	614.5 (33.5)	27.02 (1.18)	30.17 (1.17)	2.05 (0.06)
Rivaroxaban	22 ng/ml	9.67 (0)	1418.5 (27.5)	96.60 (1.19)	15.17 (0.50)	17.73 (1.83)
	55 ng/ml	11.50 (0.83)	1160.5 (115.5)	55.28 (9.69)	20.50 (3.50)	7.08 (3.18)
	118 ng/ml	14.50 (0.83)	745.5 (35.5)	29.38 (1.34)	27.67 (1.0)	2.23 (0.13)
	174 ng/ml	17.00 (0)	558.0 (1.0)	21.29 (0.23)	31.00 (0)	1.52 (0.02)
	231 ng/ml	18.00 (0.67)	513.5 (26.5)	17.93 (0.57)	32.33 (0.67)	1.25 (0.04)
	339 ng/ml	21.17 (0.50)	400.5 (18.5)	12.70 (0.10)	36.50 (0.17)	0.83 (0.02)
Direct thrombin inhibitors						
Argatroban	0.25 µg/ml	16.50 (0.17)	1262.0 (53.0)	172.03 (2.78)	19.67 (0.33)	54.43 (1.99)
	0.53 µg/ml	20.00 (0.33)	1065.0 (6.0)	117.25 (0.49)	23.50 (0.50)	33.57 (1.46)
	3.10 µg/ml	33.33 (1.0)	No tail found	6.63 (0.04)	49.00 (1.33)	0.43 (0.06)
	5.84 µg/ml	33.50 (0.83)	No tail found	3.25 (0.09)	66.17 (2.17)	0.10 (0.01)
Bivalirudin	5.9 µg/ml	42.50 (0.50)	1614.5 (39.5)	341.91 (2.52)	44.83 (0.50)	146.53 (1.08)
	13.8 µg/ml	56.50 (0.50)	1554.5 (13.5)	295.13 (2.27)	59.50 (0.50)	98.38 (0.76)
	31.0 µg/ml	74.17 (0.83)	1590.0 (21.0)	260.73 (7.31)	78.00 (1.0)	68.23 (4.87)
Dabigatran	0 ng/ml	11.67 (0)	1653.0 (28.0)	205.77 (0.11)	16.17 (0.17)	45.79 (1.67)
	44 ng/ml	19.33 (0.33)	1459.5 (2.5)	235.13 (0.07)	22.67 (0.33)	70.54 (0.02)
	92 ng/ml	24.50 (0.83)	1210.0 (19.0)	216.07 (2.74)	27.33 (1.0)	76.47 (3.53)
	148 ng/ml	28.17 (0.17)	1048.0 (10.0)	212.73 (1.34)	30.83 (0.17)	79.77 (0.50)
	176 ng/ml	31.17 (0.50)	896.5 (0.5)	180.71 (0.04)	33.83 (0.50)	67.77 (0.01)
	276 ng/ml	36.33 (1.67)	661.5 (7.5)	137.95 (2.10)	38.67 (1.67)	59.12 (0.90)
Indirect factor Xa inhibitors						
Danaparoid	0.33 U/ml	22.67 (1.33)	188.5 (7.5)	8.59 (0.22)	34.00 (1.67)	0.76 (0.04)
	0.78 U/ml	95.33 (4.0)	No tail found	1.05 (0.08)	114.33 (3.67)	0.06 (0)
	1.93 U/ml	Flat CAT traces				
Enoxaparin	0.35 U/ml	10.33 (0)	401.5 (8.5)	22.63 (0.47)	19.17 (0.17)	2.56 (0.10)
	1.06 U/ml	Flat CAT traces				
	1.95 U/ml	Flat CAT traces				
Fondaparinux	0.64 µg/ml	15.50 (0.50)	714.5 (43.5)	49.94 (2.08)	22.50 (0.17)	7.14 (0.04)
	1.64 µg/ml	21.33 (6.67)	202.5 (2.5)	9.48 (0.39)	37.67 (0.33)	0.69 (0.29)
	2.24 µg/ml	50.50 (1.50)	80.0 (11.0)	1.99 (0.38)	71.17 (4.83)	0.10 (0.03)

Table D1. Results of the different anticoagulant concentrations on the CAT at TF 1pM
Results are reported as mean (SD) of two measurements.

		CAT at TF 5pM				*CAT at TF 1pM*			
		Lag	***ETP***	***Peak***	***Time***	***Lag***	***ETP***	***Peak***	***Time***
Warfarinised plasma									
INR 2.22		1.42	0.39	0.55	1.00	1.39	0.38	0.59	1.04
INR 3.24		2.25	0.21	0.26	1.50	2.32	0.18	0.23	1.68
INR 4.11		2.79	0.17	0.20	1.79	2.55	0.15	0.17	1.83
Direct factor Xa inhibitors									
Apixaban	4 ng/ml	1.21	1.00	0.82	1.17	1.23	0.82	0.61	1.18
	42 ng/ml	1.59	0.84	0.45	1.37	1.50	0.65	0.36	1.25
	89 ng/ml	1.68	0.61	0.27	1.37	1.71	0.53	0.24	1.36
	128 ng/ml	1.85	0.51	0.20	1.46	1.59	0.57	0.29	1.28
	179 ng/ml	2.10	0.45	0.15	1.61	1.95	0.41	0.16	1.53
	266 ng/ml	2.27	0.39	0.12	1.74	2.18	0.34	0.12	1.69
Edoxaban	0 ng/ml	1.38	0.92	0.71	1.37	1.23	0.90	0.67	1.24
	15 ng/ml	1.81	0.84	0.44	2.01	1.64	0.73	0.36	1.79
	51 ng/ml	2.14	0.72	0.30	2.45	1.82	0.58	0.25	2.01
	85 ng/ml	2.40	0.60	0.23	2.77	1.91	0.60	0.28	1.99
	113 ng/ml	2.57	0.52	0.19	2.93	2.30	0.36	0.14	2.40
	188 ng/ml	2.91	0.42	0.14	3.17	2.32	0.34	0.12	2.51
Rivaroxaban	22 ng/ml	1.34	0.91	0.56	1.37	1.32	0.80	0.43	1.26
	55 ng/ml	1.89	0.65	0.23	2.49	1.57	0.65	0.25	1.71
	118 ng/ml	2.23	0.50	0.15	2.93	1.98	0.42	0.13	2.31
	174 ng/ml	2.44	0.45	0.13	3.08	2.32	0.31	0.10	2.58
	231 ng/ml	2.70	0.35	0.10	3.37	2.46	0.29	0.08	2.69
	339 ng/ml	3.04	0.26	0.07	3.63	2.89	0.22	0.06	3.04
Direct thrombin inhibitors									
Argatroban	0.25 µg/ml	1.97	0.73	0.79	1.37	2.15	0.71	0.82	1.55
	0.53 µg/ml	2.46	0.57	0.53	1.59	2.61	0.60	0.56	1.85
	3.10 µg/ml	4.59	NC	0.02	4.88	4.35	NC	0.03	3.87
	5.84 µg/ml	6.09	NC	0.01	7.58	4.37	NC	0.02	5.22
Bivalirudin	5.9 µg/ml	5.29	0.83	1.20	2.96	5.80	0.85	1.40	3.79
	13.8 µg/ml	6.91	0.86	1.09	3.84	7.71	0.82	1.21	5.03
	31.0 µg/ml	10.47	0.84	0.92	5.79	10.12	0.84	1.07	6.59
Dabigatran	0 ng/ml	1.22	1.05	1.04	1.09	1.52	0.94	0.98	1.28
	44 ng/ml	1.97	0.87	0.96	1.42	2.52	0.83	1.12	1.79
	92 ng/ml	2.68	0.75	0.91	1.68	3.19	0.69	1.03	2.16
	148 ng/ml	3.26	0.63	0.81	1.96	3.67	0.59	1.01	2.43
	176 ng/ml	3.57	0.56	0.73	2.12	4.06	0.51	0.86	2.67
	276 ng/ml	4.50	0.40	0.56	2.53	4.74	0.37	0.66	3.05
Indirect factor Xa inhibitors									
Danaparoid	0.33 U/ml	1.25	0.31	0.14	1.57	3.09	0.11	0.04	2.83
	0.78 U/ml	2.08	0.07	0.01	3.87	13.01	NC	0.00	9.53
	1.93 U/ml	NC	NC	NC	NC	NC	NC	NC	NC
Enoxaparin	0.35 U/ml	1.08	0.74	0.46	1.30	1.41	0.23	0.10	1.60
	1.06 U/ml	1.50	0.08	0.02	2.30	NC	NC	NC	NC
	1.95 U/ml	NC	NC	NC	NC	NC	NC	NC	NC
Fondaparinux	0.64 µg/ml	1.83	0.65	0.33	1.81	2.02	0.40	0.24	1.78
	1.64 µg/ml	2.96	0.23	0.09	2.65	2.78	0.11	0.05	2.97
	2.24 µg/ml	4.17	0.11	0.04	3.52	6.58	0.05	0.01	5.62

Table D2. Results of the different anticoagulant concentrations on the CAT at TF 5pM and TF 1pM, expressed as mean ratio to normal plasma

		SP (min)	TMA (min)	G parameter (dyn/cm^2)	E parameter (dyn/cm^2)	TPI (/sec)	CI
Warfarinised plasma							
INR 2.22		9.45 (0.07)	22.60 (2.83)	3.05 (0.35)	60.80 (7.35)	12.00 (4.53)	-2.70 (0.57)
INR 3.24		15.10 (3.25)	33.45 (1.77)	2.95 (0.07)	58.90 (1.27)	5.70 (0.57)	-3.95 (0.78)
INR 4.11		13.80 (5.09)	29.75 (9.83)	3.10 (0.14)	61.50 (3.39)	8.80 (3.54)	-3.70 (1.41)
Direct factor Xa inhibitors							
Apixaban	4 ng/ml	15.40 (1.27)	30.80 (5.66)	2.85 (0.35)	56.90 (7.35)	9.15 (4.60)	-4.30 (0.99)
	42 ng/ml	13.10 (0.99)	28.00 (5.52)	2.80 (0.57)	55.35 (11.53)	5.75 (1.20)	-3.45 (1.06)
	89 ng/ml	15.25 (1.06)	29.20 (4.95)	2.30 (0.28)	45.30 (5.37)	6.10 (0.14)	-5.10 (0)
	128 ng/ml	13.60 (6.51)	33.50 (3.39)	2.55 (0.78)	50.90 (15.0)	4.55 (1.06)	-4.10 (2.69)
	179 ng/ml	14.95 (1.77)	29.70 (0.71)	2.20 (0.28)	44.35 (6.43)	4.60 (0.71)	-4.90 (0.85)
	266 ng/ml	15.90 (1.98)	28.85 (4.03)	1.95 (0.35)	39.65 (6.86)	4.70 (2.83)	-5.45 (0.92)
Edoxaban	0 ng/ml	11.30 (0.14)	27.00 (2.55)	2.70 (0.71)	54.00 (14.28)	6.50 (0.42)	-3.50 (1.13)
	15 ng/ml	14.85 (1.06)	27.10 (1.41)	2.10 (0.14)	41.25 (3.04)	5.45 (1.34)	-5.15 (0.49)
	51 ng/ml	13.00 (0.57)	29.00 (1.13)	2.20 (0.57)	43.85 (11.24)	4.70 (2.55)	-4.55 (1.06)
	85 ng/ml	19.75 (7.14)	35.50 (9.62)	1.95 (0.35)	38.70 (7.50)	3.25 (2.05)	-6.35 (2.33)
	113 ng/ml	24.35 (9.69)	43.05 (11.53)	1.95 (0.49)	39.05 (9.40)	2.60 (1.41)	-7.40 (3.11)
	188 ng/ml	25.20 (2.26)	50.50 (3.68)	2.05 (0.21)	41.05 (3.18)	1.90 (0.28)	-7.40 (0.28)
Rivaroxaban	22 ng/ml	12.20 (0.57)	27.25 (1.77)	2.35 (0.21)	46.70 (3.68)	5.75 (1.34)	-4.05 (0.07)
	55 ng/ml	16.6 (2.55)	30.00 (0.57)	2.15 (0.07)	42.65 (0.78)	5.25 (1.06)	-5.45 (0.78)
	118 ng/ml	17.4 (1.13)	32.70 (3.54)	2.05 (0.21)	41.30 (4.10)	3.75 (1.48)	-5.65 (0.49)
	174 ng/ml	17.00 (1.27)	34.75 (2.47)	1.95 (0.07)	39.70 (0.99)	2.80 (0.85)	-5.60 (0.14)
	231 ng/ml	24.35 (2.90)	45.35 (0.21)	1.85 (0.07)	37.15 (0.78)	2.20 (0.42)	-7.65 (0.78)
	339 ng/ml	26.60 (4.53)	48.55 (8.98)	1.65 (0.07)	33.35 (1.48)	1.50 (0.71)	-8.30 (0.99)
Direct thrombin inhibitors							
Argatroban	0.25 µg/ml	18.25 (6.43)	31.95 (2.47)	2.35 (0.07)	46.45 (1.48)	7.20 (2.83)	-5.65 (1.63)
	0.53 µg/ml	21.65 (2.05)	38.80 (0.42)	2.05 (0.07)	41.60 (1.70)	3.65 (0.78)	-6.85 (0.21)
	3.10 µg/ml	37.85 (9.26)	57.15 (13.51)	2.40 (0.14)	47.75 (3.89)	3.85 (1.77)	-10.20 (2.55)
	5.84 µg/ml	61.95 (17.89)	85.20 (23.33)	2.35 (0.07)	46.95 (0.21)	2.70 (0.85)	-16.20 (4.38)

		SP (min)	TMA (min)	G parameter (dyn/cm^2)	E parameter (dyn/cm^2)	TPI (/sec)	CI
Bivalirudin	5.9 µg/ml	29.85 (8.84)	49.20 (9.19)	2.25 (0.07)	44.85 (0.21)	4.60 (0)	-9.00 (2.69)
	13.8 µg/ml	43.65 (2.62)	66.15 (9.55)	2.20 (0)	44.00 (0.85)	2.45 (0.92)	-11.90 (0.85)
	31.0 µg/ml	49.80 (15.41)	74.70 (12.02)	1.90 (0.14)	38.50 (2.83)	1.95 (0.21)	-13.80 (4.24)
Dabigatran	0 ng/ml	15.20 (0.57)	29.05 (0.35)	2.20 (0.14)	44.35 (3.61)	5.25 (2.76)	-4.85 (0.07)
	44 ng/ml	17.65 (1.06)	32.10 (5.09)	2.25 (0.07)	45.15 (1.20)	5.75 (2.76)	-5.70 (0.42)
	92 ng/ml	26.15 (0.92)	42.35 (0.64)	2.05 (0.07)	40.80 (2.55)	3.20 (0.14)	-8.05 (0.35)
	148 ng/ml	26.30 (9.76)	38.50 (12.30)	2.85 (0.35)	56.65 (7.14)	8.70 (0.14)	-7.00 (1.98)
	176 ng/ml	27.25 (3.46)	48.00 (0)	2.80 (0.57)	56.05 (12.52)	6.55 (5.59)	-7.05 (0.21)
	276 ng/ml	40.30 (12.16)	64.35 (31.32)	2.60 (0.42)	52.35 (7.71)	5.70 (6.36)	-10.40 (2.97)
Indirect factor Xa inhibitors							
Danaparoid	0.33 U/ml	24.30 (14.85)	57.15 (9.26)	1.90 (0.57)	37.75 (11.24)	1.05 (0.35)	-7.60 (4.81)
	0.78 U/ml	Flat TEG traces					
	1.93 U/ml	Flat TEG traces					
Enoxaparin	0.35 U/ml	18.70 (1.56)	44.55 (1.06)	1.95 (0.07)	39.25 (1.91)	1.75 (0.35)	-6.30 (0.14)
	1.06 U/ml	Flat TEG traces					
	1.95 U/ml	Flat TEG traces					
Fondaparinux	0.64 µg/ml	19.20 (1.13)	40.85 (6.86)	1.90 (0)	38.60 (0.14)	2.15 (0.49)	-6.35 (0.64)
	1.64 µg/ml	Flat TEG traces					
	2.24 µg/ml	Flat TEG traces					

Table D3. Results of the different anticoagulant concentrations on the secondary parameters of the TEG
Results are reported as mean (SD) of two measurements.

		R time	K time	Angle	MA	TMA
Warfarinised plasma						
INR 2.22		0.85	0.90	1.10	1.18	0.97
INR 3.24		1.38	1.68	0.70	1.16	1.44
INR 4.11		1.27	1.21	0.85	1.19	1.28
Direct factor Xa inhibitors						
Apixaban	4 ng/ml	1.47	1.40	0.89	1.08	1.44
	42 ng/ml	1.23	1.94	0.70	1.06	1.31
	89 ng/ml	1.53	1.42	0.74	0.93	1.36
	128 ng/ml	1.36	2.31	0.68	1.00	1.57
	179 ng/ml	1.47	1.85	0.68	0.92	1.39
	266 ng/ml	1.47	1.88	0.80	0.85	1.35
Edoxaban	0 ng/ml	1.20	1.62	0.73	1.04	1.26
	15 ng/ml	1.38	1.48	0.83	0.87	1.27
	51 ng/ml	1.34	1.96	0.64	0.91	1.36
	85 ng/ml	1.87	2.65	0.59	0.83	1.66
	113 ng/ml	2.30	3.17	0.50	0.84	2.01
	188 ng/ml	2.45	4.17	0.36	0.87	2.36
Rivaroxaban	22 ng/ml	1.19	1.60	0.76	0.95	1.27
	55 ng/ml	1.57	1.60	0.78	0.90	1.40
	118 ng/ml	1.70	2.25	0.60	0.87	1.53
	174 ng/ml	1.70	2.88	0.50	0.85	1.62
	231 ng/ml	2.37	3.33	0.45	0.81	2.12
	339 ng/ml	2.52	4.77	0.38	0.75	2.27
Direct thrombin inhibitors						
Argatroban	0.25 µg/ml	1.52	1.11	0.97	0.99	1.37
	0.53 µg/ml	1.93	1.87	0.61	0.92	1.67
	3.10 µg/ml	3.16	2.19	0.60	1.01	2.45
	5.84 µg/ml	5.11	2.92	0.47	1.00	3.66
Bivalirudin	5.9 µg/ml	3.02	1.87	0.53	0.93	2.30
	13.8 µg/ml	4.09	3.73	0.41	0.91	3.09
	31.0 µg/ml	4.60	3.79	0.45	0.83	3.49
Dabigatran	0 ng/ml	1.29	1.55	0.83	0.96	1.25
	44 ng/ml	1.57	1.42	0.82	0.97	1.38
	92 ng/ml	2.28	2.03	0.68	0.91	1.82
	148 ng/ml	2.17	1.05	1.05	1.13	1.65
	176 ng/ml	2.28	1.98	0.75	1.12	2.06
	276 ng/ml	3.30	3.55	0.72	1.07	2.76
Indirect factor Xa inhibitors						
Danaparoid	0.33 U/ml	2.51	7.06	0.23	0.81	2.67
	0.78 U/ml	NC	NC	NC	NC	NC
	1.93 U/ml	NC	NC	NC	NC	NC
Enoxaparin	0.35 U/ml	2.03	4.38	0.32	0.84	2.08
	1.06 U/ml	NC	NC	NC	NC	NC
	1.95 U/ml	NC	NC	NC	NC	NC
Fondaparinux	0.64 µg/ml	1.76	2.97	0.47	0.87	1.75
	1.64 µg/ml	NC	NC	NC	NC	NC
	2.24 µg/ml	NC	NC	NC	NC	NC

Table D4. Results of the different anticoagulant concentrations on the main parameters of the TEG, expressed as mean ratio to normal plasma

		R time	K time	Angle	MA	LY30	LY60
Warfarinised plasma							
INR 2.22		0.80	0.73	1.31	1.12	3.47	1.97
INR 3.24		1.66	1.86	0.56	0.80	2.25	1.47
INR 4.11		1.49	2.36	0.55	0.68	2.78	1.62
Direct factor Xa inhibitors							
Apixaban	89 ng/ml	1.28	1.81	0.76	0.93	2.39	1.45
	128 ng/ml	1.38	2.27	0.60	0.76	3.00	1.62
Edoxaban	51 ng/ml	1.77	1.20	0.78	0.64	4.07	2.43
	85 ng/ml	1.78	1.17	0.51	0.50	4.72	2.09
Rivaroxaban	118 ng/ml	1.56	1.16	0.68	0.81	2.31	1.47
	174 ng/ml	1.90	2.01	0.57	0.63	3.93	1.86
Direct thrombin inhibitors							
Argatroban	0.53 μg/ml	1.71	-	0.52	0.55	1.82	1.23
	3.10 μg/ml	3.12	2.17	0.32	0.51	1.85	1.17
Bivalirudin	5.9 μg/ml	3.10	-	0.90	0.50	7.98	3.49
	13.8 μg/ml	4.25	3.24	0.70	0.66	5.18	1.72
Dabigatran	92 ng/ml	1.68	1.19	0.97	0.87	2.09	1.33
	148 ng/ml	2.14	1.33	0.94	0.91	2.69	1.47
Indirect factor Xa inhibitors							
Danaparoid 0.33 U/ml		3.61	-	0.10	0.17	4.02	1.64
Enoxaparin 0.35 U/ml		2.59	-	0.25	0.27	7.61	2.21
Fondaparinux 0.64 μg/ml		2.42	-	0.16	0.26	1.58	1.14

Table D5. Results of the different anticoagulant concentrations on the TEG with TPA, expressed as mean ratio to normal plasma

Appendix E – English and Maltese versions of the DASS and the PACT-Q

English Version Date _____ Patient number _____

DASS instrument

We would like to know how your anti-clot treatment (warfarin) affects you, and what you know and feel about your anti-clot treatment. Please mark the answer by ticking or circling the phrase that best fits your situation. If a question does not apply to you, then check "not at all".

When you have anti-clot treatment you tend to bleed or bruise more easily. You may limit your activities as a result. Limit means you do less of the activity, or no longer perform the activity at all.

1a) How much does the possibility of bleeding or bruising limit you from taking part in physical activities (for example, housework, gardening, dancing, sports, or anything else you would usually do)?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

1b) How much does the possibility of bleeding or bruising limit you from traveling?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

1c) How much does the possibility of bleeding or bruising limit you from getting the medical care you need (for example, visiting a dentist, chiropractor, or doctor of your choice)?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

1d) How much does the possibility of bleeding or bruising limit your ability to work?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

1e) Overall, how much does the possibility of bleeding or bruising affect your daily life?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

Being on anti-clot treatment may mean changing some of your other habits as well.

2a) How much does anti-clot treatment limit your choice of food (diet)?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

2b) How much does anti-clot treatment limit the alcoholic beverages you might wish to drink?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

2c) How much does anti-clot treatment limit the over-the-counter medications (for example, aspirin, ibuprofen, vitamins) you might wish to take?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

2d) Overall, how much does anti-clot treatment affect your daily life?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

Being on anti-clot treatment means doing a lot of things, some every day and some less often.

Daily tasks could include: remembering to take your medicine at a certain time, taking the correct dose of your medicine, not drinking much alcohol, following a moderate diet, avoiding bruising and bleeding, and so forth.
Occasional tasks could include: traveling to the clinic for blood check-ups, contacting the clinic in case of bleeding or other important events, and so forth.

3a) How much of a hassle (inconvenience) are the daily tasks of anti-clot treatment?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

3b) How much of a hassle (inconvenience) are the occasional tasks of anti-clot treatment?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

Considering anti-clot treatment as a whole (that is, both the daily and occasional tasks), please consider the following.

3c) How complicated do you find your anti-clot treatment to be?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

3d) How time-consuming do you find your anti-clot treatment to be?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

3e) How frustrating do you find your anti-clot treatment to be?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

3f) How painful do you find your anti-clot treatment to be?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

3g) Overall, how much of a burden do you find your anti-clot treatment to be?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

3h) Overall, how confident are you about handling your anti-clot treatment

[7]Not at all	[6]A little	[5]Somewhat	[4]Moderately	[3]Quite a bit	[2]A lot	[1]Very much

These last questions ask what you know and feel about your anti-clot treatment.

4a) How well do you feel that you understand the medical reason for your anti-clot treatment?

[7]Not at all	[6]A little	[5]Somewhat	[4]Moderately	[3]Quite a bit	[2]A lot	[1]Very much

4b) How much do you feel reassured because of your anti-clot treatment?

[7]Not at all	[6]A little	[5]Somewhat	[4]Moderately	[3]Quite a bit	[2]A lot	[1]Very much

4d) How much do you worry about bleeding and bruising?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

4f) Overall, how much has anti-clot treatment had a positive impact on your life?

[7]Not at all	[6]A little	[5]Somewhat	[4]Moderately	[3]Quite a bit	[2]A lot	[1]Very much

4g) Overall, how much has anti-clot treatment had a negative impact on your life?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

4h) Overall, how satisfied are you with your anti-clot treatment?

[7]Not at all	[6]A little	[5]Somewhat	[4]Moderately	[3]Quite a bit	[2]A lot	[1]Very much

4i) Compared with other treatments you have had, how difficult is your anti-clot treatment to manage?

[1]Not at all	[2]A little	[3]Somewhat	[4]Moderately	[5]Quite a bit	[6]A lot	[7]Very much

4j) How likely would you be to recommend this form of anti-clot treatment to someone else with your disease or medical condition?

[7]Not at all	[6]A little	[5]Somewhat	[4]Moderately	[3]Quite a bit	[2]A lot	[1]Very much

Maltese Version Date _____ Patient number _____

DASS instrument

Nixtiequ nkunu nafu kif it-trattament kontra l-embolu (boċċa żgħira ta' demm magħqud li jista' jimblokka parti mis-sistema kardjovaskulari) jaffettwak, x'taf u xi tħoss fuq it-trattament tiegħek kontra l-embolu (warfarina). Jekk jogħġbok, irrispondi billi timmarka jew tagħmel ċirku madwar il-fraži li fil-fehma tiegħek, taqbel l-aktar mas-sitwazzjoni tiegħek. Jekk taħseb li l-mistoqsija ma tkunx tapplika għalik, immarka "Lanqas xejn".

Meta tieħu t-trattament kontra l-embolu jista' jkollok tendenza li titlef xi demm jew titbenġel iktar malajr. Minħabba f'hekk, jista' jkun li jkollok tillimita l-attivitajiet tiegħek. Tillimita tfisser tnaqqas din l-attività, jew ma tipprattikahiex iktar.

1a) Kemm taħseb li l-possibiltà li titlef xi demm jew li titbenġel tillimitak milli tipparteċipa f'attivitajiet fiżiċi (pereżempju, xogħol tad-dar, ġardinaġġ, żfin, sports, jew kwalunkwe ħaġa oħra li tagħmel is-soltu)?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

1b) Kemm taħseb li l-possibiltà li titlef xi demm jew li titbenġel tillimitak milli tivvjaġġa?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

1c) Kemm taħseb li l-possibiltà li titlef xi demm jew titbenġel tillimitak milli tieħu l-kura medika li għandek bżonn? (pereżempju, li tmur għand dentist, 'chiropractor', jew tabib tal-għażla tiegħek)?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

1d) Kemm taħseb li l-possibiltà li titlef xi demm jew titbenġel tillimita l-ħila tiegħek li taħdem?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

1e) B'mod ġenerali, kemm taħseb li l-possibiltà li titlef xi demm jew titbenġel taffettwa l-ħajja ta' kuljum tiegħek?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

Li tkun qed tieħu trattament kontra l-embolu jista' jfisser bdil ta' xi drawwiet oħra tiegħek ukoll.

2a) Kemm taħseb li t-trattament kontra l-embolu jillimita l-għażla tal-ikel tiegħek (id-dieta)?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

2b) Kemm taħseb li t-trattament kontra l-embolu jillimita l-konsum tal-alkoħol tiegħek?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

2c) Kemm taħseb li t-trattament kontra l-embolu jillimitak milli tieħu mediċini li tista' tixtrihom mingħajr riċetta (pereżempju, aspirini, ibuprofen, vitamini)?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

2d) B'mod ġenerali, kemm taħseb li t-trattament kontra l-embolu jaffettwa l-ħajja ta' kuljum tiegħek?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

Li tkun qed tieħu trattament kontra l-embolu jista' jfisser li tagħmel ħafna affarijiet, ġieli kuljum u ġieli inqas ta' spiss. Ezempju:

Ħidmiet ta' kuljum jistgħu jinkludu: li tiftakar tieħu l-mediċina f'ċertu ħin, li tieħu d-doża t-tajba tal-mediċina tiegħek, li ma tixrobx ħafna alkoħol, li tagħmel dieta moderata, tevita li titbenġel u li titlef xi demm u sitwazzjonijiet oħra simili.

Ħidmiet okkażjonali: tmur sal-klinika għal test tad-demm, tikkuntattja l-klinika f'każ li titlef xi demm jew għal raġunijiet importanti oħra, u sitwazzjonijiet oħra simili.

3a) Kemm huma ta' battikata (inkonvenjent) għalik il-ħidmiet ta' kuljum marbuta mat-trattament kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

3b) Kemm huma ta' battikata (inkonvenjent) għalik il-ħidmiet okkażjonali marbuta mat-trattament kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

Meta tħares lejn it-trattament kontra l-embolu bħala entità sħiħa (jiġifieri meta tqis kemm l-attivitajiet ta' kuljum kif ukoll dawk okkażjonali), jekk jogħġbok, ikkunsidra dan li ġej:

3c) Kemm issibu kkumplikat it-trattament tiegħek kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

3d) Kemm taħseb li hu ta' ħela ta' ħin it-trattament tiegħek kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

3e) Kemm hu frustranti t-trattament tiegħek kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

3f) Kemm jikkaġunalek uġigħ it-trattament li qed tieħu kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

3g) B'mod ġenerali, kemm hu ta' piż it-trattament tiegħek kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

3h) B'mod ġenerali, kemm tħossok kunfidenti meta tieħu ħsieb it-trattament tiegħek kontra l-embolu?

[7]Lanqas xejn	[6]Ftit li xejn	[5]Kemxejn	[4]Hekk u hekk	[3]Mhux ħażin	[2]Ħafna	[1]Ħafna ħafna

Dawn il-ftit mistoqsijiet tal-aħħar huma dwar dak li taf u x'taħseb fuq it-trattament kontra l-embolu.

4a) Kemm taħseb li tifhem sew ir-raġuni medika għat-trattament kontra l-embolu?

[7]Lanqas xejn	[6]Ftit li xejn	[5]Kemxejn	[4]Hekk u hekk	[3]Mhux ħażin	[2]Ħafna	[1]Ħafna ħafna

4b) Kemm tħossok rassigurat/a fuq t-trattament kontra l-embolu?

[7]Lanqas xejn	[6]Ftit li xejn	[5]Kemxejn	[4]Hekk u hekk	[3]Mhux ħażin	[2]Ħafna	[1]Ħafna ħafna

4d) Kemm tħossok inkwetat/a fuq il-fatt li titlef xi demm jew titbenġel?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

4f) B'mod ġenerali, kemm taħseb li t-trattament kontra l-embolu ħalla impatt pożittiv fuq ħajtek?

[7]Lanqas xejn	[6]Ftit li xejn	[5]Kemxejn	[4]Hekk u hekk	[3]Mhux ħażin	[2]Ħafna	[1]Ħafna ħafna

4g) B'mod ġenerali, kemm taħseb li t-trattament kontra l-embolu ħalla impatt negattiv fuq ħajtek?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

4h) B'mod ġenerali, kemm tħossok sodisfatt/a bit-trattament li qed tieħu kontra l-embolu?

[7]Lanqas xejn	[6]Ftit li xejn	[5]Kemxejn	[4]Hekk u hekk	[3]Mhux ħażin	[2]Ħafna	[1]Ħafna ħafna

4i) Meta tqabbel ma' trattamenti oħra li kellek, kemm hu diffiċli biex tieħu ħsieb it-trattament tiegħek kontra l-embolu?

[1]Lanqas xejn	[2]Ftit li xejn	[3]Kemxejn	[4]Hekk u hekk	[5]Mhux ħażin	[6]Ħafna	[7]Ħafna ħafna

4j) Kemm taħseb li tirrakkomanda dil-forma ta' trattament kontra l-embolu lil xi ħadd ieħor bl-istess marda jew kundizzjoni medika?

[7]Lanqas xejn	[6]Ftit li xejn	[5]Kemxejn	[4]Hekk u hekk	[3]Mhux ħażin	[2]Ħafna	[1]Ħafna ħafna

PACT-Q2
(Perception AntiCoagulant Treatment Questionnaire)

- The purpose of this questionnaire is to understand your expectations and to assess your satisfaction with your anticoagulant treatment (treatment that stops the blood from clotting).
- Throughout the questionnaire, the term "taking" refers to how you take your anticoagulant treatment (either by pill or injection).
- Please read each question carefully, answering as openly as you can and without help from anyone. There are no wrong answers.
- All of the information you provide will be kept confidential.
- This questionnaire will take about **10 minutes** to complete.

Convenience

Please answer the following questions to help us understand how convenient it is to take your treatment.

Please check one box per line.

B1 - How difficult is it to take your anticoagulant treatment (i.e., pills or injections, number of pills or injections, frequency of intake ...)?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B2 - How bothered are you by taking your anticoagulant treatment?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B3 - Some anticoagulant treatments may need dose adjustments; how difficult is this for you?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B4 - Certain medications CANNOT be taken with anticoagulant treatments; how difficult is this for you?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B5 - It is recommended that certain foods be avoided while taking an anticoagulant treatment; how difficult is this for you?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B6 - How difficult is it for you to take your anticoagulant treatment when you are away from home?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B7 - How difficult is it for you to plan your time around your anticoagulant treatment (i.e., appointments with nurses, doctors or labs ...)?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B8 - How bothered are you by the medical follow-up required with your anticoagulant treatment?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B9 - How difficult is it for you to take your anticoagulant treatment as directed on a regular basis?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B10 - Do you feel more dependent on others (i.e partner, family, nurse…) because of your anticoagulant treatment?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

B11 - How worried are you about having to interrupt or stop your anticoagulant treatment?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

Burden of Disease and Treatment

Please answer the following questions to help us understand how your disease and its treatment affect you.

Please check one box per line.

C1 - Because of potential side effects (i.e., minor bruises, bleeding…), do you limit your usual activities (i.e., work, leisure, social, or physical activities…)?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Extremely

C2 - How much physical discomfort do you have due to bruises or pain?

☐1	☐2	☐3	☐4	☐5
None	A little	Moderate	A lot	Extreme

Anticoagulant Treatment Satisfaction

Please answer the following questions to help us understand how satisfied you are with your treatment.

Please check one box per line.

D1 - How reassured do you feel by your anticoagulant treatment?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Somewhat	Very	Completely

D2 - Do you feel that your anticoagulant treatment has decreased your symptoms (i.e., leg pain or swelling, palpitations, shortness of breath, or chest pain…)?

☐1	☐2	☐3	☐4	☐5
Not at all	A little	Moderately	A lot	Completely

D3 - How did your experience with side effects such as minor bruises or bleeding (i.e., while shaving, cooking, after small cuts…) compare to what you expected?

☐1	☐2	☐3	☐4	☐5
It is much worse than what I expected	It is worse than what I expected	It is exactly what I expected	It is better than what I expected	It is much better than what I expected

D4 - Regarding the follow-up of your disease and anticoagulant treatment, how satisfied are you with your level of independence?

☐1	☐2	☐3	☐4	☐5
Extremely dissatisfied	Dissatisfied	Neither satisfied nor dissatisfied	Satisfied	Extremely satisfied

D5 - How satisfied are you with the methods (i.e., appointments with nurses, doctors, labs…) used to ensure the follow-up of your disease and anticoagulant treatment?

☐1	☐2	☐3	☐4	☐5
Extremely dissatisfied	Dissatisfied	Neither satisfied nor dissatisfied	Satisfied	Extremely satisfied

D6 - How satisfied are you with the form of your anticoagulant treatment (oral pill / injection)?

☐1	☐2	☐3	☐4	☐5
Extremely dissatisfied	Dissatisfied	Neither satisfied nor dissatisfied	Satisfied	Extremely satisfied

D7 - **Overall**, how satisfied are you with your anticoagulant treatment?

☐1	☐2	☐3	☐4	☐5
Extremely dissatisfied	Dissatisfied	Neither satisfied nor dissatisfied	Satisfied	Extremely satisfied

Please make sure you answered all questions.

Thank you for your time.

PACT-Q2
(Perception AntiCoagulant Treatment Questionnaire)
Kwestjonarju dwar il-perċezzjoni tat-trattament kontra l-embolu

- L-għan ta' dan il-kwestjonarju hu sabiex nifhmu l-aspettattivi tiegħek u biex nevalwaw is-sodisfazzjoni tat-trattament tiegħek kontra l-embolu (trattament li jwaqqaf id-demm milli jagħqad).

- Matul il-kwestjonarju, it-terminu "tieħu" ifisser kif inti tieħu t-trattament kontra l-embolu (jew bil-pilloli jew bit-titqiba).

- Jekk jogħġbok, aqra sew kull mistoqsija, irrispondi bl-iktar mod sinċier u mingħajr għajnuna ta' ħadd. M'hemm l-ebda tweġiba żbaljata.

- L-informazzjoni kollha li tipprovdi ser tibqa' kunfidenzjali.

- Dan il-kwestjonarju ser jieħu madwar **10 minuti** biex jitlesta.

Konvenjenza

Jekk jogħgbok, wieġeb il-mistoqsijiet li ġejjin biex tgħinna nifhmu kemm hu tkonvenjenti biex tieħu t-trattament tiegħek.

Jekk jogħġbok, immarka kaxxa waħda f'kull linja.

B1 - Kemm hu diffiċli li tieħu t-trattament kontra l-embolu (bħal, pilloli jew titqiba, numru ta' pilloli jew titqib, frekwenza ta' kemm tieħu l-mediċina ...)?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	Mhux ħażin	Estremament

B2 – Kemm idejqek il-fatt li trid tieħu -t-trattament tiegħek kontra l-embolu?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B3 - Xi trattamenti kontra l-embolu jirrikjedu li jkun hemm bżonn ta' aġġustamenti fid-doża; kemm hu diffiċli dan għalik?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B4 - Ċerti mediċini MA JISTGĦUX jittieħdu ma' trattamenti kontra l-embolu; kemm hu diffiċli dan għalik?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B5 - Huwa rrakkomandat li ċertu ikel jiġi evitat waqt li qed tieħu t-trattament kontra l-embolu; kemm hu diffiċli dan għalik?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B6 - Kemm hu diffiċli għalik biex tieħu t-trattament tiegħek kontra l-embolu meta tkun **'il** bogħod mid-dar?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B7 - Kemm hu diffiċli għalik biex tippjana l-ħin tiegħek madwar it-trattament tiegħek kontra l-embolu (**bħal**, appuntamenti mal-**infermiera, tobba jew laboratorji…**)?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B8 - Kemm tħossok li tiddejjaq bil-**viżti** tat-tabib matul t-trattament tiegħek kontra l-embolu?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B9 - Kemm hu diffiċli għalik biex tieħu t-trattament tiegħek kontra l-embolu **fuq bażi regolari** skont kif ikunu qalulek?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B10 - Tħossok iktar dipendenti fuq ħaddieħor (bħal, sieħeb/**sieħba, familja, infermiera…**) minħabba t-trattament tiegħek kontra l-embolu?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

B11 - Kemm tħossok inkwetat/a dwar il-fatt li jkollok tinterrompi jew twaqqaf it-trattament tiegħek kontra l-embolu?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

Piż tal-Marda u t-Trattament

Jekk jogħġbok, irrispondi l-mistoqsijiet li ġejjin biex tgħinna nifhmu kif il-marda u t-trattament qed jaffettwawk.

Jekk jogħġbok, immarka kaxxa waħda f'kull linja.

C1 - Minħabba l-effetti sekondarji **li jista' jkun hemm** (bħal, tbenġil żgħir, titlef xi demm…), inti tillimita l-attivitajiet tas-soltu (bħal, xogħol, divertiment, attivitajiet soċjali jew fiżiċi…)?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

C2 - Kemm **thossok** skomdu/skomda fiżikament minħabba t-tbenġil jew l-uġigħ?

☐1	☐2	☐3	☐4	☐5
Xejn	Ftit	Moderatament	**Mhux ħażin**	Estremament

Sodisfazzjon bit-Trattament Kontra l-Embolu

Jekk jogħġbok, irrispondi l-mistoqsijiet li ġejjin sabiex tgħinna nifhmu kemm inti sodisfatt bit-trattament tiegħek.

Jekk jogħġbok, immarka kaxxa waħda f'kull linja.

D1 - Kemm tħossok rassigurat bit-trattament tiegħek kontra l-embolu?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	**Mhux ħażin**	**Ħafna**	Kompletament

D2 - Tħoss li t-trattament tiegħek kontra l-embolu naqqas is-sintomi tiegħek (bħal, uġigħ f'riġlejk u nefħa, palpitazzjonijiet, qtugħ ta' nifs, jew uġigħ f'sidrek…)?

☐1	☐2	☐3	☐4	☐5
Lanqas xejn	Ftit	Moderatament	**Mhux ħażin**	Kompletament

D3 - Kif tikkompara l-esperjenza tiegħek tal--effetti sekondarji, bħal tbenġil żgħir jew li titlef xi demm (bħal meta tkun qed tqaxxar il-leħja, issajjar, wara qatgħat żgħar…), ma' dak li stennejt?

☐1	☐2	☐3	☐4	☐5
Hi ferm agħar milli stennejt	Hi agħar milli stennejt	Hi eżatt dak li stennejt	Hi aħjar milli stennejt	Hi ħafna aħjar milli stennejt

D4 - Dwar il-viżti segwenti tat-tabib marbuta mal-marda u t-trattament **tiegħek** kontra l-embolu, kemm tħossok sodisfatt bil-livell **ta' independenza?**

☐1	☐2	☐3	☐4	☐5
Estremament mhux sodisfatt	Mhux sodisfatt	La sodisfatt u lanqas mhux sodisfatt	Sodisfatt	Estremament sodisfatt

D5 - Kemm tħossok sodisfatt bil-metodi (bħal, appuntamenti mal-infermiera, tobba, **laboratorji…)** li jintużaw biex isegwu l-marda u t-trattament tiegħek kontra l-embolu?

☐1	☐2	☐3	☐4	☐5
Estremament mhux sodisfatt	Mhux sodisfatt	La sodisfatt u lanqas mhux sodisfatt	Sodisfatt	Estremament sodisfatt

D6 - Kemm tħossok sodisfatt bil-forma tat-trattament tiegħek kontra l-embolu (pilloli jew titqiba)?

☐1	☐2	☐3	☐4	☐5
Estremament mhux sodisfatt	Mhux sodisfatt	La sodisfatt u lanqas mhux sodisfatt	Sodisfatt	Estremament sodisfatt

D7 - **B'mod ġenerali**, kemm tħossok sodisfatt bit-trattament tiegħek kontra l-embolu?

☐1	☐2	☐3	☐4	☐5
Estremament mhux sodisfatt	Mhux sodisfatt	La sodisfatt u lanqas mhux sodisfatt	Sodisfatt	Estremament sodisfatt

Jekk jogħġbok, ara li weġibt il-mistoqsijiet kollha.

Grazzi tal-ħin tiegħek.

